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Modulation of P-glycoprotein expression and function by curcumin in multidrug-resistant human KB cells

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Abstract

Multidrug resistance (MDR) is a phenomenon that is often associated with decreased intracellular drug accumulation in the tumor cells of a patient, resulting from enhanced drug efflux. It is often related to the overexpression of P-glycoprotein (Pgp) on the surface of tumor cells, thereby reducing drug cytotoxicity. In this study, curcumin was tested for its potential ability to modulate the expression and function of Pgp in the multidrug-resistant human cervical carcinoma cell line KB-V1. Western blot analysis and reverse transcription–polymerase chain reaction (RT–PCR) showed that treatment with 1, 5, and 10 μM curcumin for up to 72 hr was able to significantly lower Pgp expression in KB-V1 cells. Curcumin (1–10 μM) decreased Pgp expression in a concentration-dependent manner and was also found to have the same effect on MDR1 mRNA levels. The effect of curcumin on Pgp function was demonstrated by rhodamine 123 (Rh123) accumulation and efflux in Pgp-expressing KB-V1 cells. Curcumin increased Rh123 accumulation in a concentration-dependent manner (1–55 μM) and inhibited the efflux of Rh123 from these cells, but did not affect the efflux of Rh123 from the wild-type drug-sensitive KB-3-1 cells. Treatment of drug-resistant KB-V1 cells with curcumin increased their sensitivity to vinblastine, which was consistent with an increased intracellular accumulation of Rh123. In addition, curcumin inhibited verapamil-stimulated ATPase activity and the photoaffinity labeling of Pgp with the prazosin analog [^{125}I]iodoarylazidoprazosin in a concentration-dependent manner, demonstrating that curcumin interacts directly with the transporter. Thus, curcumin seems to be able to modulate the *in vitro* expression and function of Pgp in multidrug-resistant human KB-V1 cells. In summary, this study describes the duel modulation of *MDR*1 expression and Pgp function by the phytochemical curcumin, which may be an attractive new agent for the chemosensitization of cancer cells.

Keywords: Curcumin; ATP hydrolysis; KB-V1 cells; Chemosensitizers; Multidrug resistance; P-glycoprotein

1. Introduction

When patients with cancer are treated with a cytotoxic agent, the pharmacological goal is to deliver as much active drug as possible to the molecular target in the cancer cells, causing sufficient molecular damage to lead to cell death. On the other hand, the occurrence of drug resistance renders cells resistant not only to the drug used in the chemotherapy, but also to a broad spectrum of unrelated cytotoxic drugs as well. Cancer cells may develop a multidrug-resistant phenotype. When human tumor cells express this phenotype, they often overexpress the drug export protein called plasma membrane Pgp with a molecular mass of approximately 150–170 kDa. This plasma membrane phosphoglycoprotein, which belongs to the superfamily of ATP-binding cassette (ABC) transporters, consists of two homologous halves that share a high degree of sequence similarity [1].

Uptake and/or efflux of isotope-labelled drugs or Rh123 are used frequently for the functional assay of Pgp in tumor cells. Several classes of compounds that inhibit Pgp-mediated efflux and enhance the accumulation and efficacy

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Abbreviations: Pgp, P-glycoprotein; MDR, multidrug resistance; IAAP, [^{125}I]iodoarylazidoprazosin; Rh123, rhodamine 123; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HRP, horseradish peroxidase; HBSS, Hanks' balanced salt solution; RT–PCR, reverse transcription–polymerase chain reaction.

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of anticancer compounds have been identified. MDR-reversing agents include calcium channel blockers (verapamil), calmodulin inhibitors (phenothiazines), indole alkaloids (reserpines), and nonpolar cyclic oligopeptides with immunosuppressant activity (cyclosporin A) [2]. Although Pgp mediates the transport of many structurally and functionally diverse compounds, many potent MDR-inhibiting compounds share common physical characteristics such as cyclicity, lipophilicity, and a positive or neutral charge at physiological pH. Many synthetic MDR modulators, including reversins 121 and 205 [3] and the cyclosporin D analog Valspodar (PSC 833) [4], successfully reverse the MDR phenotype *in vitro*. However, the efficacy of these compounds in animal studies and clinical trials has been disappointing due to dose-limiting toxicity. Accordingly, much effort is currently being expanded toward identifying natural compounds from plant origins that inhibit Pgp, reverse the MDR phenotype, and sensitize cancer cells to conventional chemotherapy without undesired toxicological effects. The other approach for MDR modulation is the modulation of the *MDR*1 gene. Studies of the *MDR*1 gene promoter sequence suggest that modulation of Pgp expression at the genetic level may be possible [5]. These types of MDR modulators may either block the induction of *MDR*1 gene expression or inhibit its promoter and down-regulate Pgp expression.

Curcumin is a natural phenolic coloring compound found in rhizomes of *Curcuma longa* Linn., commonly called turmeric. The curcumin 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, to color cheese and butter, as a cosmetic, and in some medicinal preparations [6,7]. Curcumin has a wide range of biological and pharmacological activities, including antioxidant [8–10] and anti-inflammatory properties [7], antimutagenic activity *in vitro* [11], anti-carcinogenic effects [12–14], hypocholesterolemic effects in rats [15], and hypoglycemic effects in humans [16]. The safety of *C. longa* and its derivatives has been studied in various animal models [17], and it is clear that turmeric is not toxic even at high doses in laboratory animals. A single feeding of a 30% turmeric diet to rats did not produce any toxic effects. In a 24-hr acute toxicity study, mice were fed dosages of 0.5, 1.0, and 3.0 g/kg of turmeric extract daily. There was no increase in the mortality rate when compared with the respective controls in either study. A 90-day treatment with turmeric extract resulted in no significant weight gain [18].

Due to its wide range of biological and pharmacological effects, lack of toxicity in animal models, cyclicity, and lipophilicity, curcumin was examined in the present study to determine possible interactions with Pgp expression and function. We demonstrated that curcumin down-regulates Pgp expression and reduces Pgp-mediated efflux in drug-resistant human cervical carcinoma cells (KB-V1). In addition, biochemical assays demonstrated that curcumin interacts directly with Pgp. In summary, these results suggest that curcumin may have chemosensitizing properties on the MDR phenotype as a result of its ability to modulate both the expression and function of *MDR*1.

2. Materials and methods

2.1. Materials

Commercial curcumin (77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin), Rh123, disodium ATP, sodium ortho-vanadate, and mouse monoclonal anti-P-glycoprotein (MDR) clone F4 were purchased from the Sigma Chemical Co. Dulbecco's Modified Eagle's Medium (DMEM), One-step RT–PCR reagent, TRIzol reagent, HBSS, and primers were purchased from GIBCO-BRL. HRP-conjugated goat anti-mouse IgG was purchased from Amersham. A SuperSignal® detection kit was purchased from Pierce. An MTT viability assay kit was purchased from Promega. IAAP, 2200 Ci/mmol, was obtained from Perkin Elmer Life Sciences.

2.2. Cell lines and culture conditions

A multidrug-resistant cervical carcinoma cell line (KB-V1) and a drug-sensitive cervical carcinoma cell line (KB-3-1) were gifts from Dr. Michael M. Gottesman (National Cancer Institute). Both cell lines were cultured in DMEM with 4.5 g of glucose/L plus 10% fetal bovine serum, L-glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL); 1 μg/mL of vinblastine was added to only the KB-V1 culture medium. These two cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°. When the cells reached confluency, they were harvested and plated for either subsequent passages or drug treatments.

The effects of curcumin on cell growth were observed by examining the morphology of the cultures with an inverted phase contrast microscope. The trypan blue exclusion test was used throughout the experiments to check cell viability. Curcumin cytotoxicity was quantified by including increasing concentrations of the compound in the culture medium and evaluating cell viability up to 4 days later.

2.3. Curcumin treatment and membrane preparations

KB-V1 or KB-3-1 cells were plated and grown to 80% confluency in T-25 cm^2 culture flasks in the presence or absence (vehicle control, 0.1% DMSO) of 1, 5, and 10 μM curcumin for 3 days. Cells were then washed with ice-cold PBS, harvested by scraping, and homogenized in 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride,

and 10 mM Tris–HCl, pH 7.4, for 30 strokes. The cell homogenates were centrifuged at 4000 *g* for 10 min at 4°. The supernatant was collected and centrifuged at 100,000 *g* for 1 hr at 4°. This pellet (plasma membrane) was resuspended in Laemmli buffer [19], and the protein concentration was measured by the method of Lowry *et al*. [20].

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

High Five insect cells in suspension culture were infected with the recombinant baculovirus carrying the human MDR1 cDNA with a six-histidine tag at the carboxy terminal end, and crude membranes were prepared as described previously [21].

2.5. RNA extraction and quantitative RT–PCR

RNA from KB-V1 cells was isolated with the TRIzol reagent®, and RT–PCR was performed using One-step RT–PCR® (GIBCO-BRL). For MDR1, the forward primer sequence used was GCCTGGCAGCTGGAAGACAAATACACAAAATT and the reverse primer sequence used was CAGACAGCAGCTGACAGTCCAAGAACAGGACT, corresponding to residues 406–437 and residues 657–688, respectively, of the published cDNA sequence [22]. Using these primers, PCR yields gave a 283-bp product. Evaluation of β-actin expression, used as a control of the RNA amount, was carried out by using the forward primer sequence CAGAGCAAGAGAGGCATCCT and the reverse primer sequence TTGAAGGTCTCAAACATGAT corresponding to residues 216–235 and residues 405–424, respectively, which yield a 201-bp product. Amplification was performed for 30 cycles of sequential denaturation (94°, 1 min); annealing (55°, 1 min); and extension (72°, 1 min). For the negative control, water was amplified for a total of 30 cycles to detect for possible contamination. A total of 10 μL of each PCR product was electrophoresed in 1× Tris/acetate/EDTA (TAE) electrophoresis buffer on a 1% agarose gel. Gels were stained with 2 μg/mL of ethidium bromide and photographed with Polaroid positive-negative film. The negative films were analyzed by scanning densitometry.

2.6. ATPase assays

ATPase activity of Pgp in crude membranes of High Five insect cells was measured by the endpoint, P_i assay as previously described [21,23]. This assay measures the amount of inorganic phosphate released over 15–20 min at 37° in the ATPase assay buffer. The crude membranes were incubated with increasing concentrations of curcumin in the presence and absence of 5 μM verapamil and 0.25 mM sodium ortho-vanadate for 5 min at 37°. The reaction was initiated by the addition of 5 mM ATP and quenched with SDS (2.5% final concentration); the amount of P_i released was quantitated using a colorimetric method [23]. Pgp-specific activity was recorded as the vanadate-sensitive ATPase activity.

2.7. Photoaffinity labeling of Pgp with IAAP

The crude membranes of High Five cells (50–100 μg) were incubated at room temperature (22–23°) in 50 mM Tris–HCl, pH 7.5, with IAAP (6 nM) for 5 min in the presence of the indicated concentration of curcumin or DMSO alone under subdued light. The samples were then illuminated with a UV lamp (365 nm) assembly (PGC Scientifics) for 10 min at room temperature (22–23°). Following SDS–PAGE on an 8% Tris-glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film at −70° for 18–24 hr. The radioactivity incorporated into the Pgp band was quantified as described [24] using a STORM 860 Phosphorimager system (Molecular Dynamics) and ImageQuant software.

2.8. Accumulation and efflux of Rh123

The measurement of Rh123 accumulation was performed as previously described [25]. Briefly, KB-V1 cells (5×10^5/sample) were incubated with 1 μg/mL of Rh123 in the dark at 37° in 5% CO_2 for 120 min. Curcumin (dissolved in DMSO) was added to cultures at the same time as Rh123. A final concentration of 0.4% DMSO (v/v) was used for all experiments and controls. Following Rh123 accumulation, cells were washed twice with ice-cold HBSS (without phenol red), placed in HBSS with 10% fetal bovine serum on wet ice, and analyzed using a FACScan flow cytometer (Becton-Dickinson) equipped with a 488-nm argon laser. The green fluorescence of Rh123 was measured by a 530 nm band-pass filter (hLi). Samples were gated on forward scatter and side scatter to exclude debris and clumps. A minimum of 10,000 events was collected for each sample.

For determination of Rh123 efflux, cells were loaded for 60 min with Rh123 in the absence of curcumin, and then the medium was replaced with drug-free medium containing curcumin, verapamil (a positive MDR reversal agent), or the vehicle (DMSO). Following efflux intervals of 60 min, the medium was removed, and the cells were washed twice with ice-cold HBSS and prepared for flow cytometry as above. As measured by trypan blue exclusion, the cells remained viable during the Rh123 accumulation and efflux studies with curcumin and verapamil.

2.9. Measurement of vinblastine cytotoxicity

Cells were incubated in 96-well plates (1.0×10^4/well), in 100 μL medium, before drug treatment at 37° for 24 hr. After 24 hr, the cells were variably treated in their respective medium (100 μL), and incubated for another 24 hr. The

metabolic activity of each well was determined by the MTT assay [26] and compared with that of untreated cells. Briefly, after removal of 100 μL medium, MTT stock dye solution was added (15 μL/100 μL medium) to each well, and the plates were incubated at 37° in 5% CO_2 atmosphere. After 4 hr, 100 μL of the solubilization/stop solution was added to each well, and mixed thoroughly to dissolve the dye crystals. Absorbance was measured by 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 wells)/(mean absorbance in control wells) × 100.

2.10. Western blot analysis

The cell membrane proteins (10 μg/lane) were separated on a 7.5% SDS–polyacrylamide gel and immunoblotted overnight onto nitrocellulose filters (GIBCO-BRL). The filters were incubated sequentially with mouse monoclonal anti-Pgp clone F4 at 1:5000 and HRP-conjugated goat anti-mouse IgG at a 1:20,000 dilution. Proteins were visualized by the SuperSignal® protein detection kit and quantitated by scanning densitometry.

2.11. Statistical analysis

Data are the means ± SEM from duplicate or triplicate samples of three independent experiments. Differences between the means were analyzed by one-way analysis of variance. Results were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Effect of curcumin on Pgp/MDR1 expression

A Western blot analysis for the level of Pgp in KB-V1 and KB-3-1 cell lines showed that the drug-resistant KB-V1 cells expressed large amounts of Pgp compared with the drug-sensitive KB-3-1 cells. Pgp was undetectable in KB-3-1 cells by the method used in our experiments (data not shown).

In preliminary experiments, KB-V1 cells were treated with 25 μM curcumin for 1–4 days. Pgp expression was found to be decreased by 11, 31, 60, and 64%, respectively, in response to 1, 2, 3, and 4 days of treatment (Fig. 1). The results clearly showed a significant decrease in Pgp expression at day 3; hence, subsequent experiments were designed to observe results at day 3. From a morphological examination of the cell cultures by the trypan blue exclusion method, it was apparent that a concentration of 25 μM curcumin did not produce toxic effects in KB-V1 cells under our experimental conditions.

The toxic effect of curcumin on KB-V1 cells for 1–4 days was then assessed by the trypan blue exclusion method. It was found that 1–30 μM curcumin was not toxic to KB-V1 cells. However, 35–100 μM curcumin caused toxicity to KB-V1 cells, as shown in Fig. 2. Since treatment of the cells with 1, 5, and 10 μM curcumin resulted in no significant effect on cell viability, we used these concentrations for further analyses. Pgp expression of KB-V1 cells decreased after treating the cells with 1, 5, and 10 μM curcumin for 3 days, as determined by Western blotting with monoclonal antibody, F4 (Sigma). Pgp levels

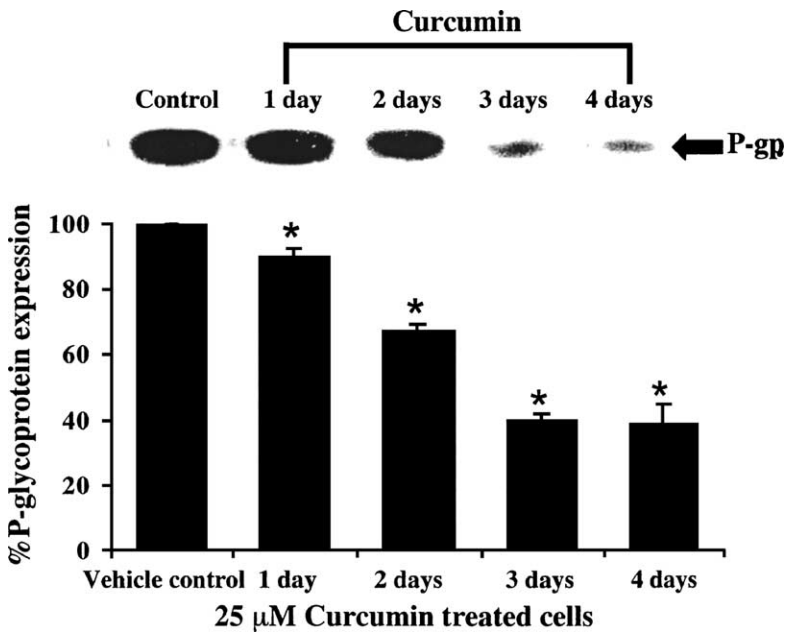

Fig. 1. Effect of curcumin on the Pgp protein level in KB-V1 cells. Pgp expression in KB-V1 cells cultured in 25 μM curcumin for 1, 2, 3, and 4 days was determined by Western blotting, using Pgp-specific MAb F4 (top), and quantitated by laser densitometry (bottom). The control culture for the top panel (0.1% DMSO) was harvested on day 4. The result of one typical experiment out of three is shown. Key: (*) significantly different from the vehicle control ($P < 0.05$).

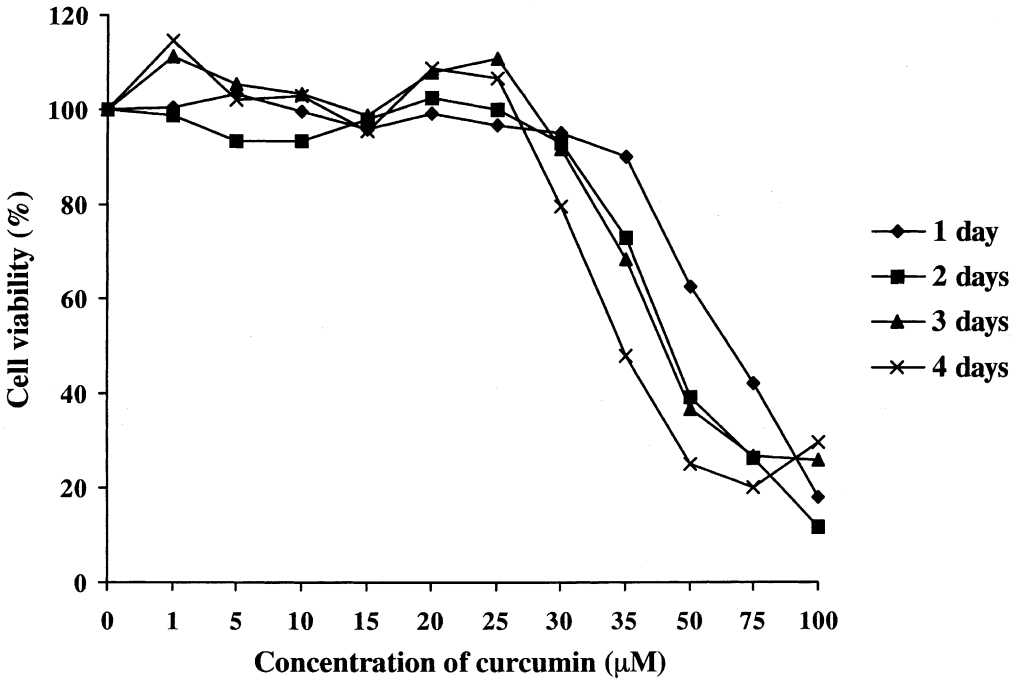

Fig. 2. Effect of curcumin on KB-V1 cell growth, as measured by the trypan blue exclusion method. Cells (1.0×10^4/mL) were cultured at various curcumin concentrations for 1–4 days. The result of one typical experiment out of three independent experiments is depicted.

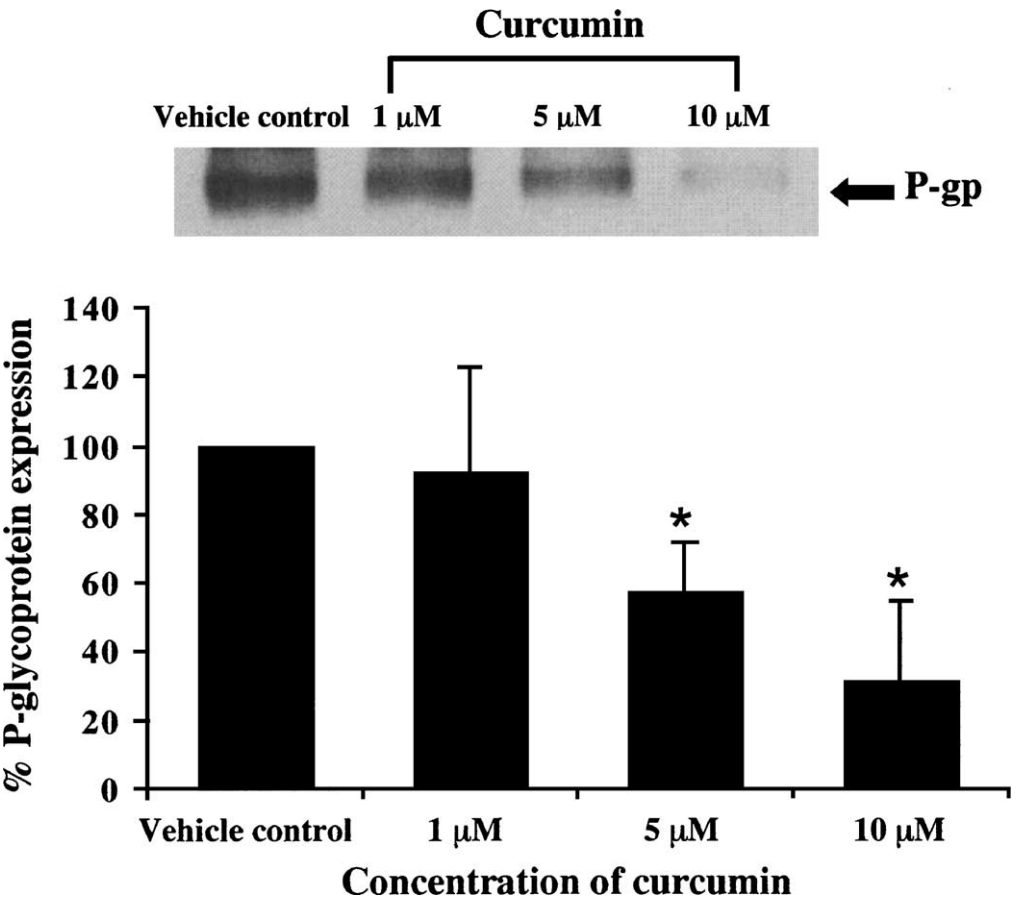

Fig. 3. Pgp expression in KB-V1 cells cultured in 1, 5, and 10 μM curcumin for 3 days. The Pgp protein level was determined by Western blotting using MAb F4 (top), and quantified by laser densitometry (bottom). Data are means ± SEM of three independent experiments. Key: (*) significantly different from the vehicle control ($P < 0.05$).

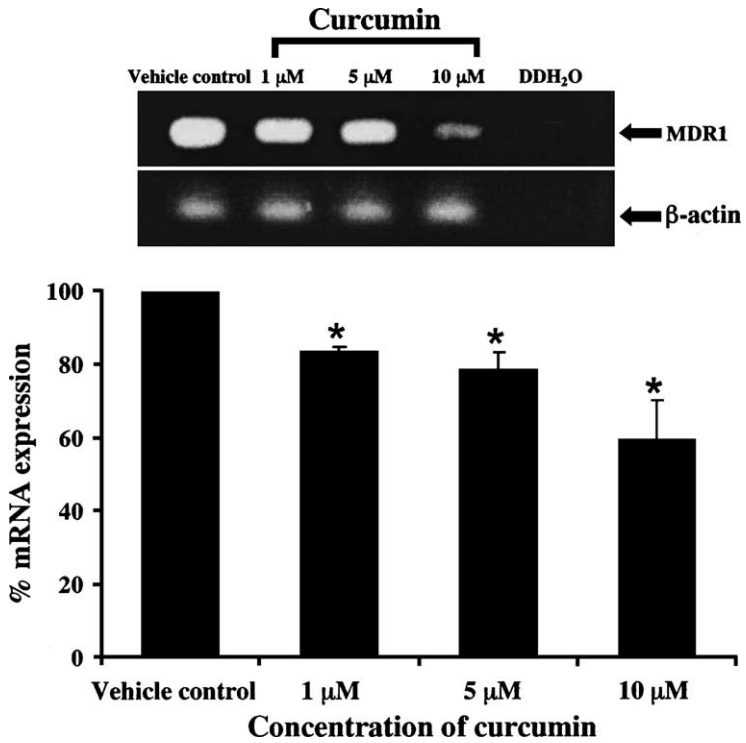

Fig. 4. Analysis of the effects of curcumin treatment on MDR1 mRNA levels in the KB-V1 cell line. The MDR1 and β-actin mRNA levels following treatment with different curcumin concentrations were determined by RT–PCR in KB-V1 cells after 3 days. The PCR products (283 bp MDR1 and 201 bp β-actin) were run on 1% agarose gel. The bands from the autoradiographs were quantitated by laser densitometry. *MDR*1 expression was measured and normalized to β-actin expression. Double-distilled water (DDH_2O) was used as a negative control. Data are means ± SEM of three independent experiments. Key: (∗) significantly different from the vehicle control ($P < 0.05$).

in KB-V1 cells were decreased by 8, 43, and 69% in response to treatment with 1, 5, and 10 μM curcumin, respectively, compared with the vehicle control at day 3 of growth (Fig. 3).

To verify if curcumin could modulate the spontaneous Pgp expression (mRNA) occurring *in vitro*, 1–10 μM curcumin was added to the culture medium and the cultures were examined at 72 hr. Expression of MDR1 mRNA was

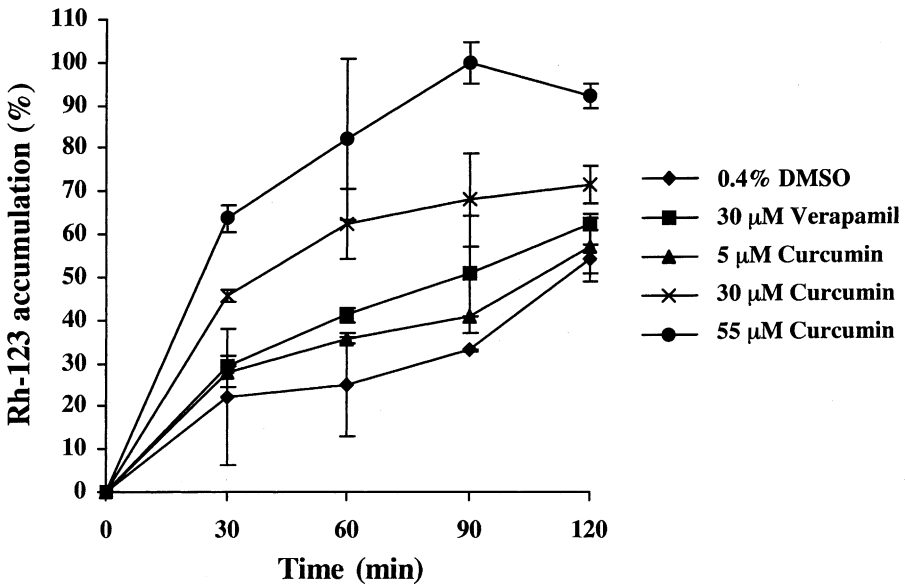

Fig. 5. Effect of curcumin on the accumulation of Rh123 in KB-V1 cells. Cells were treated with the indicated concentrations of curcumin (5, 30, and 55 μM), vehicle control (0.4% DMSO), and 30 μM verapamil (positive control). Rh123 (1 μg/mL) was added, and the cells were incubated for 120 min at 37° in the dark. Cells were then harvested and used immediately to measure Rh123 fluorescence using FACScan. Data are means ± SEM from three independent experiments, each carried out in triplicate.

determined by measuring the PCR product after amplification. Values for the expression of MDR1 mRNA (after normalization to β-actin expression) by KB-V1 cells after treatment with 1, 5, and 10 μM curcumin were decreased by 17, 22, and 40%, respectively, in three independent experiments (Fig. 4).

3.2. Modulation of intracellular Rh123 accumulation and efflux by curcumin

To examine the function of Pgp on the surface of viable cells, Rh123 accumulation and efflux studies were chosen because it appears to be a sensitive indicator of Pgp activity when assayed by FACScan [27,28]. Figure 5 demonstrates the increase in Rh123 accumulation after treatment with either verapamil (positive control) or curcumin. Rh123 accumulation presented a clear concentration-dependent relationship with curcumin (5–55 μM), compared with the vehicle control. The effect of curcumin on Pgp-mediated efflux of Rh123 in KB-V1 and KB 3-1 cells was examined. As shown in Fig. 6, curcumin caused a significant decrease in the amount of Rh123 effluxed (when KB-V1 cells were treated with curcumin, the percentage of Rh123 retention increased by 2.6-fold compared with the vehicle control). However, in KB 3-1 cells, there was no change in Rh123 retention in the presence of curcumin.

3.3. Effect of curcumin on vinblastine cytotoxicity

Because curcumin inhibited Pgp efflux activity, we examined its effect on vinblastine-induced cytotoxicity as measured by inhibition of cell growth (MTT assay). Incubation of wild-type KB-3-1 cells with increasing amounts of vinblastine resulted in a decrease in cell growth that was not affected by incubation with curcumin (data not shown). In drug-resistant KB-V1 cells, co-incubation of vinblastine with curcumin resulted in a significant increase

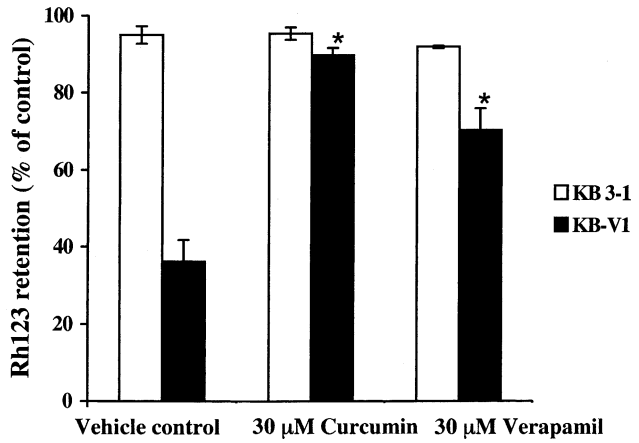

Fig. 6. Effect of curcumin on Rh123 retention in KB-3-1 and KB-V1 cells. Cells were incubated with Rh123 for 60 min, washed, and resuspended in medium with 30 μM curcumin, 30 μM verapamil (positive control), or 0.4% DMSO (vehicle control) for 60 min. Cells were then harvested, and Rh123 fluorescence was measured using FACScan. Data are means ± SEM from three independent experiments, each done in triplicate. Key: (*) significantly different from the vehicle control ($P < 0.05$).

in the cytotoxicity of vinblastine (Fig. 7). In this experiment, 20 μM curcumin was used because curcumin itself at 35–100 μM inhibited cell growth. The higher concentration used in the drug accumulation and drug efflux experiments (Figs. 5 and 6) did not affect cell viability because the incubation time was short (2 hr).

3.4. Biochemical evidence for the interaction of curcumin with Pgp: effect of curcumin treatment on ATPase activity and photoaffinity labeling of Pgp with IAAP

The results described above suggest that curcumin inhibits the transport function of Pgp. To assess whether curcumin interacts directly with the multidrug transporter, we determined its effect on ATPase activity and labeling of Pgp with the photoaffinity analog of a substrate. The data in

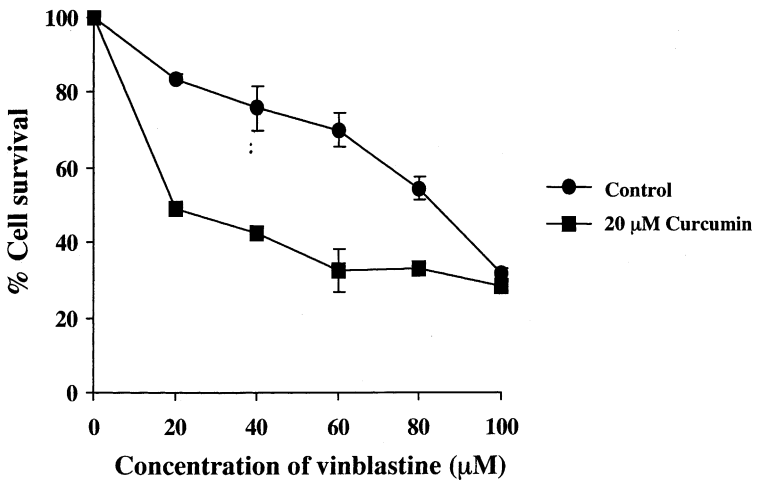

Fig. 7. Effect of curcumin on the cytotoxicity of vinblastine. KB-V1 cells (1.0×10^4/well) in 100 μL medium were grown in the presence of 1% DMSO (vehicle control) or 20 μM curcumin in the presence of different concentrations of vinblastine. The number of viable cells was determined by the MTT assay. Data represent means ± SEM, N = 3.

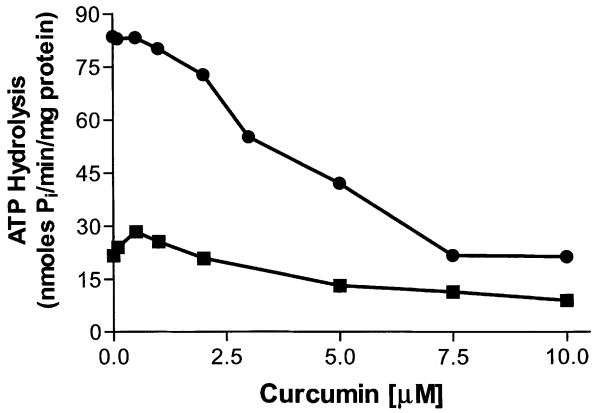

Fig. 8. Effect of curcumin on basal and verapamil-stimulated ATPase activity of Pgp. ATPase activity of Pgp in crude membranes of High Five insect cells was assayed as described in Section 2. Crude membranes (100 μg protein/mL) were incubated with the indicated concentrations of curcumin in the presence of 5 μM verapamil (●) or an equivalent volume of DMSO (■). Data are representative of three independent experiments.

Fig. 8 show the effect of curcumin on basal and verapamil-stimulated ATPase activity of Pgp in High Five insect cell membranes. Similar to a variety of compounds (reviewed in Ref. [1]), curcumin was also found to stimulate ATPase activity at low (0.5 to 1 μM) concentrations but at high concentrations it inhibited this activity. In addition, curcumin also inhibited verapamil-stimulated ATPase activity in a concentration-dependent manner (50% inhibition was observed at 3–4 μM, N = 5). We also studied the effect of curcumin on the photoaffinity labeling of Pgp with IAAP, an analog of prazosin, which has been shown to be a substrate of the transporter [1,21,24]. The data in Fig. 9 clearly demonstrate that curcumin inhibited the incorporation of IAAP into Pgp in a concentration-dependent manner. The concentration of curcumin required for 50% inhibition of IAAP incorporation was 2.75 ± 0.52 μM (mean ± SD, N = 5).

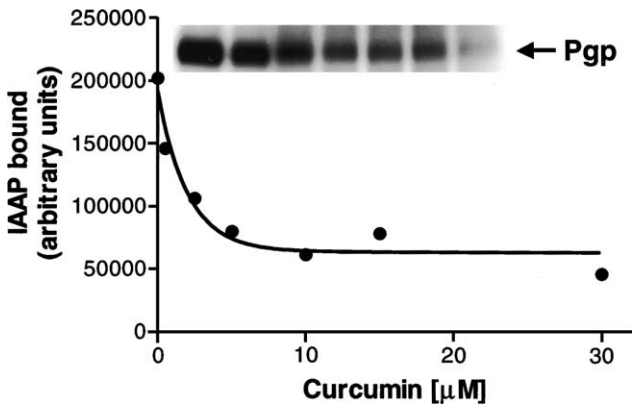

Fig. 9. Effect of curcumin on photoaffinity labeling of Pgp with IAAP. Crude membranes (1 mg protein/mL) were labeled with 6 nM IAAP after pretreatment with the indicated concentrations of curcumin for 5 min at 22° as described in Section 2. The autoradiogram (20 μg protein/lane) shows incorporation of IAAP into the Pgp band in the presence of 0 (DMSO alone), 1, 2.5, 5, 10, 15, and 30 μM curcumin, respectively. The data were fitted by non-linear least squares regression analysis using the software GraphPad Prism 2.0 for the PowerPC Macintosh and are representative of three independent experiments.

4. Discussion

Overexpression of Pgp has been well established as the cause of the MDR phenotype in many *in vitro* selected drug-resistant cell lines. In many human cancers, the presence of Pgp/*MDR*1 has been demonstrated using monoclonal antibodies or gene probes [29–31]. Due to their drug resistance, KB-V1 cells have been shown to express only Pgp at a high level [32] on their plasma membrane, but Pgp was not expressed in the drug-sensitive cells. The KB-V1 phenotype was selected for by subjecting KB-3-1 cells in a step-wise fashion to increasing concentrations of vinblastine [33]. The level of Pgp in KB-V1 cell membranes is about 1% of total plasma membrane protein [34]. As KB-3-1 and KB-V1 cell lines have been characterized extensively with respect to the phenomenon of MDR as well as the function of Pgp, we decided to use these cell lines to assess the effect of curcumin on the expression and function of Pgp.

Curcumin, a diferuloylmethane, which inhibits the proliferation of many cancer cell lines, has potential as an anticancer agent [35,36]. Curcumin has shown much promise in the prevention of chemically induced carcinogenesis in animal models [12–14]. The present study demonstrates the possibility of using curcumin in cancer chemotherapy as an MDR modulator. Curcumin at 1, 5, and 10 μM decreased Pgp expression in KB-V1 cells. From experiments that test cell toxicity by the trypan blue exclusion method, curcumin at 1 and 5 μM did not affect the growth of KB-V1 cells compared with the vehicle control.

The effect of curcumin on Pgp expression was established by western blotting and RT–PCR. Initially, a concentration of 25 μM curcumin was used in this study, since previous experiments indicated that high concentrations (50–150 μM) of curcumin induced a significant loss of cell viability in primary rat hepatocyte cultures; however, low (up to 25 μM) concentrations of curcumin showed no effect on cell viability [37]. The results of our experiments revealed that 25 μM curcumin did not cause toxicity in KB-V1 cells; however, continuous exposure of KB-V1 cells to higher concentrations (from 35 to 100 μM) of curcumin induced a significant loss of cell viability. Therefore, only a low concentration of curcumin was employed in these studies.

The experiment in Fig. 1 shows the effect of curcumin treatment for 1–4 days on Pgp expression. First we determined that curcumin clearly inhibited Pgp expression, as analyzed by western blotting. When 1, 5, and 10 μM curcumin was added to KB-V1 for 3 days, the results showed a decrease in Pgp expression with increasing

curcumin concentrations, indicating that curcumin inhibits the level of immunoreactive Pgp protein observed in KB-V1 cells and reduces the level of MDR1 mRNA under the same conditions as well. These results are quite similar to the effects seen with other MDR modulators, such as Valspodar (PSC 833) [4], reversin 121, and reversin 205 [3], which decrease MDR1 mRNA levels. No information has been reported on curcumin modulation of Pgp expression in tumor cells. This is the first report that curcumin can down-regulate the expression of a multidrug-resistance transporter in mammalian cells. Although the mechanism by which curcumin produced Pgp down-regulation is not deducible from the present data, it is of interest to recall that AP-1 transcription factor is required for the positive regulation of *MDR*1 expression [38]. Curcumin has been reported to be a potent inhibitor of the AP-1 transcription factor [39,40]. Therefore, the existence of some regulatory features in *MDR*1 gene expression could explain the inhibition of *MDR*1 expression in KB-V1 cells by curcumin.

In the present study, we also investigated the effects of curcumin on Pgp function *in vitro*. Curcumin caused a substantial increase in the accumulation of Rh123 in KB-V1 cells, and inhibited the efflux of Rh123, but had no effect on drug-sensitive cells (KB-3-1), which do not overexpress Pgp. Since Rh123 is known to be a good substrate for Pgp, we concluded that curcumin modulates intracellular Rh123 levels by inhibiting Pgp. Since the time of exposure of cells to curcumin in these experiments was short (1–2 hr), it is unlikely that curcumin acts by down-regulating *MDR*1 gene transcription and, therefore, reducing the level of cellular Pgp. However, we examined the effect of curcumin on the expression of Pgp at the protein (Western blotting) and mRNA (RT–PCR) levels. There was no difference in Pgp expression in KB-V1 cells when treated with curcumin for 1–2 hr (data not shown).

In agreement with the above data, curcumin also increased the toxicity of vinblastine in KB-V1 cells, but not in wild-type KB-3-1 cells. This demonstrates that curcumin can partially reverse MDR in cells that express Pgp. Taken together, our data indicate that curcumin increases intracellular drug levels by modulating Pgp function and expression. The modulation of the function of Pgp by curcumin was demonstrated further by its effect on the basal and verapamil-stimulated ATPase activity and the photoaffinity labeling of Pgp with IAAP. Similar to earlier studies with cyclosporin A and other modulators [41–43], curcumin also inhibited verapamil-stimulated ATPase activity of Pgp (Fig. 8). In addition, curcumin effectively inhibited photoaffinity labeling of Pgp with IAAP in a concentration-dependent manner (Fig. 9). These biochemical assays clearly demonstrate that curcumin interacts directly with the transporter and possibly binds to the same site as other agents such as prazosin and verapamil. Additional detailed work is needed to elucidate the nature of the interaction of curcumin with Pgp.

In conclusion, the present studies suggest that curcumin could be considered as a promising lead compound for the design of more efficacious MDR chemosensitizers. Although the present experiments demonstrate that curcumin is an effective inhibitor of Pgp function and expression *in vitro*, animal experimentation is required to determine if curcumin has potential as an effective and safe chemosensitizer for treating cancers expressing Pgp-mediated MDR.

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