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ORIGINAL ARTICLE

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Curcuminoids purified from turmeric powder modulate the function of human multidrug resistance protein 1 (ABCC1)

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Abstract Multidrug resistance is a major cause of chemotherapy failure in cancer patients. One of the resistance mechanisms is the overexpression of drug efflux pumps such as P-glycoprotein and multidrug resistance protein 1 (MRP1, (ABCC1)). In this study, curcumin mixture and three major curcuminoids purified from turmeric (curcumin I, II, and III) were tested for their ability to modulate the function of MRP1 using HEK293 cells stably transfected with MRP1-pcDNA3.1 and pcDNA3.1 vector alone. The IC_{50} of curcuminoids in these cell lines ranged from 14.5–39.3 μM. Upon treating the cells with etoposide in the presence of 10 μM curcuminoids, the sensitivity of etoposide was increased by several folds only in MRP1 expressing and not in pcDNA3.1-HEK 293 cells. Western blot analysis showed that the total cellular level of MRP1 protein level was not affected by treatment with 10 μM curcuminoids for three days. The modulatory effect of curcuminoids on MRP1 function was confirmed by the inhibition of efflux of two fluorescent substrates, calcein-AM and fluo4-AM. Although all the three curcuminoids increased the accumulation of fluorescent substrates in a concentration-dependent manner, curcumin I was the most effective inhibitor. In addition, curcuminoids did not affect 8-azido[α–^{32}P]ATP binding, however they did stimulate the basal ATPase activity and inhibited the quercetin-stimulated ATP hydrolysis of MRP1 indicating that these bioflavonoids interact most likely at the substrate-binding site(s). In summary, these results demonstrate that curcuminoids effectively inhibit MRP1-mediated transport and among curcuminoids, curcumin I, a major constituent of curcumin mixture, is the best modulator.

Keywords Curcumin I · Curcumin II · Curcumin III · Modulator · Multidrug resistance (MDR) · Multidrug resistance related protein (MRP1)

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Introduction

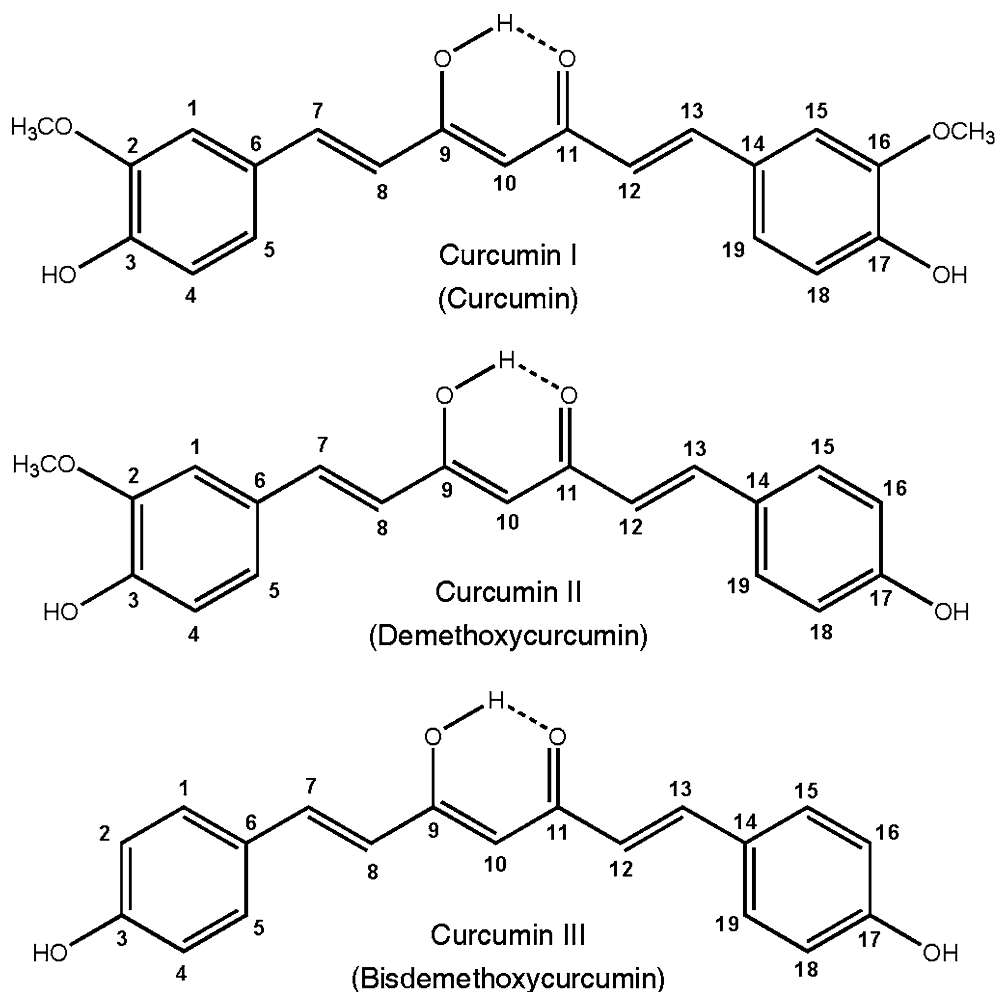
Drug resistance is a major impediment to the treatment of cancer patients receiving single or multiple drugs. Efforts to reverse drug resistance of tumor cells have been largely unsuccessful [25]. In recent years, considerable research has been directed toward understanding the underlying mechanisms that confer drug resistance. One of the major mechanisms of Multidrug resistance (MDR) is the enhanced ability of tumor cells to actively efflux drugs leading to a decrease in cellular drug accumulation below toxic levels [13]. Active drug efflux is mediated by several members of the ATP-binding cassette (ABC) superfamily. The first of ABC transporter to be identified and characterized was the 170 kDa P-glycoprotein (P-gp) [1, 2] and then Multidrug Resistance Protein 1 (MRP1) was discovered in 1992 [10]. In contrast to P-gp, MRP1 is primarily an active transporter of GSH, oxidized GSH, glucuronate and sulfate conjugated organic anions [6, 11]. Many chemicals are known to inhibit MRP1-mediated transport, such as MK571, ONO-178 and glibenclamide, however mostly specific inhibitors are still under development [9].

Turmeric belongs to the *Zingiberaceae* family and is distributed throughout tropical and subtropical regions around the world. It is widely used as a food flavoring and coloring agent (e.g., in curry) as well as in tradi-

tional medicinal applications. Three main curcuminoids have been isolated from turmeric: curcumin I (diferuloyl methane), curcumin II (demethoxycurcumin) and curcumin III (bisdemethoxycurcumin) [22]. Their chemical structures are illustrated in Fig. 1. All three impart the hallmark yellow pigmentation to the *Curcuma longa* plant and particularly to its rhizome. Ongoing experimental and clinical studies indicate that turmeric and its curcuminoid components exhibit unique antioxidant [4], anti-inflammatory [17] and antitumorigenesis properties [19]. Their potential use in the prevention of cancer and in the treatment of human immunodeficiency virus (HIV) infection is also a subject of intensive research [3].

The inhibitory effects of a mixture of curcumins I, II, and III on MRP1-mediated transport using isolated membrane vesicles of MRP1 expressing Sf9 cells were recently reported [26]. However, the mechanism of inhibition remains unknown. Moreover, it is unknown whether each curcumin form in the curcumin mixture exhibits the same effect. Previously, we reported that among the three major forms of the curcuminoids, curcumin I is the most active in modulating the function of P-gp by binding directly to the transporter and perhaps to the same binding site as verapamil or prazosin [8]. In the present study, we compare the effects of the individual curcumins I–III and the curcumin mixture with regard to their ability to inhibit MRP1 function. Moreover, we probe the mechanism to clarify the nature of the modulatory effects of the curcuminoids on MRP1. The results demonstrate that curcumins I, II, and III increase the sensitivity of etoposide in MRP1-transfected cells, and that curcumin I is the most effective of these three major curcuminoids. However, curcuminoid treatment led to no significant change in the MRP1 protein level. The inhibitory effect of the curcuminoids on MRP1 function is supported by the ability of the curcuminoids to enhance the accumulation of calcein-AM and fluo4-AM in MRP1-transfected cells. In addition, we found that purified curcuminoids I–III and the mixture stimulate basal ATPase activity and inhibit quercetin-stimulated ATP hydrolysis by MRP1, demonstrating the interaction of curcuminoids most likely at

Fig. 1 Chemical structure of the curcuminoids. The three major curcuminoid purified from the turmeric powder are curcumin I (curcumin or diferuloyl methane), curcumin II (demethoxycurcumin or p-hydroxy-cinnamoyl-feruoyl methane) and curcumin III (bisdemethoxycurcumin or pp′-dihydroxy-dicinnamoyl-methane). These curcuminoids exist in the -enol rather than the keto- form as indicated by their spectroscopic data detailed in the Results section [7]



the substrate-binding site(s) on this multidrug transporter.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), Iscove's Modified Eagle's Medium (IMDM), penicillin, streptomycin, trypsin-EDTA, Hank Balance Salt solution (HBSS) and PBS (Dulbecco's Phosphate Buffered Saline) were purchased from GIBCO-BRL. Fetal bovine serum was purchased from HyClone. Bovine Serum Albumin (BSA) was purchased from Promega. Curcumin mixture, MTT dye, etoposide, MES (2-[*N*-morpholino] ethanesulfonic acid and ouabain were purchased from Sigma-Aldrich. Calcein-AM and fluo4-AM were obtained from Molecular Probes, Inc. [$\alpha-^{32}P$] 8-azido ATP (15–20 Ci/mmole) and 8-azido ATP were obtained from Affinity Labeling Technologies, Inc. (Lexington, KY).

Extraction and isolation of curcuminoids

Curcuminoids were isolated and purified as described previously [20]. Briefly, turmeric powder was successively extracted with 95% ethanol at room temperature, then precipitated with petroleum ether to yield crude curcumin mixture consisted of 78% curcumin I, 16% curcumin II, and 5% curcumin III. The crude mixture was then fractionated by silica gel 60 column chromatography using first $CHCl_3$ and then $CHCl_3$/methanol with increasing polarity to isolate pure fractions of curcumins I, II, and III. The identity and purity of each curcuminoids were verified using by TLC, HPLC, IR, MS, and NMR analysis. The purity of curcumin I, II, and III by TLC and HPLC analysis was described previously [8]. In the present study, IR spectra were collected of samples in KBr pellets using a Nicolet FITR spectrometer. Mass spectral (MS) data were collected using a quadupole via electron impact ionization. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 500 spectrometer using DMSO-it d_6 as the solvent.

Cell lines and culture conditions

The characterization of MRP1-transfected HEK293 cells has been described previously [21]. The cell lines MRP1- and pcDNA3.1-HEK293 were maintained in DMEM supplemented with 4.5 g of glucose per liter plus 10% fetal calf serum, L-glutamine (5 mM), penicillin (50 units/ml), streptomycin (50 units/ml) and G418 (800 μg/ml). Etoposide (5 μM) was added only to the MRP1-HEK 293 cell-culture medium. The cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C [21].

MTT assay

Growth inhibition (IC_{50}) assays were performed by plating pcDNA3.1- and MRP1-HEK293 cells at density 3.0×10^3 cells/well in 96 well plates. After 24 h, curcumin I, II, III and curcumin mixture stocks prepared in DMSO were added to the culture medium and incubated in a humidified tissue-culture chamber (37°C, 5% CO_2) for another 72 h. The DMSO concentration was kept at 0.1%. Surviving cells were detected by the bromo-3(4,5-dimethyl-2-thiazoyl)2,5-diphenyltetrazolium (MTT) assay. The medium was aspirated first, followed by adding 100 μl of MTT solution to each well and incubated for 4 h at 37°C. Thereafter, the reaction was terminated by aspirating off the MTT dye and then 200 μl isopropanol was added to each well. Presence of MTT-metabolite formazan in the resulting solution was photometrically measured at wavelength of 570 nm with a reference wavelength of 650 nm. IC_{50} values were calculated from dose response curves obtained from three-triplicate experiments [8].

Effect of curcuminoids on MRP1-mediated etoposide resistance

Cytotoxicity of etoposide in pcDNA3.1- and MRP1-HEK293 cells in the absence and presence of 10 μM of curcumin mixture, or purified curcuminoids I, II, III and 10 nM of vinblastine (used as a positive control) were determined by MTT assay. Briefly, the cells (3.0×10^3 cells) were seeded into 96 well plates and cultured overnight. Various concentrations of etoposide with 10 μM of curcuminoids or 10 nM of vinblastine were then added and incubated for an additional 72 h. After incubation, the medium was aspirated, then 100 μl of MTT solution was added to each well and further incubated for 4 h. The reaction was terminated by adding 200 μl of isopropanol. Absorbance was measured using plate reader at 570 nm. The fractional absorbance was calculated by the following formula: % Cell survival = (mean absorbance in test well)/(mean absorbance in control wells)×100 as previously described [8].

Effect of curcuminoids on MRP1 expression

MRP1-and pcDNA3.1-HEK 293 cells (3×10^6) were plated in T-75 cm^2 culture flasks and cultured overnight. The following day curcumin mixture, curcumin I, II and III at 5 and 10 μM were added and cells were further incubated for 3 days. After a 72 h incubation, cells were harvested and the cell lysates were prepared as described by Mûller et al.21, with minor modifications. The expression of MRP1 was determined by immunoblotting with a specific antibody, MRPm6. Briefly, the cell lysates were separated by electrophoresis on NuPAGE gel (7% w/v) and the

proteins were electroblotted on to nitrocellulose membrane. Immunoblotting was performed by using MRPm6 (1:4000) antibody and the anti-mouse IgG-horseradish peroxidase (HRP) conjugated (1:10000) secondary antibody. HRP-dependent luminescence was developed using Western lighting chemiluminescence reagent plus (PerkinElmer life science).

Effect of curcuminoids on MRP1 fluorescent substrate accumulation detected by Fluorescence Activated Cell Sorter (FACS)

Two fluorescent substrates of MRP1, calcein-AM and fluo4-AM, were used in this study. The accumulation of the two fluorescent substrates in pcDNA3.1- and MRP1-HEK 293 were conducted as previously described with minor modifications [8, 21]. Briefly, cells were trypsinized and harvested by centrifugation at 500×g and re-suspended in IMDM supplemented with 5% FBS. Calcein-AM (0.5 μM) or fluo-4 AM (0.5 μM) was then added to 5×10^5 cells in 4 ml of IMDM in the presence or absence of indicated agents, MK-571 (20 μM) or curcuminoids (20 μM). The cells were incubated in water bath at 37°C in dark. After incubation for 45 min with fluo4-AM 10 min with calcein-AM, the cells were pelleted by centrifugation at 500×g. The pellet was re-suspended in 300 μl of PBS containing 0.1% BSA and analyzed immediately by using flow cytometer.

Preparation of crude membranes from High Five insect cells infected with recombinant baculovirus carrying the human MRP1 cDNA

High Five insect cells (Invitrogen, Carlsbad, CA) were infected with the recombinant baculovirus carrying the human *MRP*1 cDNA with a 10 Histidine tag at the C-terminal end (BV-MRP1 (His 10). Crude membranes were prepared as described previously [18] and used in all experiments. Total protein was estimated using the Amido Black protein method of Schaffner and Weissmann [24] with bovine serum albumin (BSA) as a standard.

ATPase activity of MRP1

ATPase activity of MRP1 in crude membranes of High Five insect cells infected with baculovirus-MRP1 was measured by the endpoint, $P_i$release assay as previously described [23]. Membranes (100 μg/ml) were incubated at 37°C with curcuminoids in the presence or absence of 2.5 mM sodium fluoride and 0.2 mM beryllium sulfate (to form beryllium fluoride, BeFx) for 5 min. The reaction was initiated by the addition of 5 mM ATP and terminated with SDS (2.5% final concentration); the amount of P_i released was quantified using a colorimetric method [18, 23]. MRP1-specific activity was recorded as the BeFx-sensitive ATPase activity.

Photocrosslinking of MRP1 with 8-azido [α−^{32}P]-ATP

Crude membranes of MRP1 expressing High Five insect cells (50 μg protein per assay) were incubated in the ATPase assay buffer containing 10 μM 8-azido[α−^{32}P]ATP (8–10 μCi/nmole) in the dark on ice for 5 min in the presence or absence of 20 μM curcumin mixture or purified curcumin I, II or III. The samples were then illuminated with a UV lamp assembly (PGC Scientifics, Gaithersburg, MD) fitted with two Black light (self-filtering) UV-A long wave-F15T8BLB tubes (365 nm) for 10 min on ice. Ice-cold ATP (10 mM) was added to displace excess non-covalently bound radionucleotide. Following electrophoresis on a 7% NuPAGE gel (Invitrogen, Carlsbad, California) at constant voltage, gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at −70°C for 6–24 h. The radioactivity incorporated into the MRP1 band was quantified using a STORM 860 phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and the software ImageQuaNT [23].

Statistical analysis

Data are the means ± Standard error (SE) from at least three independent experiments. Differences between the means were analyzed by one-way analysis of variance. Results were considered to be statistically significant when $P < 0.05$.

Results

Determination of purity of isolated curcumin I, II, and III

The commercially available curcumin mixture (usually labeled as curcumin) contains three major curcuminoids. To compare the modulatory effect of each form of curcuminoids in curcumin mixture, we isolated curcumin I, II, and III from turmeric powder by extraction with 95% ethanol followed by silica gel 60 chromatography as described [8]. We used TLC, HPLC, IR, MS and NMR to verify the identity and determine the purity of curcumin I, II, and III. The TLC and HPLC profiles of each curcuminoid were described previously [8]. In the present study, data from MS, NMR and IR analyses for curcumin I, II and III are also provided in Table. 1, 2, 3 respectively, demonstrating high purity (99%) and accurate identification of all samples. In particular, the data reported here are consistent with published spectroscopic analyses of curcumin I, II, and III [7]. The structures and numbering scheme used in the ^{1}H NMR data are given in Fig. 1.

Table 1 NMR, IR, and MS data for Curcumin I

Chemical Shift (ppm)	Multiplicity	Integration ratio	Assignment	Coupling constant (Hz)
^{1}H NMR[a] assignments				
9.69	s	2	3,17-OH	
7.54	d	2	7,13-H	$J_{7-8} = J_{12-13} = 16$
7.32	d	2	1,15-H	$J_{1-5} = J_{15-19} = 2$
7.15	dd	2	5,19-H	$J_{4-5} = J_{18-19} = 8$
6.82	d	2	4,18-H	$J_{4-5} = J_{18-19} = 8$
6.76	d	2	8,12-H	$J_{7-8} = J_{12-13} = 16$
6.06	s	1	10-H, enol form	
3.83	s	6	2,16-OCH_3	

^{13}C chemical shifts[b] (ppm): 183.7, 149.8, 148.5, 141.2, 126.8, 123.6, 121.6, 116.2, 111.8, 101.3, 56.2IR absorption bands (cm^{-1}): 3523 (O–H str); 1624 (conjugated C=C str); 1609 (α,β-unsaturated C=O str); 1510 (aromatic ring str); 1268 and 1050 (=C–O–CH_3 str); 1140 (C–OH str)MS (*m*/*z*, rel int): 391.12 (100%, M^{+} + Na); 333.06 (56%)[a] ^{1}H NMR spectra were collected in DMSO-it d_6 at 500 MHz[b] ^{13}C NMR spectra were collected in DMSO-it d_6 at 125.8 MHz

Table 2 NMR, IR, and MS data for Curcumin II

Chemical shift (ppm)	Multiplicity	Integration ratio	Assignment	Coupling constant (Hz)
^{1}H NMR[a] assignments				
10.07	s	1	17-OH	
9.68	s	1	3-OH	
7.58	d	2	15,19-H	$J_{15-16} = J_{18-19} = 9$
7.54	d	1	7-H	$J_{7-8} = 16$
7.53	d	1	13-H	$J_{12-13} = 16$
7.32	d	1	1-H	$J_{1-5} = 2$
7.14	dd	1	5-H	$J_{4-5} = 9$, $J_{1-5} = 2$
6.82	m	3	4,16,18-H	$J_{4-5} = J_{15-16} = J_{18-19} = 9$
6.76	d	1	12–H	$J_{12-13} = 16$
6.69	d	1	8-H	$J_{7-8} = 16$
6.04	s	1	10-H, enol form	
3.83	s	3		

2-OCH_3^{13}C chemical shifts[b] (ppm): 183.7, 183.6, 160.3, 149.8, 148.5, 141.2, 140.8, 130.8, 126.8, 126.3, 123.7, 121.5, 121.3, 116.4, 116.1, 111.7, 101.4, 56.2IR absorption bands (cm^{-1}): 3328 (O–H str); 1632 (conjugated C=C str); 1582 (α,β-unsaturated C=O str); 1511 (aromatic ring str); 1262 and 1060 (=C–O–CH_3 str); 1140 (C–OH str)MS (*m*/*z*, rel int): 361.11 (100%, M^{+} + Na); 355.05 (25%); 333.06 (54%)[a] ^{1}H NMR spectra were collected in DMSO-it d_6 at 500 MHz[b] ^{13}C NMR spectra were collected in DMSO-it d_6 at 125.8 MHz

Table 3 NMR, IR, and MS data for Curcumin III

Chemical shift (ppm)	Multiplicity	Integration ratio	Assignment	Coupling constant (Hz)
^{1}H NMR[a] assignments				
10.07	s	2	3,17-OH	
7.58	d	4	1,5,15,19-H	$J_{1-2} = J_{4-5} = J_{15-16} = J_{18-19} = 9$
7.54	d	2	7,13-H	$J_{7-8} = J_{12-13} = 16$
6.82	d	4	2,4,16,18-H	$J_{1-2} = J_{4-5} = J_{15-16} = J_{18-19} = 9$
6.70	d	2	8,12-H	$J_{7-8} = J_{12-13} = 16$
6.04	s	1	10-H, enol form	

^{13}C chemical shifts[b] (ppm): 183.7, 160.3, 140.8, 130.8, 126.3, 121.3, 116.4, 101.4IR absorption bands (cm^{-1}): 3324 (O–H str); 1624 (conjugated C=C str); 1595 (α,β-unsaturated C=O str); 1510 (aromatic ring str); 1133 (C–OH str)MS (*m*/*z*, rel int): 333.06 (100%, M^{+} + Na); 332.10 (19%); 331.09 (90%)[a] ^{1}H NMR spectra were collected in DMSO-it d_6 at 500 MHz[b] ^{13}C NMR spectra were collected in DMSO-it d_6 at 125.8 MHz

Curcuminoids may not be substrates for MRP1

Sensitivity of parental pcDNA3.1-HEK293 cells and MRP1-overexpressing MRP1-HEK293 cells was determined by exposing cells to curcuminoids at various concentrations up to 50 μM for 72 h. The IC_{50} values of curcuminoids in these cell lines were in the range of 14.5–39 μM. The curcumin mixture and curcumin I and II were more toxic compared to curcumin III in both control and MRP1 expressing cells (IC_{50}in the range of 14.5–20 and 34–40 μM, respectively). We found that there was no significant difference between the IC_{50} in pcDNA3.1 control and MRP1-HEK293 cells (Fig. 2, Table 4) for curcumin mixture or isolated curcuminoids I, II and III. Therefore, similar to P-gp, we suggest that curcuminoids are probably not MRP1 substrates.

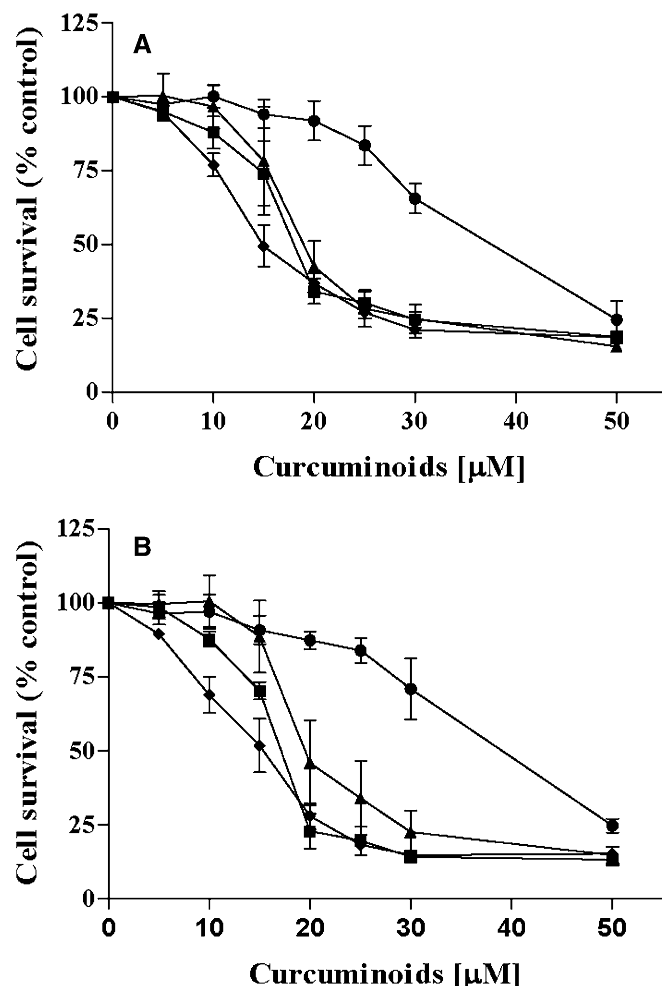


Fig. 2 Cytotoxicity of purified curcuminoids and curcumin mixture in pcDNA3.1 and MRP1-HEK 293 cells. Standard 72 h incubation MTT assay was used to investigate how curcuminoids affect the viability of the pcDNA3.1 control vector- and MRP1-transfected cells. **a** Empty vector (pcDNA3.1)-transfected HEK 293 cells and **b** MRP1-transfected HEK 293 cells. Various concentrations of the curcuminoids; curcumin mixture (*diamond*), curcumin I (*square*), curcumin II (*triangle*) and curcumin III (*circle*) were exposed to the cells for 72 h and cytotoxicity was determined by MTT assay. The IC_{50} values were calculated from dose response curves obtained from three independent experiments. The mean values from three independent experiments performed in triplicate are plotted and the error bars represent standard error (SE)

However, as expected, only the MRP1-expressing HEK293 cells were resistant to etoposide and vinblastine (relative resistance factor 80.7 and 25.64, respectively; Table 4).

Curcuminoids reversed the resistance to etoposide of MRP1–HEK293 cells

To assess the modulatory effect of curcuminoids, we used non-toxic concentrations of curcumin mixture and curcumin I, II, III as determined from the experiment shown in Fig. 2. Given that the concentrations lower than 10 µM produced minimal effects on HEK293 cells (Fig. 2), concentrations of 5 µM and 10 µM were selected to determine the ability of curcuminoids to sensitize MRP1 and pcDNA3.1-HEK 293 cells to the cytotoxic effects of etoposide. The cells were treated with increasing concentrations of etoposide in the presence of 5 and 10 µM of curcuminoids for 3 days. As a positive control, we tested also the effect of 5 and 10 nM of vinblastine on the cytotoxicity of etoposide. These data are given in Fig. 3a, b and c. The IC_{50} values and relative resistance factors determined from data in Fig. 3 are summarized in Table 5. The results indicated that curcuminoids did not have any effect on the cytotoxicity of etoposide in control pcDNA3.1-transfected HEK293 cells. On the other hand, the MRP1-HEK293's sensitivity toward etoposide was significantly enhanced in the presence of curcuminoids and vinblastine; in addition, curcumin I was the most effective component among the three forms of curcuminoids. The relative resistance to etoposide was lowered by 14- to 20-fold in the presence of 10 µM curcumin mixture and curcumin I (Table 5). Both curcumin II and III were considerably less effective in reversing the resistance of MRP1-HEK293 cells to etoposide.

Curcuminoids have no effect on MRP1 expression levels in MRP1-HEK 293 cells

It is possible that the reversal of resistance to etoposide by curcuminoids in MRP1-HEK293 cells was caused by

Table 4 Intrinsic cytotoxicity of curcuminoids, etoposide and vinblastine in pcDNA3.1 and MRP1-HEK 293 cells

Agent	Cytotoxicity (IC_{50})[a]		
	pcDNA3.1-HEK 293 cells	MRP1-HEK 293 cells	RR[b]
Etoposide alone (µM)	0.9 ± 0.11	72.67 ± 1.67	80.7
Curcumin mixture (µM)	15.37 ± 1.95	14.50 ± 1.95	0.94
Curcumin I (µM)	17.17 ± 1.09	16.67 ± 0.33	0.97
Curcumin II (µM)	17.83 ± 1.74	20.33 ± 2.83	1.14
Curcumin III (µM)	34.16 ± 2.21	39.33 ± 1.20	1.15
Vinblastine (nM)	5.33 ± 0.88	136.67 ± 8.80	25.64

[a] IC_{50} (concentration required for killing 50% cells) values were calculated from each experiment run in triplicates and the values represent the mean ± SE of three independent experiments

[b] The relative resistance was derived from the IC_{50}ratio of the compounds in MRP1-HEK 293 cells to pcDNA3.1-HEK 293 cells indicating the degree of resistance of MRP1-HEK 293 cells toward a particular drug or curcuminoid

Table 5 Reversal of MRP1-mediated resistance to etoposide with non-toxic concentrations of curcuminoids or 10 nM vinblastine

Drug and/or curcuminoid	Cytotoxicity of etoposide, IC_{50} (μM)[a]		
	pcDNA3.1-HEK 293 cells	MRP1-HEK 293 cells	RR[b]
Etoposide alone	0.9 ± 0.12	72.67 ± 1.67	80.74
+ Curcumin mixture			
5 μM	ND	22.50 ± 2.50	28.80*
10 μM	0.78 ± 0.12	4.45 ± 0.05	5.70*
+ Curcumin I			
5 μM	ND	20.25 ± 0.20	24.60*
10 μM	0.82 ± 0.08	3.25 ± 0.75	3.96*
+ Curcumin II			
5 μM	ND	62.50 ± 18.90	75.30
10 μM	0.83 ± 0.15	34.33 ± 5.48	41.36*
+ Curcumin III			
5 μM	ND	67.00 ± 7.00	77.00
10 μM	0.87 ± 0.15	43.88 ± 10.76	50.40*
+ Vinblastine			
5 nM	ND	57.50 ± 6.29	53.73*
10 nM	1.07 ± 0.24	18.33 ± 4.81	17.10*

[a] The data represent the mean values ± SE of three independent experiments performed in triplicate

[b] Relative resistance values were obtained by dividing the IC_{50} value of the MRP1-HEK 293 cells by the IC_{50} value of the empty vector transfected cell line

* Asterisks denote values that were significantly different from the etoposide control ($P < 0.05$)

ND Not determined

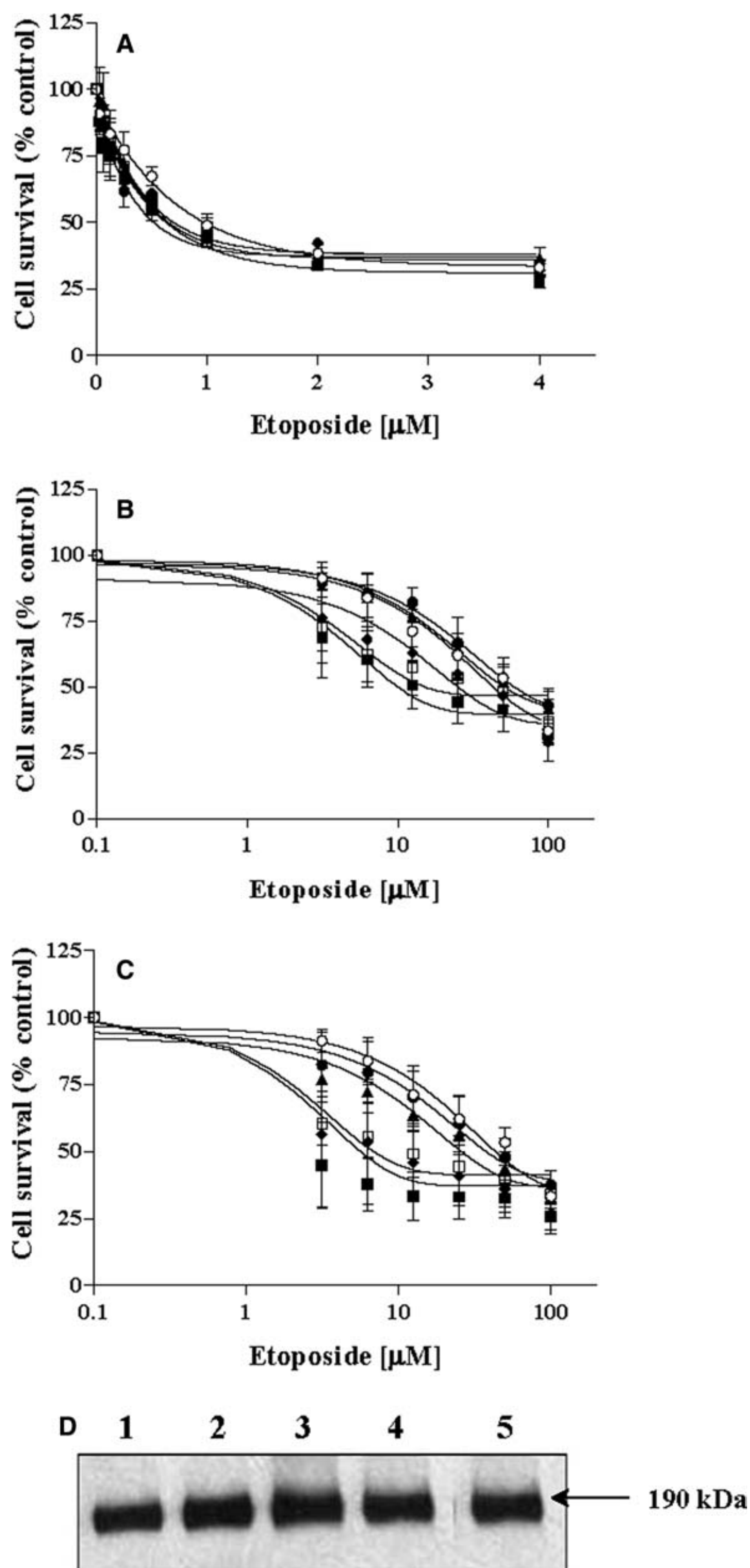
decrease in MRP1 protein expression in the presence of curcuminoids. To address this issue, we determined whether the curcuminoids are able to reduce the expression level of MRP1 in HEK293 cells, where the *MRP*1 gene is under CMV promoter instead of its native one. The MRP1-HEK293 cells were treated with 10 μM of the curcuminoids for 3 days and the MRP1 protein level was detected by Western blot analysis using monoclonal antibody to MRP1, MRPm6. It was found that the protein level of MRP1 in curcuminoid- treated cells was similar to the DMSO-treated control MRP1 cells (Fig. 3d), indicating that curcumin I, II and III as well as a mixture form has no effect on the expression level of MRP1 protein. Thus, the observed effects (reversal of resistance, Fig. 3 and Table 5) are indeed due to the inhibition of the function of MRP1.

Curcuminoids increase the accumulation of calcein -AM and fluo4-AM in MRP1-HEK 293 cells but not in pcDNA3.1-HEK 293 cells

The effect of curcuminoids on the accumulation of MRP1-fluorescent substrates was tested to characterize the inhibitory effect on MRP1-mediated transport using flow cytometry. The observed effects in MTT assay on cell killing could be due to accumulation of metabolites of curcuminoids rather than curcuminoids themselves. To rule out this possibility, short-term (10 and 45 min) transport assays were used. The MRP1-transfected MRP1-HEK293 cells and the control pcDNA3.1-HEK293 cells (5×10^5 cells) were incubated with non-fluorescent precursors; fluo4-AM or calcein-AM and the intensity of fluorescence of accumulated substrate was analyzed by FACS. The results are shown in Fig. 4. Consistent with the MDR reversing property, curcuminoids increased the accumulation of the fluorescent substrates in MRP1-transfected cells in a concentration-dependent manner (for clarity, data with only 20 μM curcuminoids are shown in Fig. 4) and among curcuminoids, curcumin I showed the greatest inhibitory effect, which was comparable to curcumin mixture. The concentrations required for 50% increase in accumulation of fluo4-AM or calcein-AM of curcumin mixture, curcumin I, II and III were 10–12.5, 10–12.5, 20–25 and 30–35 μM, respectively (data not shown).

Curcuminoids stimulate ATP hydrolysis of MRP1

The results described above demonstrate that curcuminoids inhibit the transport function of MRP1. To assess whether curcuminoids interact directly with the transporter, the effect of curcuminoids on ATPase activity of MRP1 was investigated. The crude membranes prepared from MRP1 baculovirus-infected High Five insect cells were used in this study. The inhibitory effect of BeFx on MRP1 ATPase activity was found to be higher than that of sodium orthovanadate (data not shown) and for this reason, we used BeFx to determine the MRP1-specific activity. As shown in Fig. 5a, curcumin mixture, I, II and III had similar effects on MRP1 ATP hydrolysis as to P-gp [8]. All the three forms were able to stimulate the basal ATPase activity of MRP1 at low concentrations but inhibited the activity at higher concentrations, indicating the interactions between the curcuminoids and MRP1. However, it should be noted that the extent of stimulation is considerably low at 15–45%, which is



consistent with the extent of stimulation by various substrates/modulators reported by other workers (see review Ref. [15]). By comparing all the three curcuminoids at the same concentration (1.5 μM), curcumin I

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Fig. 3 Purified curcuminoids and curcumin mixture increase the drug sensitivity in MRP1-transfected HEK293 cells but do not affect the expression levels of MRP1 protein. The curcuminoids were tested for their ability to reverse the sensitivity of (**a**) pcDNA3.1 3.1 - and (**b**,**c**) MRP1-HEK 293 cells to etoposide. The cells were grown in 5 μM (**b**), 10 μM (**c**) curcuminoids or 10 nM vinblastine in the presence of indicated concentration of etoposide. The number of viable cells after 72 h incubation was determined by MTT assay. The means of three independent experiments performed in triplicate are plotted and the *error bars* denote standard error (SE). Etoposide (*open circle*), curcumin mixture (*filled diamond*), curcumin I (*open square*), curcumin II (*triangle*) curcumin III (*filled circle*), and vinblastine (*filed square*). **d** Curcumin I, II, III and curcumin mixture had no effect on the MRP1 protein-expression level. MRP1-HEK 293 cells (3×10^6 cells) were treated for three days with DMSO (vehicle control) and 10 μM of the curcuminoids at 37°C; then the SDS-PAGE samples were prepared as described previously [21]. The samples were separated by electrophoresis on NuPAGE gel (7% w/v) and the protein were electroblotted on nitrocellulose membrane. Immunoblotting was performed using MRPm6 (1:4000) as first antibody and anti-mouse horseradish peroxidase (HRP) conjugate (1:10000) as the secondary antibody. HRP-dependent luminescence was developed using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Science) and detected with a Lumino Imagine Analyzer FAS-1000 (Toyobo). The arrow shows the position of MRP1 with a predicted molecular weight about 180–190 kDa (similar results were obtained when MRPr1 instead of MRPm6 was used for immunoblotting; data not shown). Lane 1, curcumin mixture; lane 2, curcumin I; lane 3, curcumin II; lane 4, curcumin III and lane 5, control (untreated)

showed the highest stimulation of approximately 45% (Table 6). It is shown that bioflavonoid quercetin markedly stimulates MRP1-mediated ATP hydrolysis (data not shown). Curcumin mixture, I, II and III were able to inhibit quercetin-stimulated MRP1-mediated ATP hydrolysis (see Fig. 5B) in a concentration-dependent manner.

Effect of curcuminoids on photoaffinity labeling of MRP1 by [α−^{32}P] 8-azido ATP

To determine the possible sites of interaction of curcuminoids with MRP1, photoaffinity labeling of MRP1 was performed using 8-azido [α−^{32}P]-ATP. Crude membranes were incubated on ice with 10 μM 8-Azido [α−^{32}P]-ATP (10 μCi/nanomole) in the presence of DMSO or 20 μM curcumin mixture, curcumin I, II, or III and photocrosslinked using 365 nm UV light (see Experimental Procedures for details). Curcumin mixture, I, II and III had no effect on 8-azido [α−^{32}P]-ATP labeling of MRP1 at 20 μM (Fig. 6) at a concentration where they affected both basal and quercetin-stimulated ATPase activity (Fig. 5). This lack of effect on nucleotide binding suggests that curcuminoids produce their effects most likely by interacting at the substrates binding site(s) rather than at the nucleotide-binding sites. The photocrosslinking with 8-azido [α−^{32}P]-ATP was inhibited by the presence of excess (10 mM) ATP as was to be expected for nucleotide binding to ATP sites.

Discussion

Turmeric is a perennial herb widely cultivated in the tropical regions of Asia. It contains about 1–5% curcuminoids. The major forms of curcuminoids found in Turmeric are curcumin I, II and III. The commercially available curcumin mixture (labeled as curcumin, for example, Sigma Cat# C1386) contains approximately 70–77%, 12–17% and 3–5% of curcumin I, II, and III, respectively [3, 14]. Curcuminoids modulate a variety of biological activities as outlined in the Introduction section. Previously, curcuminoids have been considered as the P-gp reversing agents and were demonstrated clearly that they inhibit both the expression and the function of P-gp [8, 20]. In the present study, we hence investigated the possible modulatory effect of curcuminoids on MRP1 at the functional levels. Furthermore, to determine which form of curcuminoid has the most prominent effect on MRP1-mediated transport, purified curcumin I, II and III were used. The structure and purity of isolated curcuminoids were determined by IR, MS and NMR spectroscopy. The structure of curcuminoids as confirmed by NMR are shown in Fig. 1 and the purity of each compound was found to be >99%.

Initially, MTT assays were used to determine the relative cytotoxicities of the curcuminoids in MRP1-HEK293 and pcDNA3.1-HEK293 cells. Curcuminoids have different IC_{50} values in the range of 14.5–39.33 μM as shown in Fig. 2a (pcDNA3.1-HEK293) and Fig. 2b (MRP1-HEK293 cells). Results indicated that curcuminoids might not be MRP1 substrate since the IC_{50} values were almost identical in both parental and MRP1-transfected cells. Additional experiments using radiolabeled or curcuminoid conjugated with a fluorescent probe will have to be carried out to resolve this issue. Concentrations with minimal toxicities then were chosen to investigate using curcuminoids as chemosensitizers. Curcuminoids at 5 μM and 10 μM substantially enhanced the sensitivity toward etoposide of MRP1 transfected cells, while they had no effect in control cells (Fig. 2, Table 5). Curcumin I showed the most significant potentiation effect while the rest of the curcumins had mild to strong reversing effect. It is likely that curcuminoids either directly inhibited MRP1-mediated etoposide efflux in MRP1-transfected cells or they reduced the expression level of MRP1 protein, thus increasing the etoposide sensitivity. The later was found not to be the case since curcuminoids had no effect on MRP1 protein level in MRP1-HEK293 cells (Fig. 3d). This suggested that the potentiation effect of curcuminoids is by interfering with the function of MRP1. The direct inhibition of curcuminoids on MRP1-mediated transport was confirmed by flow cytometry studies where they increased the accumulation of MRP1 substrates calcein-AM and fluo4-AM in a concentration-dependent manner. The potency of curcumin I was comparable to MK-571, which is known to inhibit MRP1-mediated transport with high affinity (Fig. 4).

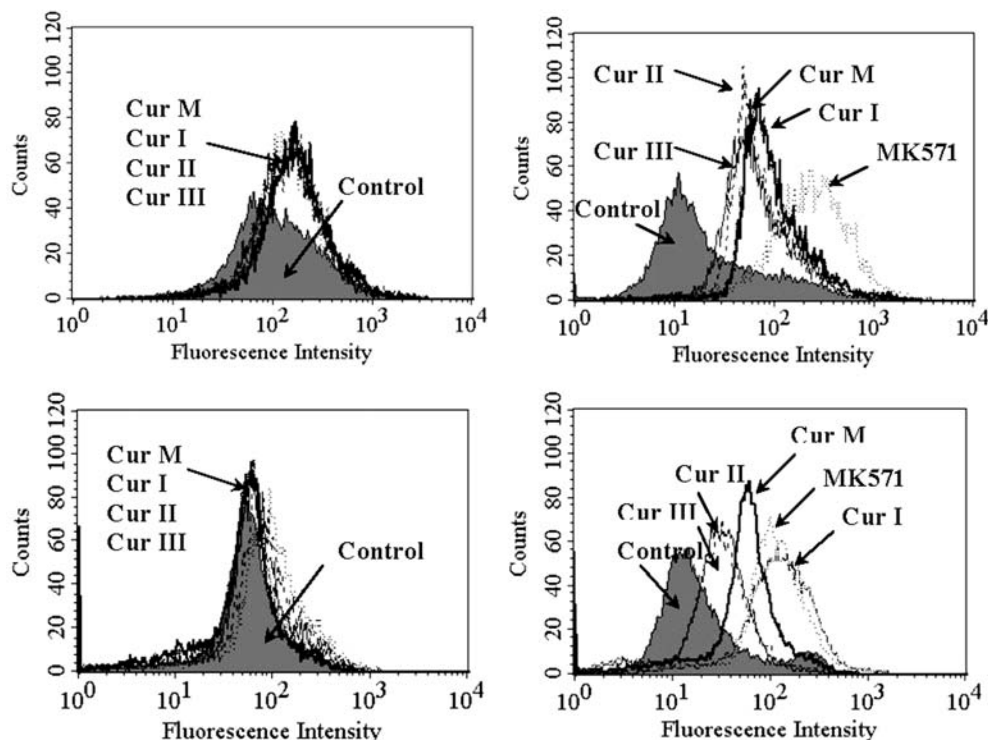

Fig. 4 Curcumin I, II, III and curcumin mixture increase calcein-AM and fluo-4AM accumulation in MRP1-HEK 293 cells but not in pcDNA3.1-HEK 293 cells. To study the effect of the curcuminoids on MRP1 transport function, the activity of MRP1 was assessed by measuring the intracellular accumulation of two fluorescent MRP1 substrates: **Top Panels** Fluo-4AM and **Bottom Panels** calcein-AM. Cells were incubated at 37°C in the dark for 45 min with 0.5 μM Fluo-4AM or 10 min with 0.5 μM calcein-AM in the presence or absence of 20 μM MK571 or various concentrations of the curcuminoids. The reaction was stopped by centrifugation and pelleted cells were re-suspended in 300 μl of ice-cold PBS containing 0.1% BSA. The accumulation of the fluorescent substrates was analyzed immediately by FACS. Representative histogram of three independent experiments is shown. The effect of 20 μM curcuminoids in pcDNA3.1- (**a, b**, *left panels*) and MRP1-HEK 293 cells (**Top Panels, Bottom Panels**, *right panels*) are depicted to show in comparison among each form of curcuminoids. The traces in each histogram are labeled. Control (*gray filled*); Cur M, curcumin mixture; Cur I, curcumin I; Cur II, curcumin II; and Cur III, curcumin III

Moreover, curcuminoids were able to stimulate the ATPase activity of MRP1 at the low concentrations and slightly inhibit the activity at high concentrations (Fig. 5a). This stimulation is most likely due to the interactions between curcuminoids at the MRP1 substrates binding site(s) and not the nucleotides binding sites since curcuminoids had no effect on 8-azido [$\alpha-^{32}$P]-ATP binding (Fig. 6). This is further supported by the fact that curcuminoids inhibited the quercetin-stimulated ATP hydrolysis by MRP1 (Fig. 5b).

Recently, other workers have reported modulation of MRP1 and MRP2 function by curcumin mixture [26, 16]. In addition, curcumin mixture appears to affect the trafficking of Δ F508 mutant of cystic fibrosis transmembrane regulator (CFTR) [12] which also belongs to ABCC subfamily (ABCC7), similar to MRP1 (ABCC1) and MRP2 (ABCC2). Curcumin mixture has been reported to stimulate the chloride channel activity of wild-type CFTR [5]. However, this is the first report to our knowledge, where the purified curcumin forms I, II, and III, rather than a mixture, have been used to assess their effect on the function of MRP1. It remains to be seen whether the function of MRP2 or CFTR is also modulated to the same extent by curcumin I alone.

Further extensive work of interest is whether curcumin I concentrations achieved in vivo are sufficient to inhibit MRP1 function and/or expression, and extensive pharmacokinetics studies with curcumin I will be

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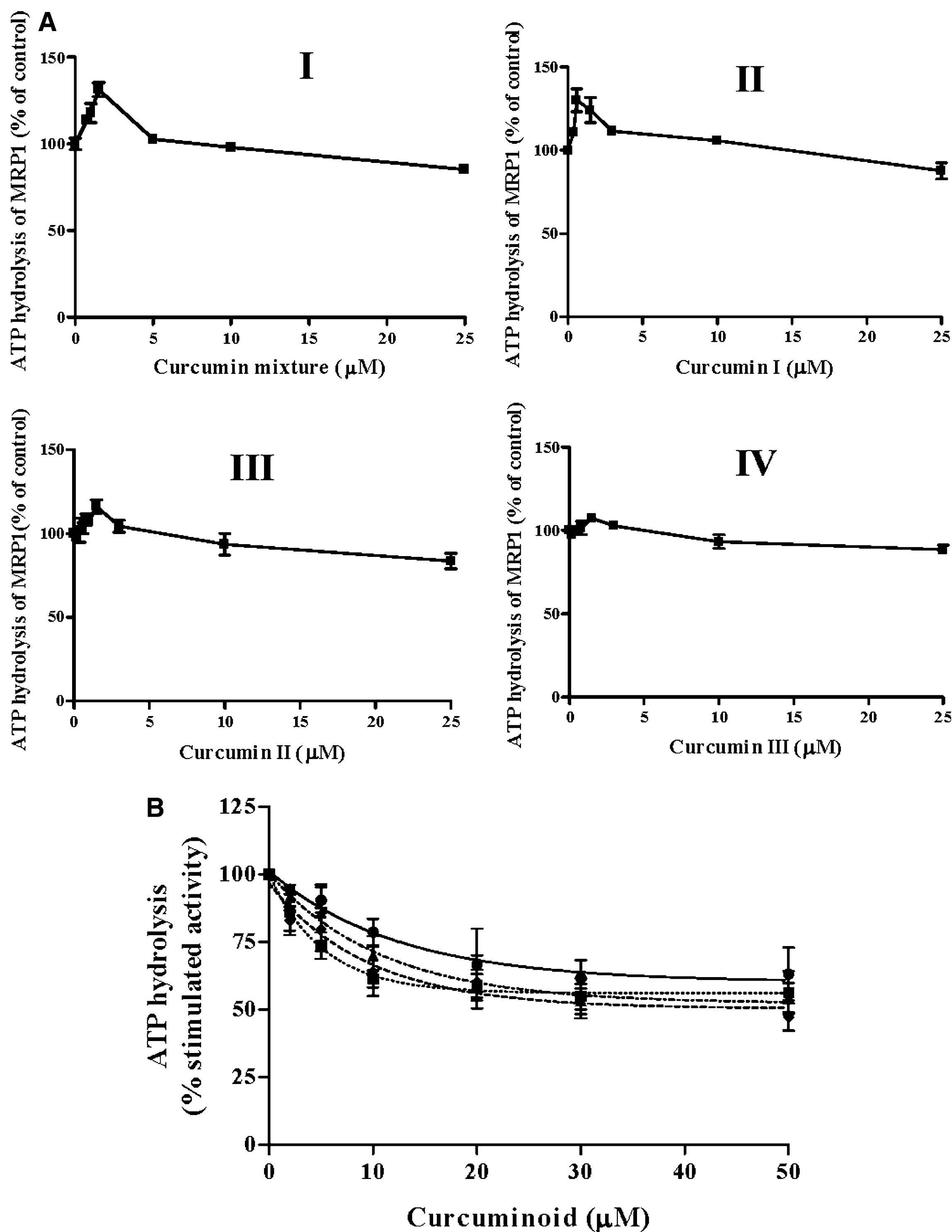
Fig. 5 Curcuminoids stimulate ATP hydrolysis of MRP1 and inhibit quercetin-stimulated MRP1 ATPase activity. **a** Crude membranes of (100 μg protein/ml) of MRP1 baculovirus infected HF insect cells were incubated at 37°C for 5 min with curcuminoids in the presence or absence of BeFx. The reaction was initiated by the addition of 5 mM ATP and terminated with SDS (2.5% final concentration) after a 20 min incubation at 37°C; the amount of P_i released was quantitated using a colorimetric method [18, 23]. MRP1-specific activity was recorded as the BeFx-sensitive ATPase activity. Values represent mean ± SE from at least three independent experiments. Control values show the basal activity measured in the absence of added compounds. The effect of curcumin mixture, and purified curcuminoids I, II and III on MRP1 ATPase activity is shown in panels I, II, III and IV, respectively. **b** The flavonoid quercetin at 10 μM is known to produce twofold increase in MRP1-mediated ATP hydrolysis. The effect of curcumin mixture (*diamond*), curcumin I (*square*), curcumin II (*triangle*) and curcumin III (*square*) at the indicated concentrations was tested on MRP1 ATPase activity in the presence of 10 μM quercetin. The mean values from three independent experiments are given and the error bars represent standard error (SE)



required to know the steady state levels of phytochemical reached in blood and tissue after its administration at pharmacological doses. However,the present studies suggested that, the curcumin mixture and all three pure forms of curcumins I, II, and III inhibit the function of MRP1. Curcumin I was the most effective form as an inhibitor of MRP1, similar to our previous results with P-gp [8]. These agents thus might have a beneficial effect on cancer chemotherapy with respect to the possibility of long-term use without concerns regarding MRP1 or MDR1 activation.

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Table 6 Effect of curcuminoids on ATP hydrolysis by MRP1

Curcuminoid	Folds stimulation	Concentration required for maximum stimulation(μM)
Curcumin mixture	1.45[a]	1.50[b]
Curcumin I	1.40	1.50
Curcumin II	1.26	1.50
Curcumin III	1.17	1.50

[a] Fold stimulation was obtained by dividing the ATPase activity in the presence of the curcuminoids by the in the basal activity in the presence of DMSO. These values were obtained from the data given in Fig. 5a

[b] The concentration of the curcuminoids giving the maximal stimulation

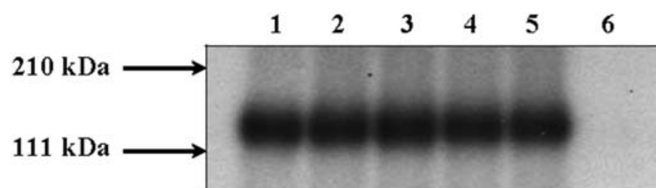

Fig. 6 Curcuminoids do not inhibit photoaffinity labeling of MRP1 with 8-azido [α−^{32}P]-ATP. Crude membranes of (50–75 μg protein) of MRP1 baculovirus infected HF insect cells were incubated at 4°C for 5 min with 10 μM [α−^{32}P]-8-AzidoATP (10 μCi/nanomole) in the presence and absence of 20 μM curcumin mixture or purified curcuminoid. The photocross linking with 366 nm UV light was carried out on ice for 10 min as described previously [23]. The incorporation of 8-azido [−^{32}P]-ATP detected by phosphorimaging after gel electrophoresis. Lane 1, 8-azido[α−^{32}P]-ATP alone, lane 2, + 20 μM curcumin I; lane 3, + 20 μM curcumin II; lane 4, 20 μM curcumin III, lane 5, 20 μM curcumin mixture and lane 6, + 10 mM ATP. The results from a representative experiment are shown; similar results were obtained in two additional experiments

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