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ORIGINAL ARTICLE

Modulatory effects of curcumin on multi-drug resistance-associated protein 5 in pancreatic cancer cells

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Abstract
Purpose Chemotherapy of pancreatic cancer often fails due to the development of intrinsic and acquired resistance during drug treatment. Recent studies have suggested that MRP5 conferred resistance to first-line drugs 5-fluorouracil and gemcitabine by active efflux of drugs from the cell. Our aim was to evaluate whether curcumin could reverse this multi-drug resistance by inhibition of MRP5-mediated efflux.
Methods MRP5 protein was detected and localized by immunocytochemistry using a monoclonal antibody in MRP5 over-expressing HEK293 (HEK293/MRP5) cells and two pancreatic cancer cell lines PANC-1 and MiaPaCa-2. The cellular accumulation of a specific MRP5 fluorescent substrate 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) into these cells was measured by flow cytometry and the cell proliferation determined by a 72-h CyQuant assay.
Results The cellular accumulation of BCECF in HEK293/MRP5 cells and in PANC-1 and MiaPaCa-2 cells was significantly increased by curcumin in a concentration-dependent manner. Curcumin and a MRP5 inhibitor MK571 had no apparent effects on cellular accumulation of BCECF in parental HEK293 cells. In the proliferation assays, curcumin caused a concentration-dependant increase in the sensitivity to the cytotoxic drug 5-fluorouracil in HEK293/MRP5 cells, PANC-1 and MiaPaCa-2 pancreatic cancer cells, but not in parental HEK293 cells.
Conclusions Our results suggest that curcumin is an inhibitor of MRP5 and may be useful in the reversal of multi-drug resistance in pancreatic cancer chemotherapy.

Keywords Curcumin · Multi-drug resistance-associated protein 5 · Pancreatic cancer · 5-Fluorouracil

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Introduction

Pancreatic carcinoma is one of the leading causes of cancer mortality worldwide. Despite combination chemotherapy with gemcitabine and capecitabine (a prodrug of 5-fluorouracil (5-FU)), treatment of pancreatic cancer becomes problematic due to both intrinsic and acquired resistance developing to these drugs during therapy [1, 2]. A major group of proteins involved in the cellular efflux of charged anti-cancer drugs are the ATP binding cassette (ABC) transporter superfamily [3]. ABC transporters highly expressed in pancreatic cancer cells include multidrug resistance-associated protein (MRP) 1, 3 and 5, and breast cancer resistance protein (BCRP) [4]. The ABC transporters pump various anticancer agents/active metabolites out of cancer cells and confer resistance to various drugs including gemcitabine and 5-fluorouracil (5-FU) [5], which are the most important drugs used to combat pancreatic cancer in the clinic. In pancreatic carcinoma tissue, the mRNA level of MRP5 was significantly higher compared to normal pancreatic tissue [4]. Recently, it was reported that knockdown of MRP5 using short interference RNA (siRNA) reversed 5-FU and gemcitabine resistance in

pancreatic carcinoma cells [6, 7] and reversed gemcitabine resistance in lung cancer cells [8]. However, there are many difficulties in the delivery of siRNA to a target organ such as the pancreas in humans, and this type of therapy may not be realistic at present.

Development of small-molecule ABC transporter inhibitors appears to have more potential with respect to clinical applications. In the last decade, >70% of the ABC transporter inhibitors reported were natural products or synthetic derivatives of these products [9]. Some phytochemicals are safe, potent and multi-spectrum inhibitors of ABC transporters [10–12]. The phytochemical curcumin (Fig. 1) is a potent inhibitor of several ABC transporters (e.g., P-gp, MRP1 and BCRP) involved into anti-cancer drug resistance [10, 13, 14]. It has been reported to potentiate antitumor activity of 5-FU and gemcitabine in in vitro and in vivo models [15–17]. Oral curcumin is well tolerated and has shown biological effects in phase II clinical trials for pancreatic cancer [18]. However, the mechanisms for curcumin's reversal of chemoresistance in pancreatic cancer cells remain unclear. MRP5 has been reported to confer resistance to 5-FU and gemcitabine by accelerating extrusion of their cytotoxic metabolites from cells [8, 19]. In this study, we investigated the modulatory effects of curcumin and two other phytochemicals, biochanin A and diindolemethane on MRP5-mediated drug efflux.

Methods

Materials

Curcumin, biochanin A, diindolemethane, 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), 5-fluorouracil, 6-thioguanine (6-TG) and two MRP5 inhibitors, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and MK571 were purchased from Sigma (Sydney, AU). Cell culture materials were purchased from Invitrogen (Life Technologies, Auckland, NZ).

Cell lines

Two pancreatic cancer cell lines PANC-1 and MiaPaCa -2 were a gift from the Auckland Cancer Society Research Centre. Parental HEK293 cells and HEK293 cells transduced with MRP5 (HEK293/MRP5) were generous gifts from Professor P. Borst (Division of Molecular Biology and Centre for Biomedical Genetics, the Netherlands Cancer Institute, Amsterdam, the Netherlands). MiaPaCa-2 pancreatic cancer cells, parental HEK293 cells and HEK293/MRP5 were grown in DMEM, supplemented with 10% fetal calf serum and 100 units of penicillin/streptomycin per mL, at 37°C in 5% CO2 humidified air.

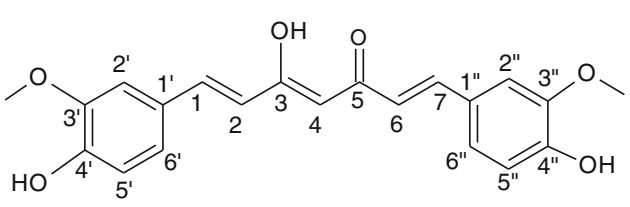

Fig. 1 Chemical structure of curcumin

Immunocytochemistry

MiaPaCa-2 pancreatic cancer cells, parental HEK293 and HEK293/MRP5 cells were cultured onto Lab-Tek Chamber Slides in DMEM supplemented with 10% fetal bovine serum. Cells were washed, fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X-100 before incubation with a rat anti-MRP5 monoclonal antibody M5I-10 (Abcam, San Diego). MRP5 staining was detected by incubation with FITC-conjugated goat anti-rat antibody. Cell nuclei were stained with propidium iodide (5 μg/mL) for 5 min. The slides were viewed using an Olympus FV1000 confocal laser scanning microscope (Olympus Inc., Tokyo).

Cellular accumulation of BCECF

The cells grown in 75-cm^2 flasks were detached with 0.05% Trypsin, 0.52 mM EDTA solution. The cells were mixed vigorously and gently passed through a 27-gauge needle to minimize cell clumping. The cells were then resuspended in phenol-red free DMEM with cell density of about 10^6 cells/ml. Cells ($\sim 10^6$/ml) were incubated with the nonfluorescent precursor, 2′,7′-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF)-AM. The latter is transported across the biomembrane by passive diffusion [20] and is rapidly converted into a fluorescent MRP5 substrate BCECF [21]. In a preliminary study, we determined the time (10 min after loading) to reach steady-state accumulation of BCECF and thus all accumulation studies were measured at steady-state. The accumulation of BCECF was performed by incubating the 1 ml cell suspension with various concentrations of phytochemicals, known MRP5 inhibitors or the vehicle (0.1% DMSO) at 37°C for 15 min, followed by addition of 0.25 μM of BCECF. After incubation for another 15 min, the accumulation was stopped by washing cells twice with ice-cold PBS. The cells were reconstituted in ice-cold 1% formaldehyde in PBS. The intracellular level of BCECF was analyzed using the BD^{TM} LSR II flow cytometer (BD Biosciences, CA) with the excitation at 488 nm and the emission at 525 nm. The cells were gated based on forward and side scatter, and only viable cells were included in the analysis.

Cytotoxicity studies

Parental HEK293 cells and the transfectants, PANC-1 and MiaPaCa cells were grown in 96-well tissue culture plates in complete DMEM. After 24 h of attachment, the cytotoxic drug was added to the growth medium in the presence and absence of curcumin (5 and 10 μM), NPPB (30 μM) and MK571 (25 μM). Cell viability was determined after 3 days of continued drug treatment using CyQuant cell proliferation kits (Invitrogen, Carlsbad, CA). The effective concentration necessary for 50% growth inhibition (IC_{50}) represents the effective concentration of drug which gives 50% inhibition of the maximum achieved and was generated by using Prism (GraphPad, La Jolla, CA). Resistance factor is the ratio of IC_{50} value of the HEK-MRP5 transfectant to that of the parental HEK293 cells.

Results

Expression and localization of MRP5

To investigate the expression and localization of MRP5, PANC-1 and MiaPaCa-2 pancreatic cancer cells, and parental HEK293 and HEK293/MRP5 cells, were stained with a monoclonal MRP5 antibody M5I-1 followed by a FITC-conjugated secondary antibody. Confocal laser scanning microscopy showed positive staining of MRP5 in PANC-1 and MiaPaCa-2 pancreatic cancer cells and HEK293/MRP5 cells except in parental HEK293 cells. Interestingly, our results indicated that MRP5 was detected at both plasma membrane and in the intracellular compartments in PANC-1 and MiaPaCa-2 pancreatic cancer cells (Fig. 2). This was further confirmed by a more detailed view after 3D-reconstruction showing X-Z and Y-Z sections (Fig. 2 i and j).

Cellular accumulation of a MRP5 substrate

The effect of curcumin on the efflux of the fluorescent substrate BCECF from MRP5-expressing cells was measured using flow cytometry with some typical results shown in Fig. 3. Accumulation in parental and MRP5-expressing HEK293 cells was assessed in the absence or presence of phytochemicals and MK571 and NPPB. In the presence of MRP5 inhibitors MK571 (25 μM) and NPPB (30 μM), the cellular accumulation of BCECF increased dramatically in MRP5-expressing cells. Similarly, the cellular accumulation of BCECF in HEK293/MRP5 cells and in PANC-1 and MiaPaCa-2 cells was significantly increased by curcumin in a concentration-dependent manner (Table 1). Curcumin, MK571 and NPPB had no effects on cellular accumulation of BCECF in parental HEK293 cells. Biochanin A and diindomethane at concentration of 50 μM had no effects on BCECF accumulation in either

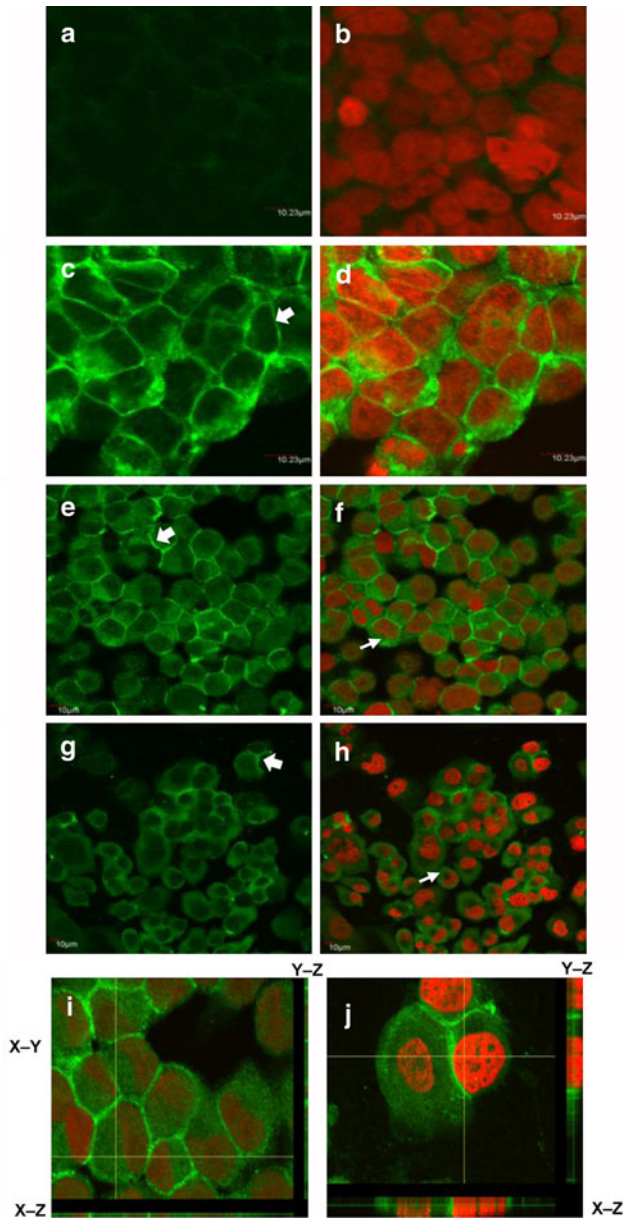

Fig. 2 Immunofluorescence detection of MRP5 in parental HEK293 (**a**, **b**, ×720 magnification) and MRP5 (**c**, **d**, ×720 magnification) transfected HEK293 cells and in pancreatic cancer MiaPaCa-2 (**e**, **f**, ×300 magnification) and PANC-1 (**g**, **h**, ×300 magnification) cells by confocal laser scanning microscopy. MRP5 was detected in cells by the primary monoclonal antibody M5I-1 in conjunction with a FITC-conjugated second antibody (*green*). Cell nuclei were stained with propidium iodide (*red*). MRP5 was detected at both plasma membrane (*thick arrow*) and in the intracellular compartments (*thin arrow*) in these cells. Detailed pictures of MiaPaCa-2 cells (i,×300 magnification) and PANC-1 cells (j, ×300 magnification) showing in the bottom and right sides optical X-Z and Y-Z sections perpendicular to the selected plane (*yellow lines* in the X-Y section image). Representative of three independent experiments

parental HEK293 or HEK293/MRP5 cells (Data not shown).

Cytotoxicity studies

The sensitivity of parental HEK293 and HEK293/MRP5 cells to 6-TG and 5-FU in the presence and absence of curcumin and a known MRP5 inhibitor NPPB was compared in a 3-day cytotoxicity assay. IC_{50} values of parental HEK293 and HEK293/MRP5 cells were determined and are presented in Table 2. Comparison of the IC_{50} values for parental HEK293 and HEK-MRP5 indicated that HEK-MRP5 is 8.9-fold resistant to 6-TG and 4.0-fold to 5-FU. Curcumin showed a concentration-dependant effect on sensitizing to both 6-TG and 5-FU, with the relative resistance factor values decreased to 5.7 and 4.7 for 6-TG, and 2.8 and 1.6 for 5-FU in the presence of curcumin 5 and 10 μM, respectively. Similarly, curcumin also potentiated the sensitivity to 5-FU in PANC-1 and MiaPaCa-2 cells in a concentration-dependant manner (Fig. 4). At 10 μM curcumin, the IC_{50} for 5-FU was reduced 24-fold from 12.0 ± 2.8 to 0.5 ± 0.1 μM for PANC-1 and fivefold from 3.5 ± 0.5 to 0.7 ± 0.1 μM for MiaPaCa-2 cells (Table 3). In addition, MRP5 appeared to confer resistance to curcumin as we found the IC_{50} value for HEK-MRP5 (121.6 ± 14.0 μM) was sixfold higher than that for parental HEK293 cells (20.1 ± 2.2 μM).

Discussion

Pancreatic cancer is infamous for its intrinsic resistance to various chemotherapeutics; 5-FU is an important drug to treat pancreatic cancer and other cancers [22, 23]. A recent study indicated that by knocking down MRP5 (a highly over-expressed ABC transporter in pancreatic cancer tissues [4]), the sensitivity to 5-FU in a pancreatic cancer cell line was significantly increased [6]. MRP5 has been reported to confer resistance to certain metallic salts, cadmium chloride and potassium antimonyl tartrate, as well as some anticancer agents, such as 5-FU, 6-TG, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), cisplatin, methotrexate and two novel compounds, raltitrexed (ZD1694) and GW1843 [19, 21, 24–26]. MK571, a potent MRP1, 2 and 4 inhibitor, appeared to inhibit MRP5 less potently [27] and may not be suitable to reverse MDR mediated by MRP5. In HEK293/MRP5 cells, NPPB has been reported to reverse 5-FU resistance at a concentration (40 μM) but also showed some toxicity to parent HEK293 cells [19]. Given the fact that many potent and less-toxic ABC transporter inhibitors have been developed from phytochemicals, we investigated some inhibitors of MRP5 from phytochemicals abundant in normal plant/food.

Immunocytochemistry confirmed that MRP5 was expressed in the transfected cell lines and the two pancreatic cancer cell lines used in this study. Interestingly, in

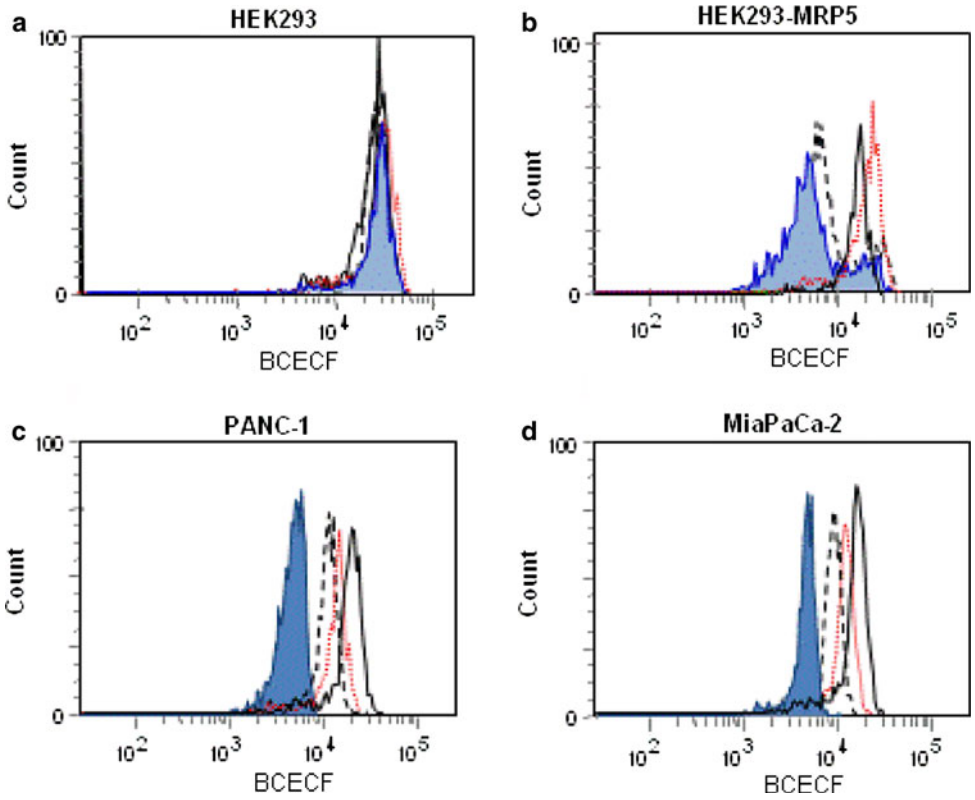

Fig. 3 Effects of 5 (*dashed line*) and 10 μM (*solid line*) curcumin, MRP5 inhibitor MK571 25 μM (*dotted line*) and vehicle (0.2% DMSO, *shadowed line*) on cellular accumulation of a specific MRP5 fluorescent substrate BCECF in parental (**a**) and MRP5-overexpressing HEK293 cells (**b**), and PANC-1 (**c**) and MiaPaCa-2 (**d**) cells. Fluorescence intensity was measured by flow cytometry with the excitation at 488 nm and the emission at 525 nm. The cells were gated based on forward and side scatter, and only viable cells were included in the analysis. The histogram derived from the FACSDiva software depicts fluorescence intensity (X *axis*) of cells, which is plotted as a function of cell number (Y *axis*)

Table 1 Effects of curcumin (5 and 10 μM), MRP5 inhibitors MK571 (25 μM) and NPPB (30 μM), and vehicle (0.2% DMSO, control) on cellular accumulation of a specific MRP5 fluorescent substrate BCECF in parental and MRP5-overexpressing HEK293 cells and in pancreatic cancer MiaPaCa-2 and PANC-1 cells

	HEK293 (%)	HEK293/MRP5 (%)	PANC-1 (%)	MiaPaCa-2 (%)
Control	100 ± 8	17 ± 4	24 ± 3	27 ± 4
Curcumin 5 μM	104 ± 9	33 ± 3[a]	35 ± 1[b]	47 ± 3[b]
Curcumin 10 μM	96 ± 11	63 ± 4[a]	64 ± 8[a]	65 ± 5[a]
MK571 25 μM	109 ± 7	99 ± 6[a]	61 ± 6[a]	55 ± 3[a]
NPPB 30 μM	112 ± 9	86 ± 7[a]	62 ± 7[a]	61 ± 4[a]

The geometric mean value of fluorescence intensity was measured by flow cytometry. Data are expressed as % of mean value determined in parental HEK293 cells pretreated with vehicle (mean ± SEM, $n = 3$–4)

[a] $P < 0.01$; [b] $P < 0.05$, both compared with control

Table 2 Effects of curcumin on drug sensitivity of parental HEK293 and HEK293/ MRP5 cells to 6-thioguanine (6-TG) and 5-fluorouracil (5-FU). IC_{50} values were determined from 3 to 4 experiments each performed in triplicate and expressed as mean ± SEM

	IC_{50} (μM)		Relative resistance
	Parental	MRP5	
Control (6-TG only)	2.8 ± 0.8	24.7 ± 2.5	8.9
6-TG + Curcumin (5 μM)	3.6 ± 1.0	20.4 ± 3.1	5.7
6-TG + Curcumin (10 μM)	2.5 ± 0.1	11.7 ± 1.6*	4.7
6-TG + NPPB (30 μM)	3.2 ± 0.2	9.7 ± 1.2*	3.0
Control (5-FU only)	4.5 ± 0.1	18.0 ± 2.0	4.0
5-FU + Curcumin (5 μM)	3.7 ± 0.3	10.2 ± 1.4*	2.8
5-FU + Curcumin (10 μM)	3.8 ± 0.4	5.9 ± 0.5*	1.6
5-FU + NPPB (30 μM)	4.1 ± 0.5	10.2 ± 1.4*	2.5

* $P < 0.05$, compared with control

addition to the predominant expression of MRP5 on the cell membrane, there was intracellular expression of MRP5. BCRP (ABCG2) has also been recently reported to be localized in mitochondria in several cancer cell lines, where it limits the mitochondrial uptake of mitoxantrone [28]. Localization of MRP5 inside an organelle in pancreatic cancer cells may have significant implication in determination of drug resistance and/or its own physiological functions. Additional work is required to further elucidate the related mechanisms and to better understand the physiological functions of MRP5.

Functional expression of MRP5 was observed using flow cytometry after loading with BCECF-AM ester, which demonstrated that MRP5 functions as a BCECF efflux pump as: (1) there was about fivefold lower accumulation of BCECF in HEK293/MRP5 cells than in parental HEK293 cells, and (2) two known MRP5 inhibitors (MK571 and NPPB) significantly increased the cellular accumulation of BCECF in HEK293/MRP5 cells, but not in parental HEK293 cells (Table 1 and Fig. 3). Our results also clearly demonstrated that curcumin inhibited the efflux

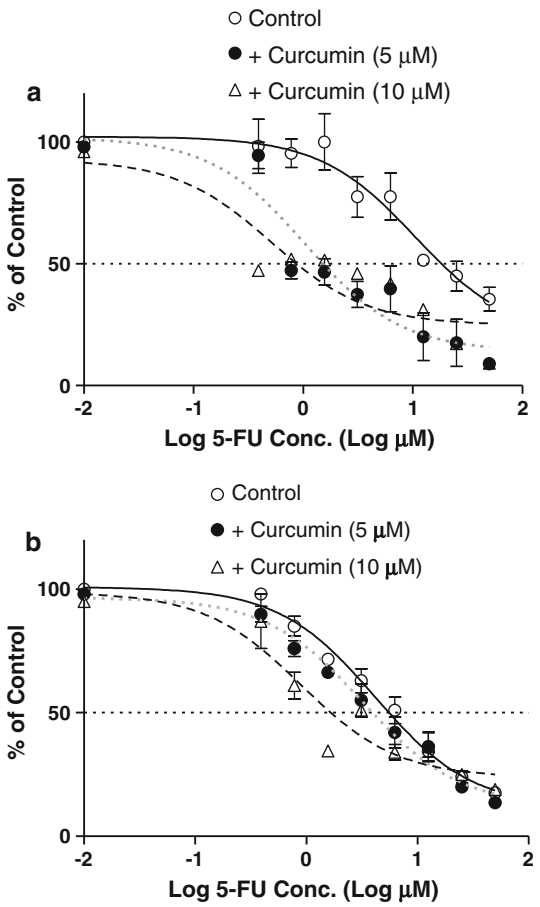

Fig. 4 Effects of 5 (*filled circles*) and 10 μM (*triangles*) curcumin, and vehicle (0.2% DMSO, *open circles*) on drug sensitivity of **a** PANC-1 and **b** MiaPaCa-2 cells to 5-FU

of BCECF mediated by MRP5, as shown by a dose-dependent increase in the accumulation of BCECF in MRP5/HEK293 but not in parental HEK293 cells. Although curcumin has been reported to alter membrane fluidity in erythrocytes [29], the lack of effect of curcumin on parental HEK293 cells ruled out the possibility that the increased accumulation of BCECF was due to enhanced

Table 3 Effects of curcumin on drug sensitivity of PANC-1 and MiaPaCa-2 cells to 5-fluorouracil (5-FU). IC_{50} values were determined from 3 experiments each performed in triplicate and expressed as mean ± SEM

	IC_{50} (μM)	
	PANC-1	MiaPaCa-2
Control (5-FU only)	12.0 ± 2.8	3.5 ± 0.5
5-FU + Curcumin (5 μM)	1.1 ± 0.2*	2.4 ± 0.7
5-FU + Curcumin (10 μM)	0.5 ± 0.1*	0.7 ± 0.1*
5-FU + NPPB (30 μM)	2.3 ± 0.6*	1.2 ± 0.2*

* $P < 0.05$, compared with control

permeation of BCECF-AM by membrane perturbations caused by curcumin. Therefore, we concluded that the observed increases in BCECF accumulation were MRP5-specific.

The 3-day curcumin cytotoxicity studies with HEK293 cells suggested that MRP5 may transport curcumin or influence cellular disposition of curcumin or its metabolites. A curcumin metabolite, tetrahydrocurcumin, has also been reported to inhibit BCRP and MRP1, suggesting that curcumin may be acting as a prodrug [13]. It is clear that additional work on the effect of curcumin metabolites is needed. Nontoxic concentrations of curcumin were chosen to investigate its potential in reversing MRP5-mediated 5-FU resistance in MRP5-overexpressing HEK293 cells and two pancreatic cancer cell lines. Curcumin also potentiated the cytotoxic effects of 6-TG, which is a well-known MRP5 probe [25]. Although MRP8 has been reported to confer resistance to 5-FU [30], its mRNA was not detected in the cell lines used (data not shown), which is consistent with a previous report [6] and rules out the possible contribution of MRP8. A recent report suggested that BCRP down-regulation could partially reverse 5-FU resistance in gastric cancer cells [31]. However, we found no differences in the sensitivity to 5-FU between parent and BCRP-overexpressing HEK293 cells, in which the latter showed ~threefold greater resistance to the BCRP substrate, mitoxantrone (data not shown). Thus the present study shows for the first time that curcumin can reverse MRP5-mediated resistance to the anticancer agent 5-FU. This, in combination with our flow cytometry results, strongly suggests that curcumin is a potent modulator of MRP5, superior to known synthetic modulators. A very recent study using both MRP5-overexpressing PANC-1 and MRP5-silenced PANC-1 cells indicated that MRP5 contributes to gemcitabine resistance [7]. While our results show the inhibitory effects of curcumin on MRP5 in PANC-1 and MiaPACa-2 cells, further study using MRP5-overexpressing PANC-1 cells may be required to better understand the effects of curcumin in reversal of MRP5-mediated 5-FU resistance in pancreatic cancer treatment.

Curcumin has shown pleiotropic effects on cancer cells including induction of apoptosis [32], inhibition of tumor cell proliferation [32], immunomodulatory effects [18] and reversal of tumor MDR [15–17]. Most of its biological effects have been associated with inhibition of nuclear factor κ-B (NF κ-B), which is also constitutively active in human pancreatic cancer [33]. Many mammalian ABC transporters are under tight transcriptional regulation by nuclear receptors via NF κ-B and other signalling pathways [34, 35], suggesting their functions are subject to environmental and dietary influences. Further studies are required to elucidate whether the modulatory effects of curcumin on MRP5 is also associated with the NF κ-B signalling pathway.

Given the apparent intrinsic antitumor effects of curcumin [36], it may represent a new type of chemosensitizing agent. However, the poor absorption and rapid metabolism resulting in low oral bioavailability continue to be a major problem with curcumin's use in the clinic [37]. To overcome this, the development of liposomal and nanoparticle formulations of curcumin has been investigated [38–40]. Such strategies to improve the bioavailability have also been recently shown to enhance curcumin distribution into pancreatic tumor tissue in a recent publication [17]. Steady-state plasma curcumin concentrations of more than 15 μg/mL (41 μM) and pancreas concentration of ~5 μg/g tissue (12 μM) were observed in mice after 4-week parenteral administration of a curcumin nanoparticle formulation. Pharmacokinetic profiles of curcumin nanoparticle formulations in humans remain unknown but parenteral administration of a curcumin nanoparticle formulation may provide much higher plasma and pancreas concentrations compared with free curcumin.

5-FU-based combination chemotherapy has been used to treat various cancers including pancreatic cancer [22]. For example, the levofolinate/5-FU combination enhances the effect of 5-FU in pancreatic cancer treatment by inhibiting thymidylate synthase, which is the enzyme involved into the DNA synthesis and repair in cancer and normal cells. However, the levofolinate/5-FU combination has been reported to be associated with some serious adverse events in pancreatic cancer patients [41], possibly due to their off-target effects. Our research indicates the possibility of overcoming 5-FU resistance by inhibitory effects of curcumin on MRP5-mediated drug efflux. Given the high expression of MRP5 in pancreatic cancers, the curcumin/5-FU combination may have more targeted effects especially when curcumin could be delivered as a nanoparticle formulation with significantly enhanced curcumin accumulation in the tumor tissue compared to free curcumin solutions due to an enhanced permeability and retention

effect [42]. However, a definitive evaluation of effects of curcumin in clinical 5-FU resistance may be complicated by pharmacokinetic and pharmacodynamic alterations, such as dihydropyrimidine dehydrogenase-mediated catabolism of 5-FU and thymidylate synthase activities [22]. In conclusion, our present results confirmed the contribution of functional expression of MRP5 in pancreatic cancer resistance against 5-FU and indicate the possibility of overcoming 5-FU resistance by inhibitory effects of curcumin on MRP5-mediated drug efflux.

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