available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Effects of capsaicin on P-gp function and expression in Caco-2 cells

Yi Han [a], Theresa May Chin Tan [b], Lee-Yong Lim [c,*]

[a] Department of Pharmacy, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
[b] Department of Biochemistry, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
[c] Pharmacy M315, School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, Crawley, WA 6009, Australia

ARTICLE INFO

Article history:
Received 22 February 2006
Accepted 30 March 2006

Keywords:
Capsaicin
P-gp
MDR1
Digoxin transport
Caco-2 cells

Abbreviations:
MDR, multidrug resistance
P-gp, P-glycoprotein
PXR, Pregnane X Receptor
PBS, phosphate-buffered saline
DMSO, dimethyl sulfoxide

ABSTRACT

Capsaicin is the pungent component of hot chilli, a popular spice in many populations. The aim of the present study was to evaluate the chronicity and reversibility of the modulating effect of capsaicin on both the P-gp expression and activity in the Caco-2 cell monolayers. Capsaicin at concentrations ranging from 10 to 100 μM, which were found to be non-cytotoxic towards the Caco-2 cells, were observed to inhibit P-gp mediated efflux transport of [^{3}H]-digoxin in the cells. The acute inhibitory effect was dependent on the capsaicin concentration and duration of exposure, with abolishment of polarity of [^{3}H]-digoxin transport attained at 50 μM of capsaicin. In contrast, longer term (48 and 72 h) co-incubation of the Caco-2 cells with capsaicin (50 and 100 μM) increased P-gp activity through an up-regulation of cellular P-gp protein and *MDR1* mRNA levels. The up-regulated protein was functionally active, as demonstrated by higher degree of [^{3}H]-digoxin efflux across the cell monolayers, but the induction was readily reversed by the removal of the spice from the culture medium. The induction of P-gp protein and mRNA levels was also influenced by capsaicin concentration and duration of exposure, with higher expression levels, in particular of the mRNA, seen at higher spice concentrations over prolonged period of incubation. Our data suggest that caution should be exercised when capsaicin is to be consumed with drugs that are P-gp substrates. In particular, the oral bioavailability of these drugs may be influenced by the P-gp status of populations that rely heavily on hot chilli in their diets.

1. Introduction

The P-glycoprotein (P-gp) is an active drug transporter belonging to the ATP-binding cassette transporter family. In humans, it is the product of the multi-drug resistance gene, *MDR1*, and it has been shown to possess a very wide substrate specificity. The P-gp was first characterized as an ATP-dependent transporter responsible for the efflux of chemotherapeutic agents from drug-resistant cancer cells [1]. However, it is also abundantly expressed in the apical membrane of many pharmacologically important epithelial barriers, including the intestinal epithelium, hepatocytes and renal tubular cells, suggesting an important role in drug absorption, distribution and elimination [2,3].

A wide panel of substrates has been evaluated both in vitro and in clinical trials for their ability to reverse the P-gp-mediated multi-drug resistance (MDR) in cancer cells. Besides the calcium channel blocker, verapamil [4], many other synthetic MDR modulators, including the novel hydrophobic peptide chemosensitizers, reversins 121 and 205 [5], and the

* *Corresponding author*. Tel.: +61 8 64884413; fax: +61 8 64881148.
E-mail address: limly@cyllene.uwa.edu.au (L.-Y. Lim).
0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.03.024

cyclosporine D analog, Valspodar (PSC 833) [6], have been found to successfully reverse the MDR phenotype in vitro. However, subsequent animal and clinical studies have yielded disappointing results, mainly because of the dose-limiting toxicity of these compounds. More recently, certain dietary phytochemicals and herbal supplements, e.g. piperine and curcumin [7,8], have been found to inhibit the P-gp function. These natural compounds may be less toxic than the synthetic inhibitors, although their clinical applications remain to be verified.

Like piperine and curcumin, capsaicin has also been recently shown to be an inhibitor of the P-gp function [9]. It increased the cellular accumulation of daunorubicin, decreased the efflux of rhodamine 123, and increased the cytotoxicity of vinblastine in the KB-C2 cell model. Capsaicin is the principal pungent and irritating constituent of hot chilli, a widely consumed spice amongst Asian populations. Capsaicin has generated much research interest in the past decade because of its interesting pharmacological and toxicological profiles [10,11]. For example, it appeared to play dual roles in the carcinogenic and mutagenic processes. It is a suspected carcinogen or co-carcinogen [12,13], yet capsaicin also exhibits apparent chemopreventive and chemoprotective properties [14,15]. It has been shown to inhibit the human malignant melanoma cell proliferation by attenuating the constitutive and induced activation of nuclear factor-kappa B (NF-κB) in these cells [15], and it induced apoptosis in human mammary and Jurkat cells [16].

The inhibitory effect of capsaicin on P-gp function suggests that it can potentially give rise to P-gp-mediated drug interactions. This is of considerable concern because hot chilli is consumed on a daily basis in many populations. Moreover, given that many P-gp function modulators are often found to influence the expression of the transporter in cells, it is important to not only establish the effects of capsaicin on the function of P-gp, but also to evaluate its effects on the P-gp expression. The aims of the present study were to delineate the acute and chronic effects of capsaicin on cellular P-gp function and expression using the Caco-2 cell monolayer as the surrogate human intestinal epithelium.

2. Materials and methods

2.1. Materials

Caco-2 cells were obtained from the RIKEN Cell Bank (RIKEN RCB0988, Saitama, Japan). The following materials were used: minimal essential medium (MEM), fetal bovine serum (FBS), non-essential amino acids (NEAA) and Opti MEM from Gibco BRL Life Technology (Grand Island, NY, USA); cell culture flasks from NUNC (Rochester, NY, USA) and Transwell polycarbonate inserts (12 mm diameter, 0.4 μm pore size) from Corning Costar Corp. (Bedford, MA, USA); 3-(4,5 dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and phenylmethanesulfonyl fluoride (PMSF) from BDH Chemicals Ltd. (Poole, England); [^{3}H]-digoxin (37.0 Ci/mmol) from PerkinElmer Life Sciences (Wellesley, MA, USA); C219 (primary antibody) from Signet Laboratories, Inc. (Dedham, MA, USA); anti-mouse horseradish peroxidase antibody (secondary antibody) and liquid scintillation cocktail from Amersham Biosciences (Little Chalfont, Buckinghamshire, England); SuperSignal West Pico chemiluminescent substrate from Pierce, Inc. (Rockford, IL, USA); sodium dodecyl sulphate (SDS) and β-mercaptoethanol from Merck (Damstadt, Germany); PVDF membrane, protein assay dye reagent, dithiothreitol (DTT), bromophenol blue, acrylamide/bis solution and Triton X-100 from Bio-Rad Laboratories, Inc. (Hercules, CA, USA); mouse anti-β-actin primary antibody, capsaicin, dimethyl sulfoxide (DMSO), streptomycin, penicillin, Hank's balance salt solution (HBSS), N-[2-Hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), digoxin, verapamil, bovine serum albumin (BSA), Kodak film and *N*,*N*,*N*′,*N*′-tetramethy-ethylenediamine (TEMED) from Sigma-Aldrich Inc. (St. Louis, MO, USA); RNeasy Mini Kit for RNA extraction from Qiagen (Hilden, Germany); Taqman reverse transcription reagents, Taqman universal master mix and Taqman probes for *MDR1* and *18S* were commercial gene expression assays from Applied Biosystems (Foster City, CA, USA).

2.2. Cell culture

Caco-2 cells (passage 52–60) were cultured in 5 ml of MEM in 25 cm^2 tissue culture flasks. The cells were incubated at 37 °C in 5% CO_2/95% air atmosphere in a CO_2 incubator (NuAire, Plymouth, MN, USA), with medium exchange on every other day until the cells reached 80–90% confluency, usually in 5–7 days. Cell viability was assessed by diluting the cell suspension with an equal volume of 0.4% trypan blue solution and counting the cells using a hemacytometer (Tiefe, Germany). The culture cycle was repeated if the cell density was in the range of 10^5 cells/ml, and the cell viability was greater than 98%.

2.3. In vitro cytotoxicity and anti-proliferation studies

MTT is a tetrazolium salt that is cleaved by mitochondrial succinate dehydrogenase in living cells to yield formazan, a dark blue product that is quantified by colorimetric measurement after cell lysis. As damaged or dead cells exhibit reduced or no dehydrogenase activity, this colorimetric assay can be used to determine cell viability (mitochondrial activity) [17].

Caco-2 cells were cultured in 96-well plates for 48 h at a seeding density of 10^4 cells/well. Capsaicin dissolved in DMSO was diluted with HBSS-HEPES to prepare samples with 0–100 μM of capsaicin and 0.5% of DMSO. Control samples included 0.5% of DMSO in HBSS-HEPES (vehicle), 0.1% of SDS in HBSS-HEPES (positive control), and 0.1% of dextran in HBSS-HEPES (negative control). Experiments were initiated by replacing the culture medium in each well with 100 μl of sample or control solutions, and incubating the cells for 4 h at 37 °C in the CO_2 incubator. The solution in each well was then aspirated and the cells incubated for a further 4 h with 100 μl of MTT solution (1 mg/ml in HBSS-HEPES). The intracellular formazan was solubilized with 150 μl of DMSO, and quantified at 590 nm (Spectra Fluor plate reader, Tecan, Austria). Percent cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the control vehicle.

For the anti-proliferation studies, the Caco-2 cells were cultured on 96-well plates as described in the previous

paragraph. Following cell attachment at 24 h, the medium in each well was replaced with culture medium containing 0–100 μM of capsaicin. The cells were incubated for another 72 h, washed thrice with PBS buffer after medium aspiration, and subjected to the MTT assay as described for the cytotoxicity experiments.

2.4. Cell cycle analysis

Caco-2 cells, at the number of 3×10^6, were seeded onto 75 cm^2 culture flasks and cultured for 24 h in the CO_2 incubator with 15 ml of MEM medium. The medium was replaced with culture medium sterilely supplemented with 50 or 100 μM of capsaicin, and the cells cultured for another 24–48 h. Floating cells were collected from the spent medium via centrifugation at 5000 × *g*, 4 °C, for 5 min using a Hettich Micro 22 microcentrifuge machine (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany). Attached cells were trypsinized, washed with cold PBS, and pooled with the floating cells. The cells were fixed with 75% ethanol, stored at −20 °C overnight, washed again with PBS before staining (200 μg/ml of RNase A, 20 μg/ml of propidium iodide and 0.1% of Triton X-100 in PBS). Cells were analyzed in a Cyan LX (DakoCytomation, Fort Collins, CO, USA) instrument and at least 10^4 events were recorded. Distribution of cells into the phases of the cell cycle was obtained using the WinMDI2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, California, USA).

2.5. [^{3}H]-Digoxin transport studies

Caco-2 cells were cultured on Transwell inserts (12 mm diameter, 0.4 μm pore size) at the seeding density of 2×10^5 cells per well in 0.5 ml of MEM medium, which was sterilely supplemented with capsaicin (50 and 100 μM) on day 18 or day 19. Control cells received medium devoid of capsaicin. Transport experiments were conducted on day 21 on cells with transepithelial electrical resistance (TEER) of 300–400 Ω cm^2 (Millicell-ERS, Millipore, Bedford, MA, USA). Monolayer TEER was measured immediately before and after the transport experiments.

To start the transport experiments, culture medium in the apical and basal chambers was replaced with 0.7 ml of Opti MEM, a serum-free transport medium without capsaicin. After 60 min of incubation in the CO_2 incubator, the Opti MEM in either the apical (for apical-to-basal transport) or basal (basal-to-apical transport) chamber was replaced with digoxin (5 μM, 0.5 μCi/ml in Opti MEM) solution. To delineate the effects of acute spice exposure, an additional series of experiments were conducted on the control cells, which received digoxin solution supplemented with 0–100 μM of capsaicin in the donor chamber, and the corresponding capsaicin concentration in Opti MEM in the receiver chamber. In addition, parallel experiments were conducted to validate the presence of functional P-gp in the control cells by adding 100 μM of verapamil (an established P-gp inhibitor) in place of capsaicin into the donor and receiver chambers.

At 0, 1, 2, 3 and 4 h, 50 μl-aliquots were removed from the receiver chambers, incubated overnight with 5 ml of scintillation cocktail and analyzed in a liquid scintillation counter (LS 3801, Beckman Instruments, Inc., CA, USA). Withdrawn samples were replaced with equal volumes of prewarmed transport medium to maintain the liquid volume in each chamber. Apparent permeability coefficient (P_{app}) was calculated as $P_{app} = (dQ/dt)/(AC_0)$ (cm/s) where dQ/dt (nmol/s) was the flux rate, A (cm^2) the effective surface area of the cell monolayer, and C_0 (nmol/ml) the initial drug concentration in the donor chamber. Net flux was expressed as the quotient of P_{app} (BA) to P_{app} (AB).

2.6. P-gp and MDR1 expression

Caco-2 cells were cultured on Transwell inserts as described in the previous section, with supplementation of the MEM medium with capsaicin (50 and 100 μM) on day 19 or day 18. Control cells received medium devoid of capsaicin. On day 21, after exposure to capsaicin for 48 and 72 h, respectively, the cells were collected into eppendorf tubes by scraping with a small spatula, and lysed in 1% Triton X-100 in PBS for P-gp protein analysis or extracted for total cellular RNA using the RNA extraction kit. Protein extraction was conducted on ice to minimize any potential protein degradation.

2.7. Western Blot analysis of P-gp

Protein concentrations were measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Western Blot for the immunodetection of P-gp was obtained using 20 μg of extracted membrane proteins in loading buffer (0.15 g/ml Tris base pH 6.8, 15% glycerol, 0.015 g/ml SDS, 0.005 g/ml bromophenol blue and 20% β-mercaptoethanol). Protein samples were separated on a 7.5% SDS-polyacrylamide gel (Sub-Cell GT system, Bio-Rad) and transferred onto a PVDF membrane (Trans-Blot SD Semi-Dry Cell, Bio-Rad). The membrane was blocked by incubating for 2 h at room temperature with Tris-buffered saline containing 0.1% Tween 20 and 5% dried skim milk, then incubated for 2 h with the primary monoclonal antibody, C219 (1:1000). The membrane was washed (4 × 5 min), incubated with anti-mouse IgG horseradish peroxidase conjugate (1:3000) for 1 h, washed again (4 × 5 min) and incubated with chemiluminescence detection reagent for 5 min. The protein was visualized by exposing the membrane to a Kodak film for 2 min in a dark room. β-actin served as internal standard, and it was similarly detected using a mouse anti β-actin as primary antibody and anti-mouse IgG as secondary antibody. Band intensity was analyzed with the Quantity One software (Bio-Rad Laboratories, Inc.) and P-gp expression was presented as the ratio of P-gp band intensity to β-actin band intensity in the same blot (P-gp/actin [%]).

2.8. Real time RT-PCR analysis of MDR1 mRNA

RNA extraction was conducted on cells co-incubated with capsaicin for 48 and 72 h on transwell inserts. Total cellular RNA was extracted using the RNeasy Mini Kit, and quantified at 260 nm (UV–vis-1601 spectrophotometer, Shimadzu, Japan). The purity of the RNA preparation was high, with OD (optical density) 260 nm/280 nm ratio in the range of 1.8–2.0. Reverse

transcription of the total RNA (0.2 μg) was carried out in a Flexigene thermal cycler (Techgene Ltd., Cambridge, United Kingdom) using Taqman reverse transcription reagents and random hexamers as primers according to the manufacturer's protocol.

For real time quantitative PCR, 1 μl (equivalent to 0.02 μg of total initial RNA) of the reverse transcription reaction mixture was used. Amplification and detection of samples were performed in a ABI 7500 sequence detection system using the ABI Assay-By-Design Kit (Hs00184491_m1), which contained the forward primer, reverse primer and FAM labeled Taqman probe for *MDR1* measurements (Foster City, CA, USA, http://www.appliedbiosystems.com). The endogenous control, *18S* mRNA, was detected using the ABI *18S* Kit (Hs99999901_s1). Reactions were multiplexed and performed in triplicates using the following protocol: 50 °C for 2 min, 95 °C for 5 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 45 s, and 72 °C for 45 s. Relative quantification was obtained by the comparative threshold cycle ($\Delta\Delta C_T$) method [18] (RQ software, version 1.2.3, Applied Biosystems). Briefly, $N_1 = N_0 \times €C$ where N_1 describes the number of molecules resulting from amplification of N_0 template molecules after C number of cycles. Each cycle results in the addition of €C molecules where € is the 'efficiency' of the amplification. Comparing two different targets, A and B, the difference between N_{0A} and N_{0B} can be described as 2^{-DCt}, where −DCt is the difference between amplification cycles at which $N_{1A} = N_{1B}$. Normalization using 18S was calculated as −DCt with *MDR1* levels at 0 h, 0 μM capsaicin serving as a calibrator, which resulted in the relative expression levels being described by 2^{-DDCt}. Values reported have a 95% confidence interval as determined by the ABI RQ software.

2.9. *Reversibility of cellular P-gp expression*

Caco-2 cells cultured on Transwell inserts were exposed to capsaicin (50 and 100 μM) on day 18. On day 21, after 72 h of capsaicin exposure, the medium was reverted to MEM devoid of capsaicin, and the cells were cultured for another 3 days. Cellular P-gp level was then determined by Western Blot analysis, and compared to that in control cells not exposed to capsaicin during the course of culture.

2.10. *Statistical analyses*

Differences between mean values were analyzed for significance by one-way ANOVA using the SPSS 10.0 software. p values ≤ 0.05 were considered significantly different.

3. Results

3.1. *In vitro cytotoxicity, anti-cell proliferation activity and effects on cell cycle*

After 4 h of exposure to capsaicin at concentrations of up to 200 μM, no toxic effect in relation to mitochondrial enzyme activity of the Caco-2 cells was observed (Fig. 1). The cell viability data were comparable ($p > 0.05$) to those obtained for 0.1% dextran (negative control). In contrast, the 0.1% SDS (positive control) sample showed significant toxicity towards all 3 cell types ($p < 0.001$).

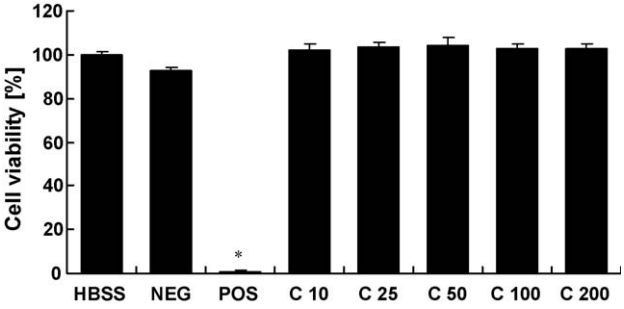

Fig. 1 – Effects of capsaicin on Caco-2 cell viability as evaluated by the MTT assay after 4 h of exposure. Data are expressed as mean ± S.D. ($n = 8$). *, significantly different from control group ($p < 0.001$). HBSS, vehicle; NEG, 0.1% dextran (negative control); POS, 0.1% SDS (positive control); C 10, capsaicin 10 μM; C 25, capsaicin 25 μM C 50, capsaicin 50 μM; C 100, capsaicin 100 μM; C 200, capsaicin 200 μM.

To evaluate the effects of capsaicin on cell proliferation, Caco-2 cells were cultured in capsaicin-supplemented culture media after attachment, and the MTT assay was applied after 72 h of incubation. Caco-2 cell proliferation was adversely affected by capsaicin in a concentration-dependent manner (Fig. 2). While the cell proliferation was unaffected by capsaicin at 10 μM, cell viability was only 80 and 45% that of control when the cells were cultured in 50 and 100 μM of capsaicin, respectively. The poor proliferation rate was, however, not associated with considerable changes in cell cycle progression. As shown in Fig. 3, the distribution of cells into the G and S phases was not significantly affected after 24 or 48 h of co-incubation with 100 μM of capsaicin although co-incubation with 50 μM of capsaicin for 48 h resulted in a significant increase of cell numbers only in the S phase.

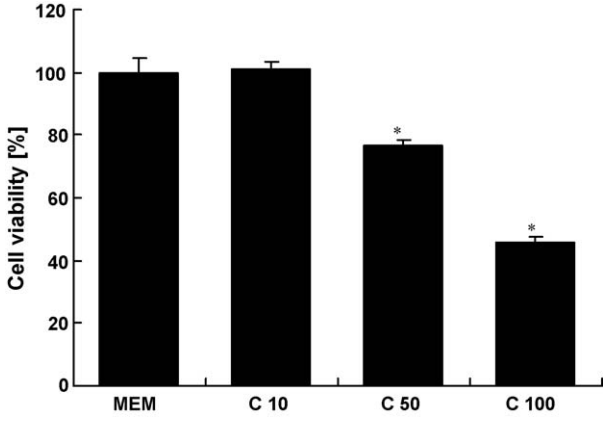

Fig. 2 – Effects of capsaicin on Caco-2 cell proliferation as evaluated by the MTT assay after 72 h co-incubation. Data are represented as mean ± S.D. ($n = 8$). *, significantly different from control group ($p < 0.05$). MEM, culture medium; C 10, capsaicin 10 μM; C 50, capsaicin 50 μM; C 100 capsaicin 100 μM.

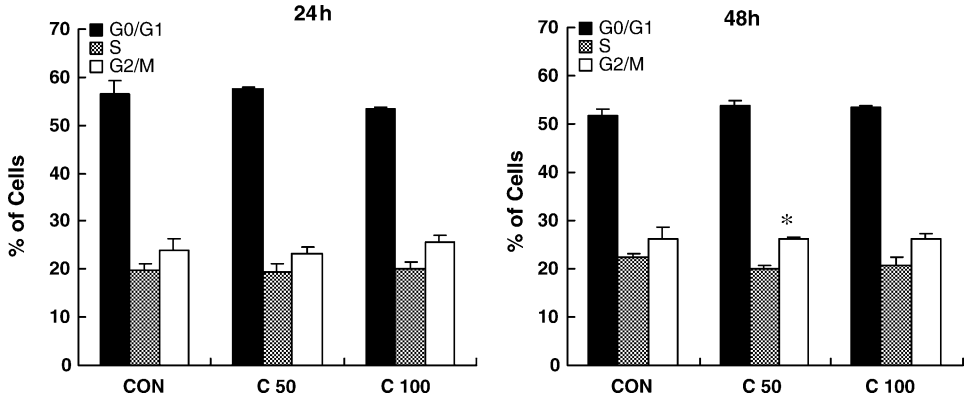

Fig. 3 – Cell cycle distribution of Caco-2 cells co-cultured with capsaicin for 24 h (left) and 48 h (right). CON, control; C 50, capsaicin 50 μM; C 100, capsaicin 100 μM. Data are expressed as mean ± S.E.M. ($n = 3$). *, significantly different from control group ($p < 0.05$).

3.2. *[^{3}H]-digoxin transport studies*

P-gp function was evaluated by measuring transepithelial transport of [^{3}H]-digoxin across Caco-2 cell monolayers. TEER measured before (0 h) and after (4 h) completion of transport studies showed no significant differences (data not shown). Digoxin transport in the Caco-2 cells was polarized, the efflux ratio being 4.11 (Table 1). In the presence of 100 μM of verapamil, AB digoxin transport was enhanced while that in the BA direction was inhibited, and the efflux ratio was reduced to 1.1. This indicates that the digoxin transport across the Caco-2 cell monolayers was polarized by P-gp mediated efflux activity.

Like the P-gp inhibitor, verapamil, capsaicin also increased AB digoxin transport and inhibited BA digoxin transport across the Caco-2 cells (Table 1). The effect of capsaicin on digoxin P_{app} was concentration-dependent over the range of 10–50 μM. Maximal inhibition of P-gp function appeared to occur at 50 μM of capsaicin, which produced comparable results with 100 μM of verapamil. Increasing the capsaicin concentration to 100 μM did not cause any further changes in digoxin P_{app}. The corresponding P_{app} values and efflux ratios are given in Table 1.

Table 1 – Apparent permeability coefficient ($P_{app} \times 10^{-6}$ cm/s) and net efflux of bi-directional [^{3}H]-digoxin (loading dose of 5 μM) transport across polarized Caco-2 cell monolayers

Sample	P_{app} ($\times 10^{-6}$ cm/s)		Net efflux
	AB	BA	
Control	1.90 ± 0.07	7.81 ± 0.20	4.11
Verapamil (100 μM)	3.32 ± 0.06*	3.66 ± 0.05*	1.10
Capsaicin (μM)			
10	2.49 ± 0.08*	5.82 ± 0.04*	2.34
25	2.64 ± 0.09*	5.05 ± 0.11*	1.91
50	3.63 ± 0.08*	3.77 ± 0.11*	1.04
100	3.65 ± 0.05*	3.64 ± 0.11*	1.00

Data are presented as mean ± S.E.M, $n = 4$–6. AB, apical to basal transport; BA, basal to apical transport. Net efflux was calculated as the ratio of mean basal-to-apical P_{app} to mean apical-to-basal P_{app}.

* $p < 0.05$, significantly different from control group.

3.3. *P-gp and MDR1 expression*

P-gp protein and *MDR1* mRNA levels in the Caco-2 cells cultured on Transwell inserts were studied by Western Blot and RT-PCR, respectively, on day 21 after 50 or 100 μM of capsaicin was added to the culture medium for 48 or 72 h. As shown in Fig. 4, capsaicin significantly up-regulated the P-gp membrane expression level in a time- and concentration-dependent manner. The cellular P-gp protein was elevated to comparable levels, which were twice that of control, following 72 h co-incubation with capsaicin at 50 and 100 μM. When the co-incubation period was shortened to 48 h, significant induction of the P-gp membrane protein was seen only at the higher concentration (100 μM) of capsaicin, which produced a P-gp protein level equivalent to 1.5-fold that of control.

Real time RT-PCR analysis suggested that capsaicin also up-regulated the *MDR1* expression in the Caco-2 cells (Fig. 5). While the induction of the gene product was again influenced by capsaicin concentration and duration of exposure, there were subtle differences when compared to the induction of the P-gp protein. Increasing the spice concentration from 50 to 100 μM led to significantly higher *MDR1* expression, regardless of whether the co-incubation time was 48 or 72 h. The difference in *MDR1* expression between the two capsaicin concentrations was, however, more pronounced (two-fold difference) when the exposure time was prolonged to 72 h.

To determine whether the capsaicin-mediated induction in P-gp expression was accompanied by changes in P-gp function, bi-directional digoxin transport was performed on the Caco-2 cells after they had been co-cultured with capsaicin. Drug transport was, however, conducted after the removal of capsaicin from the Transwell inserts to eliminate the acute effects of capsaicin on the P-gp function. Digoxin transport in these cells was characterized by higher net efflux ratios caused by lower AB transport and enhanced BA efflux (Table 2), suggesting that the P-gp mediated efflux of digoxin was enhanced in these cells. The implication is therefore that

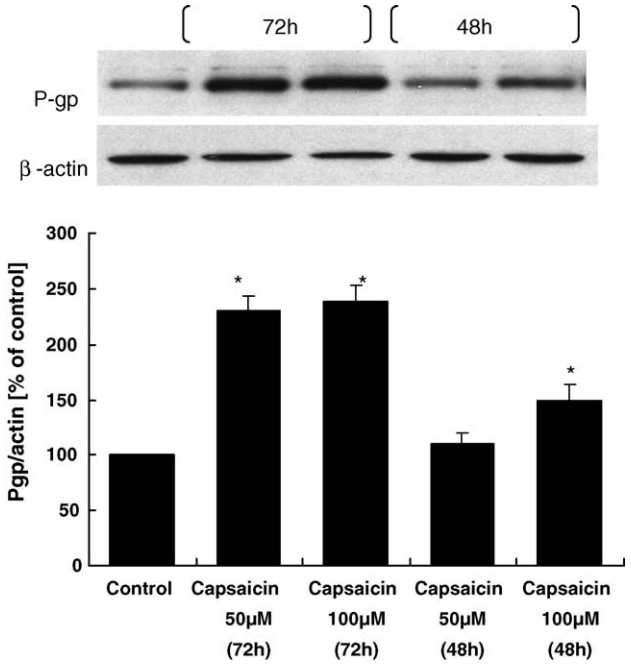

Fig. 4 – P-gp expression levels in Caco-2 cells cultured in Transwell inserts and exposed to 50 or 100 μM of capsaicin for 48 or 72 h. P-gp membrane protein level was determined by Western Blot using C219 as primary antibody. β-actin was used to confirm equal protein loading. Upper bands, P-gp; lower bands, β-actin. From left to right, control (culture medium), capsaicin 50 μM for 72 h, capsaicin 100 μM for 72 h, capsaicin 50 μM for 48 h and capsaicin 100 μM for 48 h. The result of one typical experiment out of three is shown. The bar graph shows the quantification of band intensity; data are expressed as mean ± S.E.M. ($n = 3$). *, significantly different from control group ($p < 0.05$).

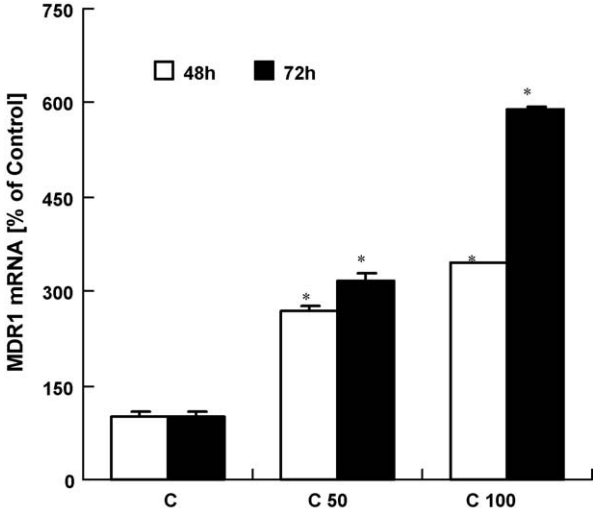

Fig. 5 – Real time RT-PCR analysis of *MDR1* mRNA in Caco-2 cells exposed to capsaicin for 48 or 72 h. *MDR1* mRNA was quantified using real time RT-PCR analysis (Taqman) standardizing against the endogenous control *18S*. C, control; C 50, capsaicin 50 μM; C 100, capsaicin 100 μM. Data were normalized to controls and expressed as percentage ratio to control levels ($n = 3$). * $p < 0.05$, significantly different from control group.

capsaicin had induced in the Caco-2 cells functionally active P-gp protein. P-gp function in the capsaicin-co-cultured cells also showed a dependence on the spice concentration and exposure time that mirrored the trends observed for the *MDR1* expression level.

The induction effect of capsaicin on P-gp expression level in the Caco-2 cells was, however, reversible. Upon the removal of the spice, the P-gp level in the Caco-2 cells previously exposed to either 50 or 100 μM of capsaicin was found to revert to baseline levels after 3 days of culture under standard conditions (Fig. 6). There were insignificant differences between the P-gp protein levels in these cells compared to control cells not exposed to capsaicin in the course of culture.

4. Discussion

Intestinal P-gp plays a significant role in the absorption and pre-systemic elimination of many peroral xenobiotics, including drugs. Therefore, a study of the factors that regulate its function and expression is important both from the pharma-

Table 2 – Apparent permeability coefficient (P_{app}) and net efflux ratio of bi-directional [^{3}H]-digoxin (loading dose of 5 μM) transport across polarized Caco-2 cell monolayers co-cultured with 50 or 100 μM of capsaicin for 48 or 72 h prior to incubation with digoxin in the absence of capsaicin

Sample	48 h			72 h		
	P_{app} ($\times 10^{-6}$ cm/s)		Net efflux	P_{app} ($\times 10^{-6}$ cm/s)		Net efflux
	AB	BA		AB	BA	
Control	1.90 ± 0.06	7.81 ± 0.20	4.11	1.90 ± 0.06	7.81 ± 0.20	4.11
Capsaicin (μM)						
50	1.65 ± 0.06*	8.42 ± 0.25*	5.09	1.42 ± 0.07*	9.76 ± 0.33*	5.45
100	1.54 ± 0.02*	10.05 ± 0.33*	6.73	1.36 ± 0.05*	12.07 ± 0.11*	9.63

Data are presented as mean ± S.E.M, $n = 4$–6. AB, apical to basal transport; BA, basal to apical transport. Net efflux was calculated as the ratio of mean basal-to-apical P_{app} to mean apical-to-basal P_{app}.
* $p < 0.05$, significantly different from control group.

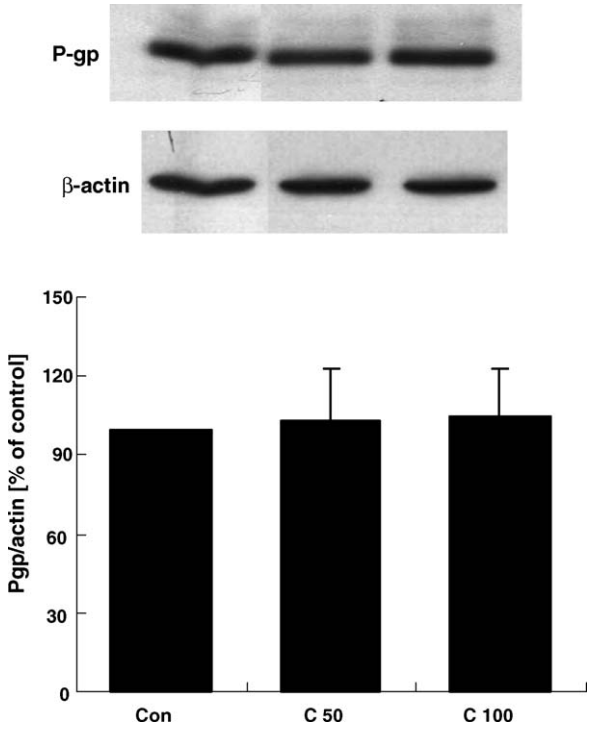

Fig. 6 – Reversibility of P-gp up-regulation by capsaicin - Caco-2 cells exposed to 50 or 100 μM of capsaicin for 72 h were cultured for another 3 days under standard conditions following the removal of capsaicin. P-gp protein level was determined by Western Blot using C219 as primary antibody. β-actin was used to confirm equal protein loading. Upper bands, P-gp; lower bands, β-actin. From left to right, control (culture medium), capsaicin 50 μM treated cells, capsaicin 100 μM treated cells. The result of one typical experiment out of three is shown. The bar graph shows the quantification of band intensity; Con, control; C 50, capsaicin 50 μM; C 100, capsaicin 100 μM; data are expressed as mean ± S.E.M. ($n = 3$). *, significantly different from control group ($p < 0.05$).

cological and toxicology perspectives. In this study, we evaluated the effects of capsaicin exposure on the P-gp function and expression in the Caco-2 cells. To our knowledge, this is the first report to examine the chronicity and reversibility of the modulating effect of capsaicin, a popular spice in Asian cuisines, on both the P-gp expression and activity in the surrogate human intestinal epithelia.

Nabekura et al. has previously demonstrated that capsaicin could reverse doxorubicin resistance in human multidrug-resistant carcinoma KB-C2 cells by inhibiting the P-gp efflux pump [9]. We affirmed the finding in the present study through [^{3}H]-digoxin transport studies carried out on Caco-2 cell monolayers. Capsaicin at sub-cytotoxic concentrations was capable of inhibiting the P-gp efflux activity to a comparable level as the established P-gp inhibitor, verapamil. Furthermore, the P-gp inhibitory effect was observed to be dependent on the concentration and duration of exposure to capsaicin.

Interestingly, 48 and 72 h exposure to capsaicin appeared to produce opposing effects on P-gp activity in the Caco-2 cells through its modulation of the P-gp expression level in the cells. Up-regulation of both the P-gp protein and *MDR1* levels were observed, the parallel changes suggesting a capsaicin-mediated transcriptional modulation. The underlying mechanism remains to be confirmed. However, it is unlikely to involve an activation of the Pregnane X Receptor (PXR) because the Caco-2 cells are regarded to be deficient in PXR [19] and they have indeed been used as a PXR-negative cell line [20]. In this respect, capsaicin might differ from other P-gp inducers, such as rifampin [19] in regulating the expression of P-gp by a PXR-independent mechanism. Other mechanisms implicated in the induction of P-gp expression include the activation of the NF-kB [21], protein kinase C [22] and the PI3-kinase/Akt [23] pathways. Identification of the specific mechanisms involved in the capsaicin-mediated induction of cellular P-gp will be an important area of research for our laboratory. The present study has, however, shown that both the P-gp protein and mRNA levels in the Caco-2 cells were influenced by the capsaicin dose and duration of exposure, although the mRNA level appeared to be more sensitive to these parameters. In addition, [^{3}H]-digoxin transport data have suggested the capsaicin-induced P-gp protein to be functional active. The induction of the P-gp protein was, however, readily reversed by the removal of the spice from the culture medium.

The in vitro acute and chronic cytotoxicity profiles of capsaicin have also been established in the present study. At concentrations of up to 100 μM, capsaicin did not exhibit cytotoxicity after 4 h exposure. However, capsaicin at 50 and 100 μM moderately lowered cell viability when the co-culture period was prolonged to 72 h. Nonetheless, we do not expect the modulation of capsaicin on P-gp expression to be substantially contributed by the selection of cells that highly expressed the P-gp protein. This is because capsaicin produced marginal effects on the cell cycle progress compared to established cell cycle modulators, such as curcumin [24,25].

The dose of capsaicin in a typical Indian or Thai diet was reported to be about 128 μg/kg human body weight [26]. Taking the gastric fluid volume to be 1–3 l for an adult human with a body weight of 60 kg, the concentrations of capsaicin in the gastric fluid would be equivalent to 8–25 μM. It is therefore possible for dietary capsaicin to affect the bioavailability of co-administered drugs by interfering with the intestinal P-gp function. It is also likely that the capsaicin dose will be higher in households that regularly consume a large amount of hot chilli, and whether this will lead to an up-regulation of the intestinal P-gp expression level remains to be verified. Until the clinical significance of these findings is established, caution is advised for patients who are likely to co-consume capsaicin with drugs of narrow therapeutic index whose bioavailability is strongly influenced by P-gp efflux activity.

5. Conclusion

In conclusion, the data of this study have shown that an acute dose of capsaicin can inhibit P-gp function. Given on a long term basis, capsaicin has the potential to up-regulate P-gp expression and enhance P-gp efflux activity in the intestinal epithelium. Until the clinical significance of these data is established, doctors should exercise caution when prescribing

drugs, which are P-gp substrates to populations that rely heavily on capsaicin in their diet.

Acknowledgements

The instrument and reagents for real time PCR analyses were kindly provided by ABI Singapore. This study was supported by a research grant (01/1/21/19/142) from the Biomedical Research Council of Singapore.

REFERENCES

[1] Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62:385–427.
[2] Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 1987;84:7735–8.
[3] Yu DK. The contribution of P-glycoprotein to pharmacokinetic drug-drug interactions. J Clin Pharmacol 1999;39:1203–11.
[4] Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 1981;41:1967–72.
[5] Sharom FJ, Yu X, Lu P, Liu R, Chu JWK, Szabo K, et al. Interaction of the P-glycoprotein multidrug transporter (MDR1) with high affinity peptide chemosensitizers in isolated membranes, reconstituted systems, and intact cells. Biochem Pharmacol 1999;58:571–86.
[6] Sikic BI. Modulation of multidrug resistance: a paradigm for translational clinical research. Oncology (Williston Park) 1999;13:183–7.
[7] Anuchapreeda S, Leechanachai P, Smith MM, Ambudkar SV, Limtrakul Pn. Modulation of P-glycoprotein expression and function by curcumin in multidrug-resistant human KB cells. Biochem Pharmacol 2002;64:573–82.
[8] Bhardwaj RK, Glaeser H, Becquemont L, Klotz U, Gupta SK, Fromm MF. Piperine, a major constituent of black pepper, inhibits human P-glycoprotein and CYP3A4. J Pharmacol Exp Ther 2002;302:645–50.
[9] Nabekura T, Kamiyama S, Kitagawa S. Effects of dietary chemopreventive phytochemicals on P-glycoprotein function. Biochem Biophys Res Commun 2005;327:866–70.
[10] Kang JY, Teng CH, Chen C, Wee A. Effect of capsaicin and chilli on ethanol induced gastric mucosal injury in the rat. Gut 1995;36:664–9.
[11] Park JS, Choi MA, Kim BS, Han IS, Kurata T, Yu R. Capsaicin protects against ethanol-induced oxidative injury in the gastric mucosa of rats. Life Sci 2000;67:3087–93.
[12] Surh YJ, Lee SS. Capsaicin in hot chili pepper: carcinogen, co-carcinogen or anticarcinogen? Food Chem Toxicol 1996;34:313–6.
[13] Surh YJ. Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. Food Chem Toxicol 2002;40:1091–7.
[14] Brar SS, Kennedy TP, Whorton AR, Sturrock AB, Huecksteadt TP, Ghio AJ, et al. Reactive oxygen species from NAD(P)H:quinone oxidoreductase constitutively activate NF-{kappa}B in malignant melanoma cells. Am J Physiol Cell Physiol 2001;280:C659–76.
[15] Han SS, Keum YS, Seo HJ, Chun KS, Lee SS, Surh YJ. Capsaicin suppresses phorbol ester-induced activation of NF-[kappa]B/Rel and AP-1 transcription factors in mouse epidermis. Cancer Lett 2001;164:119–26.
[16] Macho A, Blazquez MV, Navas P, Munoz E. Induction of apoptosis by vanilloid compounds does not require de novo gene transcription and activator protein 1 activity. Cell Growth Differ 1998;9:277–86.
[17] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[18] Berrill A, Tan HL, Wang SC, Fong WJ, Choo ABH. Assessment of stem cell markers during long-term culture of mouse embryonic stem cells. Cytotechnology 2004;44:77–91.
[19] Geick A, Eichelbaum M, Burk O. Nuclear Receptor Response Elements Mediate Induction of Intestinal MDR1 by Rifampin. J Biol Chem 2001;276:14581–7.
[20] Mitin T, von Moltke LL, Court M, Greenblatt DJ. Levothyroxine up-regulates P-glycoprotein independent of the Pregnane X Receptor. Drug Metab Dispos 2004;32:779–82.
[21] Thevenod F, Friedmann JM, Katsen AD, Hauser IA. Up-regulation of multidrug resistance P-glycoprotein via nuclear factor-kappa B activation protects kidney proximal tubule cells from cadmium- and reactive oxygen species-induced apoptosis. J Biol Chem 2000;275:1887–96.
[22] Flescher E, Rotem R. Protein kinase C [var epsilon] mediates the induction of P-glycoprotein in LNCaP prostate carcinoma cells. Cell Signal 2002;14:37–43.
[23] Nwaozuzu OM, Sellers LA, Barrand MA. Signalling pathways influencing basal and H(2)O(2)-induced P-glycoprotein expression in endothelial cells derived from the blood–brain barrier. J Neurochem 2003;87:1043–51.
[24] Chen H, Zhang ZS, Zhang YL, Zhou DY. Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. Anticancer Res 1999;19:3675–80.
[25] Moragoda L, Jaszewski R, Majumdar AP. Curcumin induced modulation of cell cycle and apoptosis in gastric and colon cancer cells. Anticancer Res 2001;21:873–8.
[26] Rumsfield JA, West DP. Topical capsaicin in dermatologic and peripheral pain disorders. Ann Pharmacother 1991;25:381–7.