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Biochemical mechanism of modulation of human P-glycoprotein (ABCB1) by curcumin I, II, and III purified from Turmeric powder

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Abstract

P-glycoprotein (Pgp, ABCB1) is an ATP-dependent drug efflux pump linked to development of multidrug resistance (MDR) in cancer cells. Previously [Biochem Pharmacol 2002;64:573–82], we reported that a curcumin mixture could modulate both function and expression of Pgp. This study focuses on the effect of three major curcuminoids — curcumin I, II and III purified from a curcumin mixture — on modulation of Pgp function in a multidrug resistant human cervical carcinoma cell line (KB-V1). The similar IC_{50} values for cytotoxicity of curcuminoids of KB-V1, and KB-3-1 (parental drug sensitive cell line) suggest that these curcuminoids may not be substrates for Pgp. Treating the cells with non-toxic doses of curcuminoids increased their sensitivity to vinblastine only in the Pgp expressing drug resistant cell line, KB-V1, and curcumin I retained the drug in KB-V1 cells more effectively than curcumin II and III, respectively. Effects of each curcuminoid on rhodamine123, calcein-AM, and bodipy-FL-vinblastine accumulation confirmed these findings. Curcumin I, II and III increased the accumulation of fluorescent substrates in a dose-dependent manner, and at 15 μM, curcumin I was the most effective. The inhibitory effect in a concentration-dependent manner of curcuminoids on verapamil-stimulated ATPase activity and photoaffinity labeling of Pgp with the [^{125}I]-iodoarylazidoprazosin offered additional support; curcumin I was the most potent modulator. Taken together, these results indicate that curcumin I is the most effective MDR modulator among curcuminoids, and may be used in combination with conventional chemotherapeutic drugs to reverse MDR in cancer cells.
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Keywords: ABC transporter; ATP hydrolysis; Chemosensitizers; Curcumin; Multidrug resistance; P-glycoprotein

1. Introduction

P-glycoprotein (Pgp)[1] is a 170-kDa membrane glycoprotein belonging to the superfamily of ATP-binding cassette (ABC) transport proteins [1]. It is known that this efflux pump is present in several physiological barriers in the body, including the blood brain barrier and the maternal–fetal barrier [2]. Although the exact physiological function remains to be elucidated, this efflux mechanism is believed to play a major role in multidrug resistance (MDR) in cancer [3–4], since many anticancer drugs (e.g. vinblastine, doxorubicin, paclitaxel) have been described to be substrates for this efflux pump and several drugs belonging to other classes (e.g. steroids, antiviral drugs and cardiac drugs) are also known to be transported by Pgp [5–7].

Because of the clinical importance of this efflux mechanism for multidrug resistance and cancer treatment, the inhibiting properties of several compounds on Pgp activity have been investigated. The calcium channel blocking agent verapamil was the first drug described as an inhibitor [8]. After this discovery, several other second and third-generation compounds have been studied for their inhibitory effect on Pgp (e.g. valspodar [PSC833], GF120918, XR9576 [4,9–10]. Although these inhibiting agents are effective, one of the major problems with most of these inhibitors is that the in vivo plasma concentrations

Abbreviations: ABC, ATP-binding cassette; AEBSF, 4-(2-aminoethyl)-benzene-sulfonylfluoride; Curcumin I, curcumin 1; Curcumin II, demethoxycurcumin; Curcumin III, bisdemethoxycurcumin; DMSO, dimethyl sulfoxide; IAAP, [^{125}I]-iodoarylazidoprazosin; MDR, multidrug resistance; PAGE, polyacrylamide gel electrophoresis; Pgp, P-glycoprotein; Vi, sodium orthovanadate

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required to obtain an inhibiting effect on Pgp, are too high, resulting in severe toxic side effects. Another issue is the possible pharmacokinetic interaction between the inhibiting agent and anticancer drugs [2,4].

Recently, many efforts have been made on a global scale to discover new drugs using plant extracts as screening libraries. These phytochemicals have the advantage of being dietary compounds that are less toxic to animals, plentiful, and inexpensive [11]. This study focuses on curcuminoids, which are natural phenolic coloring compounds found in the rhizomes of *Curcuma longa* Linn., commonly known as turmeric. The rhizomes contain three major pigments of curcuminoids: curcumin I, II, and III. Curcuminoid content in turmeric is about 1–5%, and it has been identified as the major yellow pigment in turmeric. It has been widely used as a spice, a coloring agent for cheese and butter, and as an ingredient in cosmetic and medicinal preparations [12]. Curcuminoids have a wide range of biological and pharmacological activities, including antioxidant, anti-inflammatory and anti-mutagenic activity in vitro, anti-carcinogenic effects [13–18] and hypocholesterolemic effects in rats [19], and hypoglycemic effects in humans [20]. The safety of *C. longa* and its derivatives has been studied in various animal models [21]. These studies showed that a commercial grade mixture of curcuminoids (Sigma cat # C1386), which is commonly known as curcumin, is able to modulate both expression and function of Pgp in rat hepatocytes [22]. Moreover, our recent studies also demonstrated that the commercial grade curcuminoids down-regulated both *MDR*1 gene expression and Pgp function [23]. Commercial preparations of curcuminoids usually contain approximately 77%, 17% and 3% of curcumin I, II and III, respectively [11,12].

In this paper, we further report a study on the purification of the three major curcuminoids and characterize their effects on Pgp function using human multidrug resistant KB-V1 cells and crude membranes of Pgp overexpressing HighFive insect cells. We demonstrate that all three forms of curcuminoids can inhibit Pgp function; however, we found that curcumin I is the most active form of the curcuminoids present in turmeric. This information may be useful to design more efficacious MDR chemosensitizers in combination with conventional chemotherapeutic drugs.

2. Materials and methods

2.1. Chemicals

Silica gel 60 and petroleum ether were purchased from Merck. Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), Trypsin–EDTA, Hank balance salt solution (HBSS) and Dulbecco's phosphate buffered saline (PBS) were purchased from GIBCO-BRL. Fetal bovine serum was purchased from HyClone. The MTT viability kit and bovine serum albumin were purchased from Promega. Rhodamine123, MES (2-[*N*-morpholino] ethanesulfonic acid, sodium orthovanadate, and ouabain were purchased from Sigma–Aldrich. Calcein-AM and Bodipy-FL-vinblastine were obtained from Molecular Probes Inc. [^{125}I]-iodoarylazidoprazosin (IAAP, 2200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences.

2.2. Extraction and fractionation of curcuminoids

Turmeric rhizomes purchased from a local market in Chiang Mai, Thailand were dried and blended to a powder form. The powder was extracted with 95% ethanol for 24 h. The ethanolic extract was filtered through Whatman filter paper no. 2 and ethanol was removed by using a rotary evaporator. One kilogram of turmeric powder yielded about fifty grams of laboratory made crude curcuminoids. The crude curcuminoids were then purified by precipitation with petroleum ether. The precipitate was removed by filtration through Whatman filter paper no. 2 and dried at 60 °C. HPLC analysis showed that this crude curcuminoid mixture contained 78% curcumin I, 16% curcumin II and 5% curcumin III. The curcuminoids were further fractionated by silica gel 60 column chromatography first using $CHCl_3$ and then $CHCl_3$/methanol with increasing polarity to yield pure fractions of curcumin I, II and III. The fractions were collected and spotted on TLC aluminum sheets coated with Silica gel 60 F254. Fractions that showed the same pattern on TLC were pooled and the organic solvent was removed to obtain the powder form. The purity of curcumin I, II and III by HPLC analysis was in the range of 95–99%. These curcuminoids were used in the experiments described here.

2.3. Cell lines and culture conditions

The multidrug resistant cell lines, KB-V1 (multidrug resistance cervical carcinoma cell line) and KB-3-1 (drug sensitive cervical carcinoma cell line), were generous gifts from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD). Both cell lines were cultured in DMEM with 4.5 g of glucose/l plus 10% fetal calf serum (2 mM), L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml); 1 μg/ml of vinblastine was added only to the KB-V1 culture medium [23]. These two cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C. When the cells reached confluency, they were harvested and plated for consequent passages, for drug treatments and MTT assays.

2.4. Preparation of crude membranes from HighFive insect cells infected with recombinant baculovirus carrying the human MDR1 gene

HighFive insect cells (Invitrogen) were infected with the recombinant baculovirus carrying the human *MDR*1 cDNA

with a six-Histidine tag at the C-terminal end and crude membranes were prepared and stored at −70 °C as described previously [24].

2.5. MTT assay

Cytotoxicity of curcuminoid extracts and the effect of these compounds on vinblastine cytotoxicity in KB-3-1 and KB-V1 cells were determined by MTT assay. Briefly, the cells (5.0×10^3 cells) were seeded into 96 well plates and cultured overnight. Various concentrations of curcuminoids or vinblastine with 15 μM of curcuminoid extracts were then added and incubated further for 72 h. After incubation, 100 μl of MTT solution was added to each well and further incubated for 3–4 h and the reaction was terminated by adding 200 μl of isopropanol. Absorbance was measured using an ELISA plate reader at 570 nm with a reference wavelength of 650 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 [23].

2.6. Fluorescent drug accumulation assay by fluorescence activated cell sorter (FACS)

A FACSort flow cytometer equipped with Cell Quest software (Becton-Dickinson) was used for FACS analysis [25]. Three fluorescent substrates of Pgp for accumulation assay were used in drug resistant, KB-V1 cells and their wild type KB-3-1 cells. Briefly, cells were harvested after trypsinization by centrifugation at 500 × *g* and resuspended in IMDM supplemented with 5% FBS. Rhodamine 123 (0.5 μg/ml), Bodipy-FL-vinblastine (0.5 μM) or Calcein-AM (0.25 μM) was added to $(3–5) \times 10^5$ cells in 4 ml of IMDM in the presence or absence of reversing agent, cyclosporin A (10 μM) or curcuminoid (15 μM). The cells were incubated in a water bath at 37 °C in dark. After incubation for 45 min with rhodamine123 or bodipy-FL-vinblastine and 10 min with calcein-AM, the cells were pelleted by centrifugation at 500 × *g*. The cell pellet was resuspended in 300 μl of PBS containing 0.1% BSA and analyzed immediately by using flow cytometer [25].

2.7. ATPase assays

ATPase activity of Pgp in crude membranes of HighFive insect cells was measured by the endpoint, P_i release assay as previously described [24,26]. This assay measures the amount of inorganic phosphate released for 20 min at 37 °C in the ATPase assay buffer in the presence and absence of 0.25 mM sodium orthovanadate (Vi). Crude membranes (100 μg protein/ml) were incubated with increasing concentrations of curcumin mixture, curcumin I, II or III in the presence and absence of 5 μM verapamil. 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 quantitated using a colorimetric method [24,26]. Pgp-specific activity was recorded as the vanadate-sensitive ATPase activity.

2.8. Photoaffinity labeling of Pgp with IAAP

The crude membranes of HighFive insect cells (50–100 μg protein) were incubated with increasing concentrations of curcumin mixture, curcumin I, II or III at room temperature in 50 mM Tris–HCl, pH 7.5, for 3 min. IAAP (5–10 nM) was added and further incubated for additional 5 min under subdued light. The samples were then illuminated with a UV lamp (365 nm) assembly (PGC Scientifics) fitted with two Black light (self-filtering) UV-long wavelength – F15T8BLB tubes for 10 min at room temperature (21–23 °C). Following SDS-PAGE on an 8% Tris–glycine gel at constant voltage, gels were dried and exposed to BioMax MR film (Eastman Kodak, Rochester, NY) at −70 °C for 6–24 h to obtain an autoradiogram. The radioactivity incorporated into the Pgp band was quantified using the STORM 860 Phosphorimager system (Molecular Dynamics) and software ImageQuaNT [27].

2.9. Statistical analysis

Data are the means ± S.D. from duplicate or triplicate samples of at least three independent experiments. Differences between the mean values were analyzed by one-way analysis of variance and results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Preparation of curcumin mixture and purification of curcumin I, II, and III

In order to compare the modulatory effect of each form of curcuminoids, a purification method was developed for the isolation, separation, and identification of major forms of curcuminoids from curcumin mixture. We started with extraction in 95% ethanol to extract all the polar and non-polar compounds from the turmeric powder. All three forms of curcuminoids were extracted at this step in the form of crude curcuminoids. The resultant crude extract was further subjected to purification by silica gel 60 column chromatography to yield the pure form of curcumin I, II and III. The structures of three curcuminoids are shown in Fig. 1C. To determine the purity of the compounds, we conducted the TLC (Fig. 1A), and HPLC (Fig. 1B) with the conditions as described in Section 2. As expected, the curcumin mixture showed three peaks, with a retention time of 7.078, 8.127 and 9.277 min corresponding to curcumin I, II, and III, respectively. This was verified by subjecting purified curcuminoids to HPLC analysis and curcumin I, II, and III eluted at 7.088, 8.115, and 9.287 min, respectively,

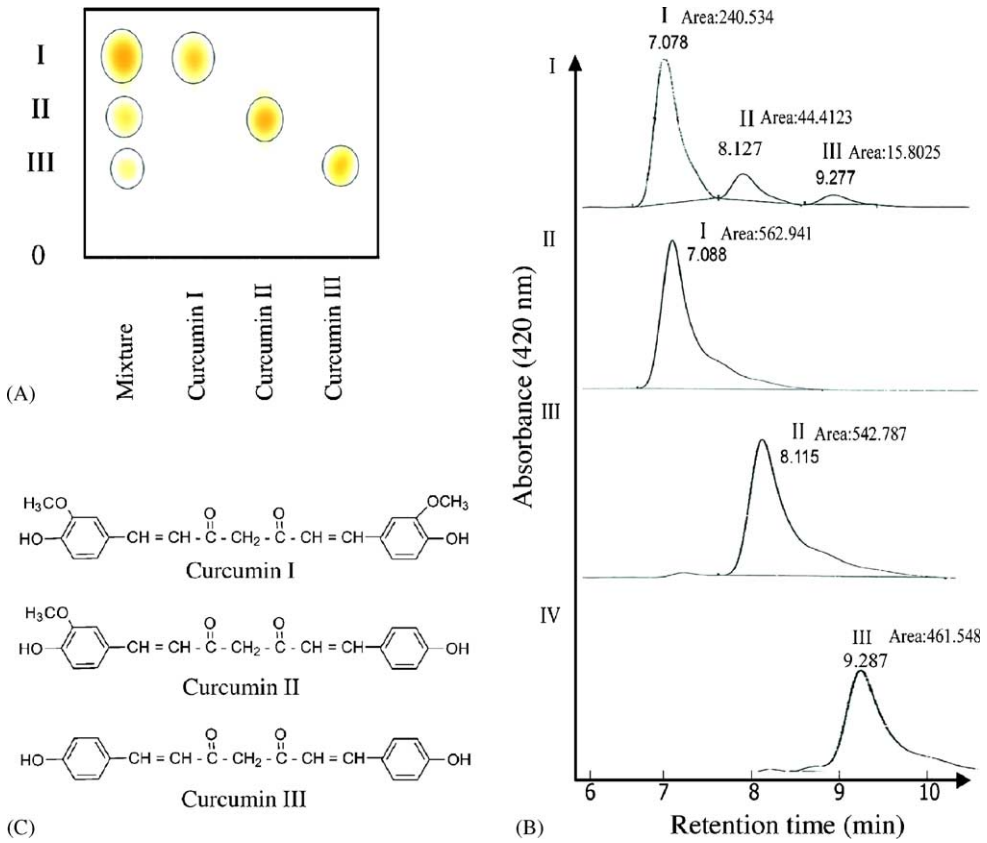

Fig. 1. Structure and determination of purity of curcuminoids by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). The crude ethanolic extract or curcumin mixture and pure form of curcuminoids collected from silica gel 60 column chromatography after eluting with $CHCl_3$ or $CHCl_3$/MeOH were subjected to TLC and HPLC analysis. (A) The extracts were spotted on Silica gel 60 F254 and conducted the TLC in solvent system of $CHCl_3$:Ethanol:acetic acid (94:5:1). I, II, and III indicates the position of curcumin I, II and III, respectively and O denotes the origin. (B) The HPLC elution profile of curcuminoids. The HPLC histogram of curcumin mixture, I, II and III as depicted in Panels I, II, III and IV, respectively. (C) The structures of curcumin I, II, and III.

demonstrating that the curcumin mixture has three major compounds: curcumin I, II, and III and that the purity of each curcuminoid is in the range of 95–99%.

3.2. Cytotoxicity of curcuminoid extracts in KB-3-1 and KB-V1 cells

To examine whether each form of curcuminoids affects the viability of cells, KB-3-1 and KB-V1 cells were exposed to various concentrations of curcuminoid extracts (without drug) for 72 h and cytotoxicity was determined by MTT assay. Dose response cytotoxicity profiles for curcuminoids were established for both drug-resistant KB-V1 and wild type KB-3-1 cells. Fig. 2A and B show the cytotoxicity effect of curcuminoids. The percent of viable cells was calculated to determine the IC_{50}. The IC_{50} values (mean ± S.D., n = 3) of curcumin I, II and III in KB-3-1 cells were 24.0 ± 1.7, 33.3 ± 2.9 and 85.0 ± 8.7 μM, respectively, whereas in KB-V1 the values (mean ± S.D., n = 3) were 23.5 ± 5.6, 35.8 ± 3.7, and 93.0 ± 9.5 μM, respectively. Similarly, the IC_{50} values of curcumin mixture were 26.3 ± 4.7 and 22.8 ± 4.4 μM for KB 3-1 and KB-V1, respectively. This suggests that although these three forms of curcuminoids interact with Pgp but the multidrug transporter may not transport them because the IC_{50} values were not higher in Pgp expressing KB-V1 cells compared to KB-3-1 cells.

3.3. Effect of curcumin I, II and III on cytotoxicity of vinblastine in KB-3-1 and KB-V1 cells

To study the effect of curcuminoids on vinblastine cytotoxicity in the drug resistant KB-V1 cells and the drug sensitive KB-3-1 cells, the growth inhibition of cells was investigated in response to increasing concentrations of vinblastine (nanomolar for KB-3-1 and micromolar for KB-V1) with and without addition of curcuminoids. Based on Fig. 2, 15 μM of curcuminoids, which was the concentration at IC_{70}-IC_{100} in KB cells, was used to assess their effect on toxicity of vinblastine and 20 μM verapamil as a modulator [1] was also included. Fig. 3A and B showed that curcumin I, II, and III at 15 μM increased the vinblastine cytotoxicity only in KB-V1 cells: the IC_{50} shifted drastically from 1.7 to 0.3 μM for curcumin I or to 0.4 μM for curcumin II and to 1.5 μM for curcumin III, respectively (Fig. 3B). However, this effect of curcuminoids was not observed in parental KB-3-1 cells (Fig. 3A). Furthermore, these results showed that among curcumi-

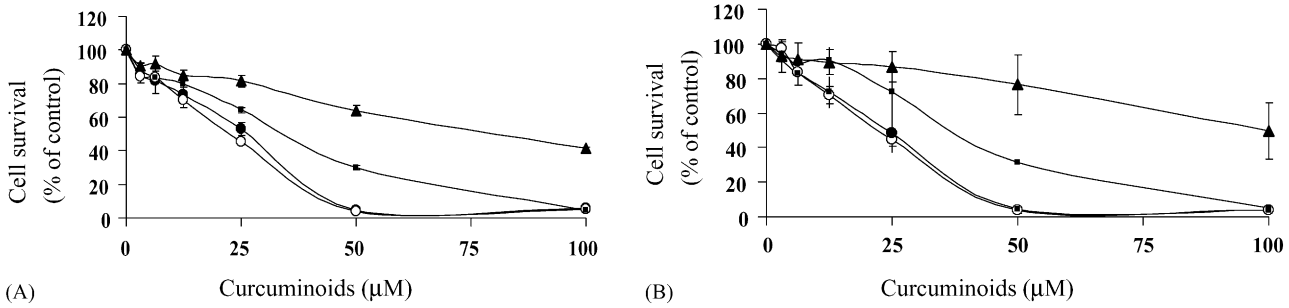

Fig. 2. Cytotoxicity of curcumin I, II and III in multidrug resistant KB-V1 cells. KB-3-1 and KB-V1 cells (5.0×10^3 cells) were seeded into 96 well plates and cultured overnight. Various concentrations of curcuminoids (0–100 μM) were then added and incubated further for 72 h in a 37 °C incubator, 100 μl of MTT solution was added to each well and incubated for 3–4 h, at which point 200 μl of isopropanol was added to stop the reaction. Absorbance was measured using a micro plate spectrophotometer, SpectraMAX 250 (Molecular Devices, Sunnyvale, CA) at 570 nm with a reference wavelength of 650 nm. Cell survival (% of control) = (mean absorbance in test well)/(mean absorbance in control wells) × 100. The experiments were conducted in triplicate and the values represent mean ± S.D. of three independent experiments. The data of KB-3-1 and KB-V1 as mean ± S.D. ($n = 3$) are shown in Panel A and B, respectively. The curcumin mixture (○), curcumin I (●), II (■) and III (▲) do not show significant difference in IC_{50} values for cytotoxicity in KB-3-1 and KB-V1 cells, respectively.

noids, curcumin I is the most effective form (almost to the same level as verapamil, $IC_{50} = 0.2$ μM) for increasing the sensitivity to vinblastine in the KB-V1 cells.

3.4. Effect of curcuminoids on calcein-AM, rhodamine123 and bodipy-FL-vinblastine accumulation

To further confirm the effect of curcuminoids on Pgp function, we investigated the effect of curcuminoids on transport of three fluorescent substrates of Pgp: calcein-AM, rhodamine123, and bodipy-FL-vinblastine using flow cytometry. These fluorescent substrates were chosen for this study to determine whether the inhibitory effects of curcuminoids are substrate specific or not. In addition, another Pgp expressing cell line, NIH 3T3-MDR1-G185 (multidrug resistant NIH3T3 cells transfected with the *MDR*1 gene [28]) was also used in this study to determine whether the effect is cell type dependent or not. Curcumin I, II, and III increased the accumulation of fluorescent substrates in a dose-dependent manner (0–25 μM) (data not shown). Fig. 4 shows the effect of curcumin I, II, and III in comparison to the curcumin mixture at 20 μM on the accumulation of calcein, rhodamine 123, and bodipy-FL-vinblastine (Panels A–F; similar results were also obtained with NIH3T3-MDR1-G185 cells, data not shown). Curcuminoids had a similar inhibitory effect on the accumulation of all three substrates. These results further confirmed previous findings using MTT assays that curcumin I was the most active form present in the curcumin mixture.

3.5. Effect of curcuminoids on ATPase activity of Pgp

The transport of a drug substrate by Pgp is coupled to ATP hydrolysis and there is evidence for stimulation of ATPase activity of Pgp by drug substrates or modulators from diverse systems (reviewed in reference [29]). This has led to the use of the stimulation by substrate or modulator of ATP hydrolysis by Pgp as a surrogate assay to determine drug–Pgp interaction [30]. The curcumin mixture, and curcuminoids I, II, and III, stimulate the Vi-sensitive Pgp ATPase activity at low concentrations (0.5–1 μM) but inhibit to the level of basal activity at higher concentrations (Fig. 5). In addition similar to our previous findings with curcumin mixture [23], purified curcuminoids also

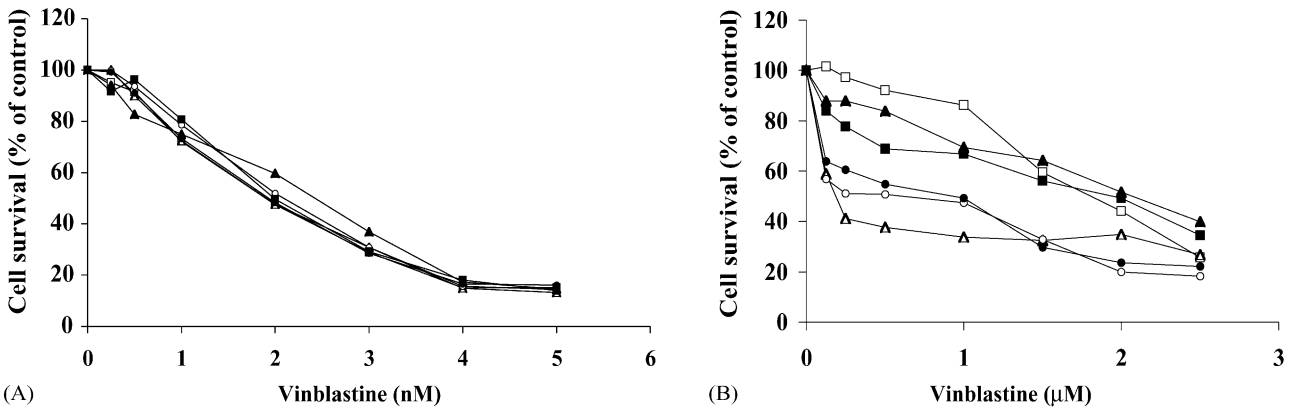

Fig. 3. Effect of curcumin I, II and III on the cytotoxicity of vinblastine. KB-3-1 (Panel A) and KB-V1 (Panel B) cells were grown in the presence of various concentrations of vinblastine as indicated alone (□) or with 15 μM of curcumin I (●), II (■), III (▲), curcumin mixture (○) and 20 μM verapamil (△). The number of viable cells was determined by MTT assay in triplicate. The result of one typical experiment out of three independent experiments is depicted.

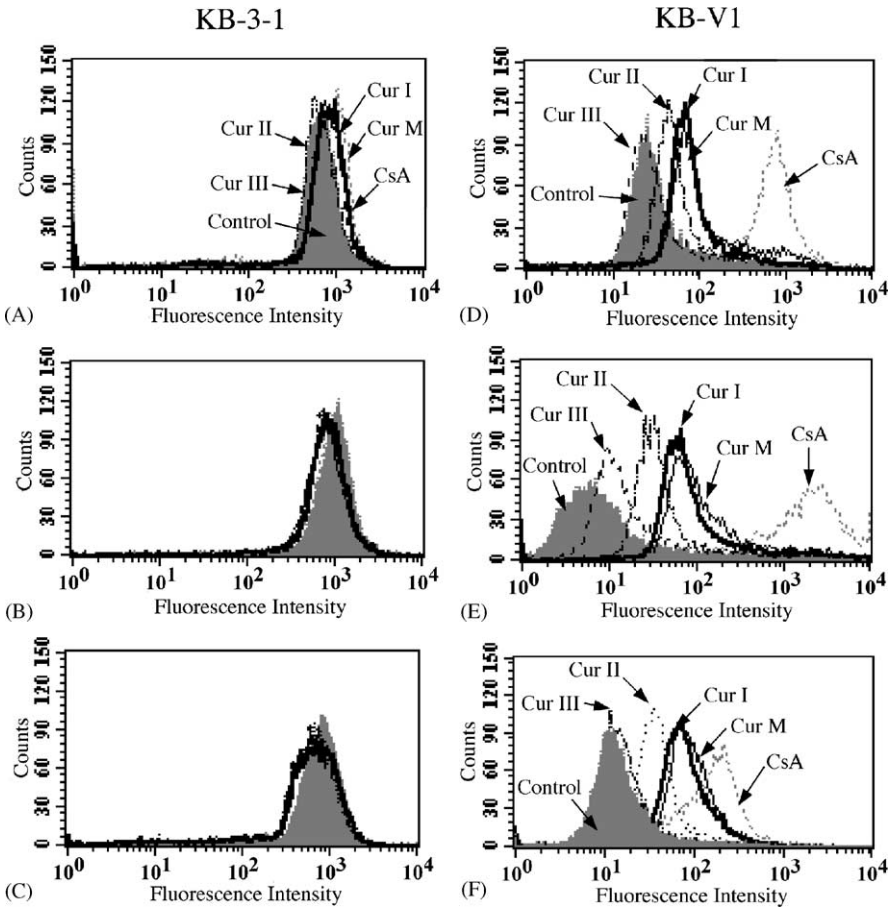

Fig. 4. Effect of curcumin I, II, III and curcumin mixture on Calcein-AM, Rhodamine 123 and Bodipy-FL-vinblastine accumulation in KB-3-1 and KBV-1 cells. Cells were resuspended in IMDM supplemented with 5% FBS. Rhodamine 123 (0.5 μg/ml) or bodipy-FL-vinblastine (0.5 μM) or calcein-AM (0.25 μM) was added to (3–5) × 10^5 cells in 4 ml of IMDM in the presence or absence of reversing agent cyclosporin A (10 μM) or indicated curcuminoid extract (15 μM). The cells were incubated at 37 °C in dark. After 45 min for rhodamine123 and bodipy-FL-vinblastine or 10 min incubation time for calcein-AM, the cells were pelleted by centrifugation at 500 × *g* and resuspended in 300 μl of PBS containing 0.1% BSA. Samples were analyzed immediately by using flow cytometer. The results of a representative experiment of three independent experiments for KB-3-1 and KB-V1 cells are shown in Panels A–C and D–F, respectively. Panels A, D, B, E and C, F show the effect of curcuminoids on calcein, rhodamine 123 and bodipy-FL-vinblastine accumulation, respectively. Control (DMSO) (gray filled); CsA (- - -); curcumin I (——); curcumin II (- - . .): curcumin III (- . - . -); and curcumin mixture (—). The traces are labeled only in Panels A, D–F for clarity. Cur I, II and III, curcumin I, II and III; Cur M, curcumin mixture; and CsA, cyclosporin A.

inhibited verapamil-stimulated ATPase activity in a concentration-dependent manner (Fig. 5); the IC_{50} values ranged from 5 to 15 μM for curcumin I, II and III (Table 1). In this case also among curcuminoids, curcumin I was as effective as the curcumin mixture (IC_{50} values 5.90 and 5.24 μM, respectively).

Table 1
Effect of curcumin and its derivatives on ATPase activity of Pgp

Compound	ATP hydrolysis (nanomoles/min/mg protein)[a]	
	Basal (fold-stimulation)	+Verapamil IC_{50} (μM)[b]
Curcumin I	1.4 ± 0.1[c]	5.9 ± 1.7
Curcumin II	2.5 ± 0.2	10.4 ± 2.1
Curcumin III	2.4 ± 0.6	14.6 ± 3.0
Curcumin mixture	2.0 ± 0.9	5.2 ± 1.0

[a] The Vi-sensitive ATPase activity was measured in the presence of various concentrations of curcuminoids as described in the legend to Fig. 5.
[b] IC_{50} (concentration required for 50% inhibition) of verapamil (5 μM)-stimulated ATPase activity.
[c] Values are mean ± S.D. of three independent experiments.

3.6. Effect of curcuminoids on photoaffinity labeling of Pgp with [^{125}I]-iodoarylazidoprazosin

To assess whether curcuminoids interact directly with the substrate-binding site(s) of Pgp, their effect on photoaffinity labeling of Pgp by IAAP was tested. IAAP is an analog of prazosin, which is transported by Pgp [31]. The data in Fig. 6 demonstrate that curcuminoids effectively inhibit photoaffinity labeling of Pgp with IAAP in a concentration-dependent manner. The IC_{50} values for inhibition of IAAP binding were 5.8, 9.7, 23.3 and 5.0 μM for curcumin I, II, III and curcumin mixture, respectively (Table 2). Thus, comparing the IC_{50} (concentration required for 50% inhibition), it was revealed that curcumin I is the most effective form among curcuminoids but slightly less effective than curcumin mixture (Table 2). It is also clear that the IC_{50} values for the inhibition of both IAAP binding and verapamil-stimulated ATPase activity are in a similar range (see Tables 1 and 2) suggesting that

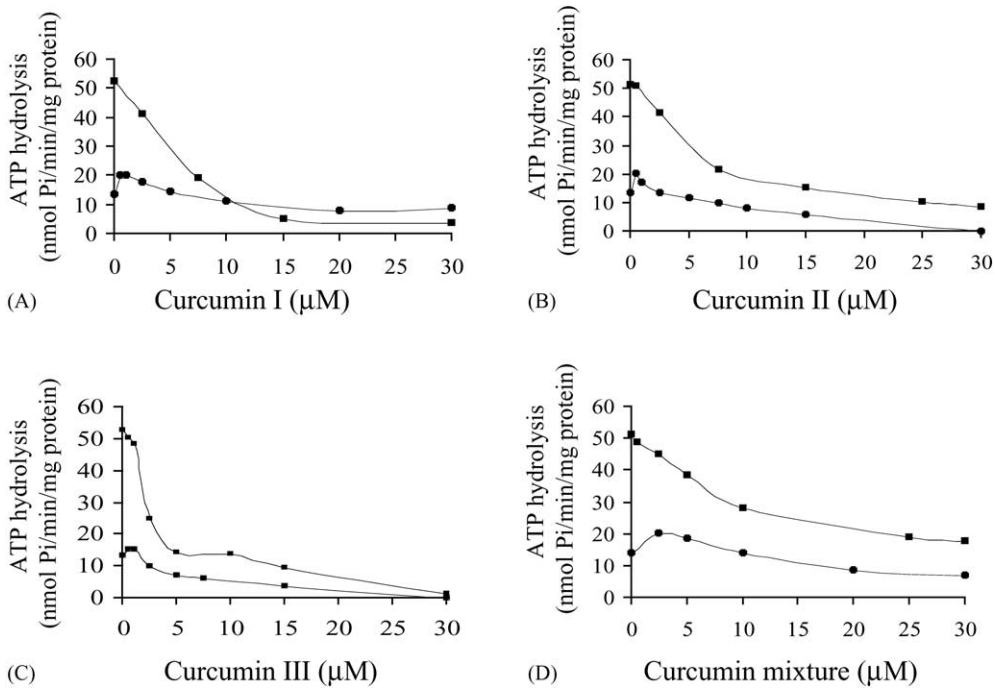

Fig. 5. Effect of curcumin I, II and III and curcumin mixture on basal and verapamil-stimulated of Pgp ATPase activity. Crude membranes of HighFive insect cells expressing MDR1 (100 μg protein/ml) were incubated with increasing concentrations of curcumin I, II, III or the curcumin mixture (0–30 μM) in the presence and absence of 5 μM verapamil in the ATPase assay buffer. The assays were carried out in the presence and absence of 0.25 mM Vi and the Pgp-specific activity was recorded as the Vi-sensitive ATPase activity as described [26]. Panels A, B, C and D: (●) Pgp basal activity (DMSO control); and (■) verapamil-stimulated Pgp ATPase activity in the presence of curcumin I, II, III, and curcumin mixture, respectively. The result of a typical experiment of at least three independent experiments is shown.

the curcuminoids exert their inhibitory effect most likely by binding to the substrate-binding sites on the transporter.

4. Discussion

Drug resistance is a major problem in cancer chemotherapy. Several mechanisms responsible for MDR have been described. The mechanism that has been most extensively investigated is the expression of the *MDR*1 gene product, Pgp. A variety of compounds have been shown to reverse Pgp-mediated MDR and the clinical use of these MDR modulators has been hampered by the toxic side effects that occur when the non-physiological doses, which are required to achieve a significant reversal of MDR, are used [4]. Therefore, the search for novel and more potent MDR modulators without side effects is of major importance. In this study, we focused on curcuminoids, which are polyphenolic pigments found in the spice turmeric. The major curcuminoids are curcumin I, II, and III and these compounds comprise 1–5% of *C. longa* rhizome powder. The curcuminoids have been found to have a number of antioxidant activities, including scavenging of such reactive oxygen species as superoxide anions and hydrogen peroxide, inhibition of lipid peroxidation and inhibition of the oxidation of low-density lipoprotein [19,32]. We had reported that curcumin mixture could reverse the multidrug resistance phenomenon in KB-V1 cells by inhibiting both function and expression [23]. The investigation in this study provides more information about the modulatory effect of various curcuminoids and further compares which form is the most effective for blocking the function of Pgp. This knowledge should be useful for drug design based on ‘‘structure–activity relationships’’ in combination with conventional chemotherapy. The preliminary studies of the effect of pure curcumin I, II, III on the expression of Pgp indicate that curcumin III instead of I is more inhibitory [33]. Clearly, additional work is needed to elucidate the effect of purified curcuminoids on the expression of Pgp.

The experiments reported here were carried out using the three purified curcuminoids (structures shown in Fig. 1C). In the first set of experiments, all three pure

Table 2
Effect of curcuminoid extracts on photoaffinity labeling of Pgp with IAAP

Compound	IC_{50} (μM)[a]
Curcumin I	5.8 ± 0.4[b]
Curcumin II	9.7 ± 1.0
Curcumin III	23.3 ± 1.5
Curcumin mixture	5.0 ± 0.2

[a] IC_{50} (concentration required for 50% inhibition). The photoaffinity labeling of Pgp with IAAP in the presence of curcuminoids at various concentrations ranging from 0.1 to 30 μM was carried out and IAAP incorporation into the Pgp band was quantified as described in the legend to Fig. 6.

[b] Values are mean ± S.D. of at least three independent experiments.

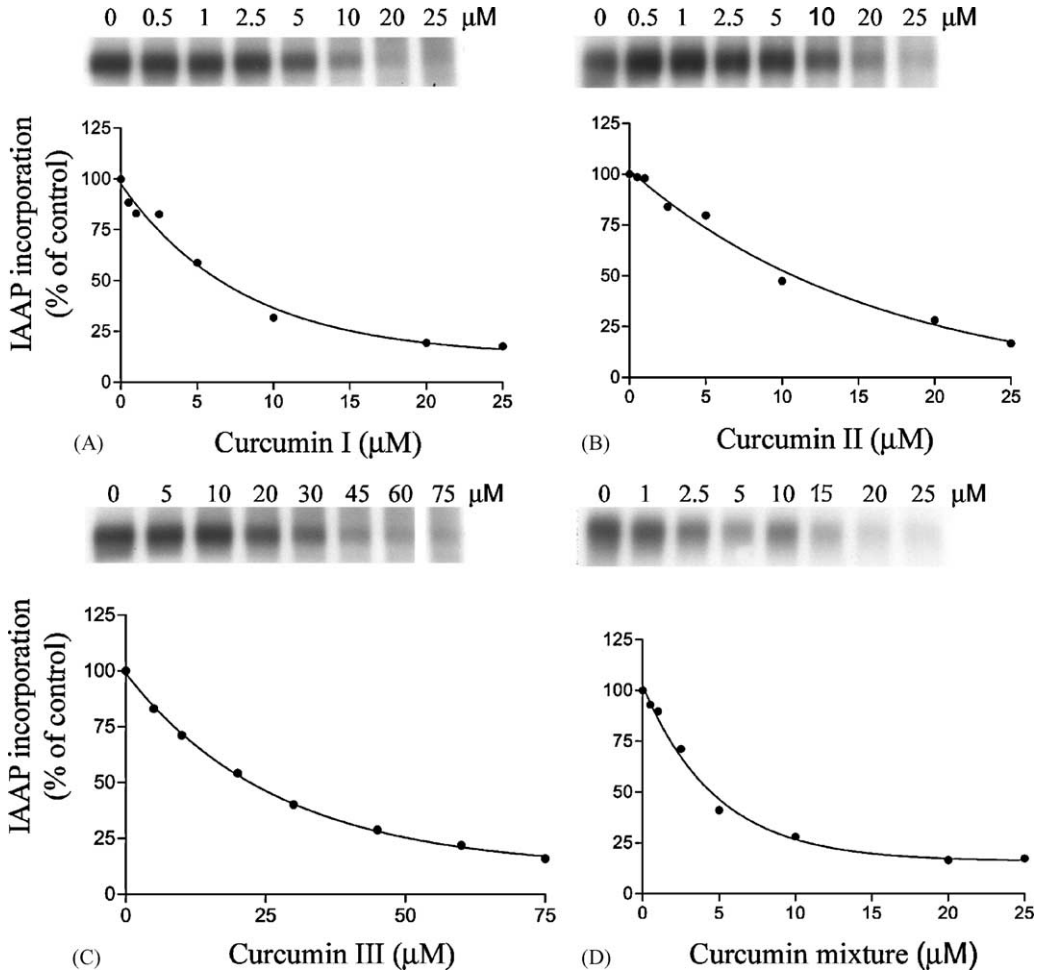

Fig. 6. Effect of various concentrations of curcumin I, II, III and the curcumin mixture on photoaffinity labeling of Pgp with IAAP. The crude membranes of HighFive insect cells (0.5–1 mg/ml protein) were incubated with increasing concentrations of curcumin mixture, curcumin I, II or III at room temperature in 50 mM Tris–HCl, pH 7.5, for 3 min. IAAP (5–10 nM) was added and further incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp (365 nm) for 10 min at room temperature (21–23 °C). Following SDS-PAGE on an 8% Tris–glycine gel, gels were dried and exposed to BioMax MR film at −70 °C for 6–24 h. The radioactivity incorporated into the Pgp band was quantified using the STORM 860 phosphorimager system [27]. Upper Panel: the autoradiograms show incorporation of IAAP into the Pgp band in the presence of various concentrations of curcumin I, II, III and the curcumin mixture as indicated in Panels A, B, C and D, respectively. The data in lower Panels (A–D) were fitted by non-linear least squares regression analysis using the software GraphPad Prism 2.0 for the PowerPC Macintosh. Data from a typical experiment are depicted and similar results were obtained in additional two independent experiments.

forms of curcuminoids were tested for their toxicity in KB-3-1 and KB-V1 cells. The IC_{50} of curcumin I, II and III from this experiment is not statistically different compared to KB-V1 (expressing high levels of Pgp) and KB-3-1 cells (parental drug sensitive) suggesting that Pgp does not confer resistance to curcumin I, II or III; in other words, these curcuminoids most likely are not transported by Pgp (Fig. 2).

The effect of curcuminoids on cytotoxicity of vinblastine in KB-V1 cells and KB-3-1 revealed that curcumin I, II and III increased the sensitivity of vinblastine in KB-V1 cells, but not wild type, KB-3-1 cells. These results showed clearly that curcumin I strongly increased the sensitivity or decreased the IC_{50} in KB-V1 cells but curcumin II and III only slightly changed the sensitivity to vinblastine. Taken together, these data indicate that curcumin I is the most active form for increasing the intracellular levels and cytotoxicity of vinblastine in KB-V1 cells by modulating Pgp function.

Consistent with the effect of curcuminoids on vinblastine accumulation in intact cells, these agents also blocked the efflux of three fluorescent substrates - rhodamine123, calcein-AM and bodipy-FL-vinblastine. Curcuminoids caused a substantial increase in the accumulation of these substrates in KB-V1 cells but had no effect on drug sensitive (KB-3-1) cells, which do not overexpress Pgp. Moreover, these results demonstrated that this effect is not specific to a particular substrate; curcuminoids affected the accumulation of all three substrates in the same manner. This study also demonstrated that this effect was found not only in KB-V1 but also in NIH 3T3-MDR1-G185, which is a mouse fibroblast cell line transfected with the human *MDR*1 gene, suggesting that the modulation of Pgp by curcumin is not cell type dependent.

The structure of curcumin I may make it more suitable for binding to the drug binding site of Pgp than the structure of curcumin II and III, because curcumin I has a balance of two hydroxyl and methoxyl groups on each side, and the presence of two methoxyl groups in the curcumin I molecule might help its inhibitory activity on the Pgp function (Fig. 1C). Since curcumin I is a major component (>70%) of curcumin mixture [11,12], and its efficacy as a modulator is similar to the curcumin mixture indicating that the presence of both curcumin II, and III in the mixture does not diminish the effect of curcumin I. When curcumin I was varied in doses for testing, it was found that it inhibited the Pgp function in a dose-dependent manner. An ATPase assay (Fig. 5) showed clearly that curcuminoids could stimulate ATPase activity at a low concentration (0.5–1 μM) and inhibit its activity at higher concentrations. These assays also demonstrated that curcuminoids inhibit verapamil-stimulated ATPase activity at higher concentrations. These results suggest that the binding site for the three curcuminoids may overlap with that of verapamil (Fig. 5). Thus, binding of curcuminoids may change the conformation of Pgp and affect the binding of the other drug substrates such as verapamil or vinblastine. These findings were further supported by the effect of curcuminoids on the photoaffinity-labeling analog, IAAP (Fig. 6 and Table 2). Thus, these biochemical results (Figs. 5 and 6 and Tables 1, 2) demonstrate that curcuminoids interact directly with Pgp and possibly bind to the same binding sites as other agents such as prazosin, vinblastine and verapamil.

In conclusion, our results show that all three forms of curcuminoids, curcumin I, II and III can inhibit Pgp function; however, curcumin I is the most potent MDR modulator compared with other forms of curcuminoids. Therefore, it may have a beneficial effect on cancer chemotherapy with respect to the possibility of long-term use without causing concern about drug transporting function of Pgp. Thus we suggest that curcumin I may be considered as a promising lead compound for the design of more efficacious MDR chemosensitizers.

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