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Modulation of function of three ABC drug transporters, P-glycoprotein (ABCB1), mitoxantrone resistance protein (ABCG2) and multidrug resistance protein 1 (ABCC1) by tetrahydrocurcumin, a major metabolite of curcumin

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Abstract

Many studies have been performed with the aim of developing effective resistance modulators to overcome the multidrug resistance (MDR) of human cancers. Potent MDR modulators are being investigated in clinical trials. Many current studies are focused on dietary herbs due to the fact that these have been used for centuries without producing any harmful side effects. In this study, the effect of tetrahydrocurcumin (THC) on three ABC drug transporter proteins, P-glycoprotein (P-gp or ABCB1), mitoxantrone resistance protein (MXR or ABCG2) and multidrug resistance protein 1 (MRP1 or ABCC1) was investigated, to assess whether an ultimate metabolite form of curcuminoids (THC) is able to modulate MDR in cancer cells. Two different types of cell lines were used for P-gp study, human cervical carcinoma KB-3-1 (wild type) and KB-V-1 and human breast cancer MCF-7 (wild type) and MCF-7 MDR, whereas, pcDNA3.1 and pcDNA3.1-MRP1 transfected HEK 293 and MXR overexpressing MCF7AdrVp3000 or MCF7FL1000 and its parental MCF-7 were used for MRP1 and MXR study, respectively. We report here for the first time that THC is able to inhibit the function of P-gp, MXR and MRP1. The results of flow cytometry assay indicated that THC is able to inhibit the function of P-gp and thereby significantly increase the accumulation of rhodamine and calcein AM in KB-V-1 cells. The result was confirmed by the effect of THC on [^{3}H]-vinblastine accumulation and efflux in MCF-7 and MCF-7MDR. THC significantly increased the accumulation and inhibited the efflux of [^{3}H]-vinblastine in MCF-7 MDR in a concentration-dependent manner. This effect was not found in wild type MCF-7 cell line. The interaction of THC with the P-gp molecule was clearly indicated by ATPase assay and photoaffinity labeling of P-gp with transport substrate. THC stimulated P-gp ATPase activity and inhibited the incorporation of [^{125}I]-iodoarylazidoprazosin (IAAP) into P-gp in a concentration-dependent manner. The binding of [^{125}I]-IAAP to MXR was also inhibited by THC

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suggesting that THC interacted with drug binding site of the transporter. THC dose dependently inhibited the efflux of mitoxantrone and pheophorbide A from MXR expressing cells (MCF7AdrVp3000 and MCF7FL1000). Similarly with MRP1, the efflux of a fluorescent substrate calcein AM was inhibited effectively by THC thereby the accumulation of calcein was increased in MRP1-HEK 293 and not its parental pcDNA3.1-HEK 293 cells. The MDR reversing properties of THC on P-gp, MRP1, and MXR were determined by MTT assay. THC significantly increased the sensitivity of vinblastine, mitoxantrone and etoposide in drug resistance KB-V-1, MCF7AdrVp3000 and MRP1-HEK 293 cells, respectively. This effect was not found in respective drug sensitive parental cell lines. Taken together, this study clearly showed that THC inhibits the efflux function of P-gp, MXR and MRP1 and it is able to extend the MDR reversing activity of curcuminoids *in vivo*. (Mol Cell Biochem **296:** 85–95, 2007)

Key words: ABC transporter, ATP hydrolysis, chemosensitivity, drug transport, multidrug resistance, P-glycoprotein, tetrahydrocurcumin

Abbreviations: ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP1, multidrug resistance protein 1; MXR, mitoxantrone resistance protein; THC, tetrahydrocurcumin; [I^{125}]-IAAP, iodoarylazidoprazosin.

Introduction

During the past five decades, the development and strategic use of anticancer drugs has become one of the most important ways of controlling malignant disease. However, the emergence of drug resistance has made many of the currently available chemotherapeutic agents ineffective. Many studies using tumor cell lines as model systems have demonstrated that exposure of cells to one drug often results in cross-resistance to many other structurally, chemically, and functionally distinct agents. This phenomenon is broadly known as the multidrug resistance (MDR) phenotype [1, 2].

One of the major mechanisms of MDR is the enhanced ability of tumor cells to actively efflux drugs, leading to a decrease in cellular drug accumulation below toxic levels. Active drug efflux is mediated by several members of the ATP-binding cassette (ABC) superfamily of membrane transporters, which have now been subdivided into seven families designated A through G [3, 4]. Among these ABC families, the classical MDR is attributed to the elevated expression of ABCB1 (P-gp), ABCC1 (MRP1), and ABCG2 (MXR) [5, 6].

The clinical importance of P-glycoprotein (P-gp), MRP1 and mitoxantrone resistance protein (MXR) for MDR and cancer treatment has led to the investigation of the inhibiting properties of several compounds on these transporters. The calcium channel blocking agent verapamil was the first drug described as an inhibitor of the P-gp efflux mechanism [7]. After this discovery, several other compounds have been studied for their inhibitory effects, for examples, valspodar, GF120918, and LY335979 [8–10]. Although these agents are effective, one of the major problems with most of them is that the *in vivo* plasma concentrations required to inhibit P-gp are too high, and result in severe toxic side effects. At present, due in part to the disappointing results associated with the many side effects of modulators that have been used in clinical trials, current research efforts are directed towards the identification of novel compounds with attention to dietary natural products. The advantage is that they exhibit little or virtually no side effects, and do not further increase the patient's medication burden.

Tetrahydrocurcumin (THC) is the ultimate metabolite of the curcumins *in vivo* [11–14]. It has been proposed that after absorption most curcumins are reduced to dihydrocurcumin and THC by endogenous reductase system, and subsequently are converted to monoglucuronoside conjugates by UDP-glucuronosyl transferase [11, 13, 15]. To date curcumin-glucuronide, dihydrocurcumin-glucuronoside, THC-glucuronoside, and THC have been demonstrated as the major curcumin metabolites *in vivo* [11, 13, 14, 16]. However, since THC was very stable in various pH values of 0.1 M PBS and more easily absorbed from the gastrointestinal tract [11], it might play crucial roles in curcumin-induced biological effects.

Previously, we reported that curcumin I is the most active form of curcuminoids purified from turmeric to modulate the function of P-gp [17], MRP1 [18] and MXR [19]. This study is an extended report to show that major metabolite of curcumin, THC, is also able to inhibit the function of P-gp, MRP1 and MXR. These findings provide additional evidences to develop curcumin and THC as MDR modulator to use in combination with conventional chemotherapy.

Materials and methods

Chemicals and media

DMEM (Dulbecco's Modified Eagle's Medium), Trypsin-EDTA, HBSS (Hank Balance Salt solution), and PBS (Dulbecco's Phosphate Buffered Saline) were purchased

from GIBCO-BRL (Grand Island, NY). Fetal Bovine Serum was purchased from Hyclone (Logan, UT). The MTT viability kit and BSA (Bovine Serum Albumin) were purchased from Promega (Madison, WI). Calcein-AM was obtained from Molecular Probes, Inc. (Eugene, Oregon). [^{125}I]-iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Mitoxantrone, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] dye, ouabain and all other chemicals were purchased from Sigma (St. Louis, MO).

Preparation of THC

Curcumin was converted to THC by hydrogenation with 10% PtO_2 as the catalyst according to the method of Venkateswarlu *et al.* [20]. After hydrogenation, THC was purified by preparative TLC (5% MeOH in $CHCl_3$, Rf = 0.86). The identity and purity of THC was confirmed by using MS, IR and NMR spectra [21]. THC: MS(m/z, rel int): 372 (55%, M^+), 137 (100%); IR (KBr) ν_{max} 3418(OH), 1603 (C=O), 1033 (OCH_3) cm^{-1}; ^{1}H NMR (CDCl3, 500 MHz): δ 6.8 (2H, d, J = 8.2 Hz, 5′, 5″), δ 6.67 (2H, s, 2′, 2″), 6.65 (2H, d, J = 7.2 Hz, 6′, 6″), 5.50 (2H, broad, OH), 5.42 (1H (enol), s, 4), 3.85 (6H, s, OCH_3), 3.50 (2H (keto), s, 4), 2.75–2.89 (4H, m, 2, 6), 2.54 (4H, t, J = 8.1 Hz, 1, 7). Chemical structure of THC is shown in Fig. 1.

Cell lines and culture conditions

Human cervical carcinoma KB-3-1 and KB-V-1 were cultured in DMEM with 10% fetal calf serum, L-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml); 1 μg/ml of vinblastine was added only to the KB-V-1 culture medium. MCF-7 and MCF-7 MDR (maintained in 60 ng/ml of colchicine) cells. were grown as monolayer culture at 37 °C in 5% CO_2 using DMEM and supplemented with 1 mM or 110 mg/l sodium pyruvate, plus 10% FBS, 5 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml) [22]. pcDNA3.1-HEK 293 and MRP1-HEK 293 (the drug resistant HEK cells transfected with pcDNA3.1 plasmid carrying MRP1 cDNA) were used. The stable transfectants were selected in G418, (800 μg/ml), (Mediatech Inc., Herndon, VA), and the selected clones were cultured in 5 μM etoposide (VP-16) [23]. MCF7 cells were cultured in RPMI with 10% FBS whereas MCF7FLV1000 (482R-ABCG2) or MCF7AdrVp3000 (482T-ABCG2) cells overexpressing ABCG2 were cultured in the presence of 1 μg/ml flavopiridol or 3 μg/ml doxorubicin and 5 μg/ml verapamil, respectively [24–26]. These cell lines were provided by Dr. Susan Bates (NCI/NIH). The cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C.

MTT assay and chemosensitivity testing

Cytotoxicity of THC and the effect of THC on anticancer drugs cytotoxicity in drug resistance KB-V-1, MRP1-HEK 293 and MCF-7 AdrVp3000 cells were determined by MTT assay. The MTT assay was performed by plating cells in 96-well plate (5.0×10^3 cells), in 100-μl medium, and incubating before drug treatment at 37 °C for 24 h. After 24 h, various drugs and/or THC were added in medium (100 μl) and incubated for another 72 h. The metabolic activity in each well was determined by the MTT assay [17, 18] and compared to untreated cells. After removal of 100 μl medium, MTT stock dye solution (5 mg/ml in PBS) 20 μl was added to each well. The plates were incubated at 37 °C under 5% CO_2 for 4 h. Most of the medium from each well was removed, leaving about 10–20 μl medium and purple formazan crystals. DMSO (200 μl) was added in the well to dissolve the crystals and the plates were shaken for 10 min. The absorbance at 540 nm with a reference wavelength of 630 nm was read on ELISA plate reader. 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 [17, 18].

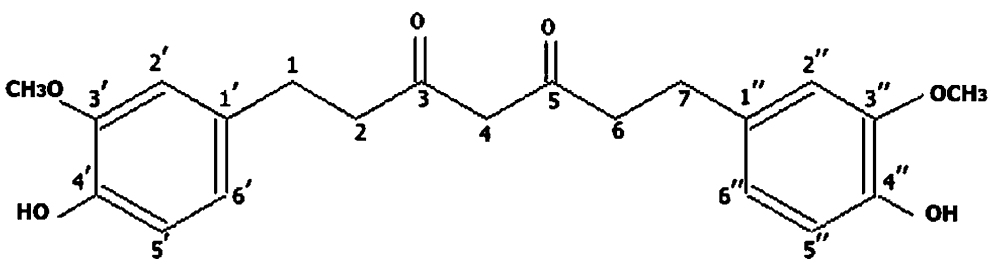

Fig. 1. Chemical structure of tetrahydrocurcumin (THC).

Fluorescent drug accumulation assay by FACS

Accumulation assays with KB-3-1, KB-V-1 (substrate used; rhodamine 123, 0.5 μg/ml or calcein-AM, 0.5 μM), or pcDNA 3.1-HEK, MRP1-HEK 293 (substrate used; calcein-AM, 0.5 μM) or MCF7, MCF7FLV1000 and MCF7AdrVp3000 (substrate used; pheophorbide A, 5 μM or mitoxantrone, 5 μM) cells in presence or absence of specific inhibitors (P-gp; 10 μM cyclosporin A, MRP1; 20 μM MK571, ABCG2; 10 μM FTC) or THC 50 μM were performed as described previously [17, 18, 25]. For all samples, 10,000 events were counted and the analysis was performed with Cell Quest software (Becton–Dickinson Immunocytometry systems). The mean fluorescence intensity was calculated using the histogram stat program in Cell Quest software.

Radiolabeled drug accumulation

The effect of THC on P-gp mediated drug transport was confirmed by monitoring the intracellular radiolabeled drug accumulation. The method was modified from Plouzek *et al.* [27]. MCF-7MDR cells were seeded at 500,000 cells per well in 6-well plates and incubated overnight. The cells were exposed to 0.05 μCi ^{3}H-vinblastine/ml (specific activity: 10.8 Ci/mmol) in the presence of THC (12.5, 25, 50, and 75 μM) or 0.5% DMSO (control) for 60 min at 37 °C. The medium was removed and the plates were washed with ice-cold PBS (pH 7.4). The cells were then harvested by centrifugation at 10,000 rpm at 4 °C for 2 min. Cells were dissolved with 200 μl of 3 N NaOH, then neutralized with 100 μl of 6 N HCl. Cell lysate, 250 μl was pipetted into the scintillation vial and 3 ml of scintillation fluid was added to each vial. The radioactivity was counted by β-scintillation counter. The protein concentration was determined by Bradford method using 10 μl of cell lysate in 96-well plate. The amount of intracellular radioactivity (counting unit) was calculated in the terms of percentage of vehicle control.

To determine the drug efflux [28], cells were plated out as described for drug accumulation experiments. In order to load cells with radiolabeled drug, the cells were incubated for 60 min at 37 °C with 0.05 μCi ^{3}H-vinblastine/ml in the presence of 20 μM verapamil. The cells were then washed with ice-cold PBS (pH 7.4), following which medium containing THC (12.5, 25, 50, and 75 μM) or 0.5% DMSO was added. After incubation at 37 °C for 30 min, cells were washed with ice cold PBS (pH 7.4) and harvested. The amount of intracellular radioactivity was determined by scintillation counter.

Preparation of crude membranes from High Five insect cells infected with recombinant baculovirus carrying the human MDR1 or ABCG2 gene

High Five insect cells (Invitrogen, Carlsbad, CA) were infected with the recombinant baculovirus carrying the human MDR1 cDNA or human G482-ABCG2 cDNA with a six-Histidine tag at the C-terminal end and crude membranes were prepared and stored at −80 °C, as described previously [29–31].

ATPase assays

The ATPase activity in crude membranes of high five insect cells expressing P-gp or ABCG2 was measured by the endpoint, P_i release assay as described previously [29, 32]. This assay measures the amount of inorganic phosphate released for 20 min at 37 °C in the ATPase assay buffer (0.05 mM KCl, 5 mM sodium azide, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 50 mM MOPS pH 7.5). Crude membranes (100 μg protein/ml) were incubated with increasing concentrations of THC in presence or absence of 0.3 mM sodium orthovanadate (for P-gp) or BeFx (0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) (for ABCG2). 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. P-gp or ABCG2 specific activity was recorded as the vanadate-sensitive or BeFx sensitive ATPase activity respectively [33].

Binding of P-gp or ABCG2 with [^{125}I]-IAAP

The crude membranes of P-gp expressing high five insect or 482R-ABCG2 expressing MCF7FLV1000 cells (50–100 μg protein) were incubated with increasing concentrations of THC (0–100 μM) at room temperature in 50 mM Tris–HCl, pH 7.5, for 10 min. [^{125}I]-IAAP (3–6 nM; specific activity: 2200 Ci/mmol) 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, Gaithersburg, MD) fitted with two Black light (self-filtering) UV-long wavelength – F15T8BLB tubes for 10 min at room temperature (21–23 °C). The labeled ABCG2 was immunoprecipitated by adding 800 μl of RIPA buffer containing 1% aprotinin followed by addition of 10 μg of BXP-21 antibody. Protein A sepharose beads (100 μl) were added and further incubated at 4 °C for 16 h. The protein A sepharose beads were pelleted at 4 °C and washed with RIPA containing 1% aprotinin. SDS-PAGE

sample buffer (50 μl) was added and incubated at 37 °C for 1 h. The samples were separated on a 7% Tris–acetate gel at constant voltage. The gels were dried under vacuum and were exposed to X-ray film for 12–24 h at −80 °C. The radioactivity incorporated into the P-gp or ABCG2 band was quantified using the STORM 860 Phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and software ImageQuaNT [30].

Results

THC increased the accumulation of rhodamine 123 and calcein-AM in P-gp expressing cells

We have first evaluated the inhibitory effect of THC on P-gp function by measuring the intracellular accumulation of two fluorescence substrates of P-gp: rhodamine and calcein-AM. As shown in Fig. 2a–d, THC (50 μM) inhibited the efflux function of P-gp and thereby increased the accumulation of rhodamine and calcein-AM in KB-V-1 cells. This inhibitory effect was observed only in drug resistant KB-V-1 but not in drug sensitive KB-3-1. A potent modulator of P-gp, cyclosporin A (CsA) was also included in this experiment as positive inhibitor. As shown in Fig. 2a–d, 10 μM CsA inhibited the efflux thereby increased the accumulation of calcein in KB-V-1 but not in KB-3-1. Thus, the results confirm the inhibitory effect of THC on P-gp efflux function.

THC inhibited the efflux of mitoxantrone and pheophorbide in 482 R-ABCG2 expressing MCF7FLV1000 cells

The effect of THC on the accumulation of MXR-fluorescent substrates was tested to characterize the inhibitory effect on MXR-mediated transport using flow cytometry. The accumulation of mitoxantrone and pheophorbide was studied in the control MCF7 and 482R-ABCG2 overexpressing MCF7FLV1000 cells. The cells were incubated with 5 μM of mitoxantrone and pheophorbide A in the presence of 10 μM of FTC or 50 μM of THC at 37 °C for 45 min. The accumulated mitoxantrone and pheophorbide A in MCF7 and MCF7FLV1000 cells after 45 min was analyzed as described in 'Materials and methods'. As shown in Fig. 2e–h, the presence of 50 μM of THC inhibited the efflux of mitoxantrone (Fig. 2f) or pheophorbide A (Fig. 2h) in the 482R-ABCG2 overexpressing MCF7FLV1000 cells while it did not show any significant effect on the accumulation of the two substrates in the control MCF7 cells (Fig. 2e, g). Similar result were obtained when 482T-ABCG2 overexpressing MCF7AdrVp3000 were used instead of MCF7FLV1000 cells (data not shown).

THC increased the accumulation of calcein-AM in MRP1-HEK293 cells

As shown in Fig. 2i–j, THC (50 μM) inhibited the efflux function of MRP1 and thereby increased the accumulation of calcein-AM in MRP1-HEK293 cells. This inhibitory effect was observed only in drug resistant MRP1-HEK293 not in its parental pcDNA3.1-HEK293 cells. A potent modulator of MRP1, MK571 was also included in this experiment as positive inhibitor. As shown in Fig. 2i–j, 20 μM MK571 inhibited the efflux thereby increased the accumulation of calcein AM in MRP1-HEK293 not in pcDNA3.1-HEK293. Thus, the results confirm the inhibitory effect of THC on MRP1 efflux function.

THC increased the accumulation and inhibited the efflux of [^{3}H]-vinblastine in MCF-7MDR

To confirm the effect of THC on the P-gp function, the activity of P-gp was assessed by determining the intracellular retention of radiolabeled drug, [^{3}H]-vinblastine in drug resistant MCF-7MDR and drug sensitive MCF-7 cells. This method had been claimed to be the appropriate model to test the actual vinblastine accumulation in the intact cells [27]. In drug resistant MCF-7MDR cells, exposure to THC resulted in an significant ($p < 0.05$) increase of [^{3}H]-vinblastine accumulation in cells, in a dose-dependent manner (12.5–75 μM), compared with DMSO control (Fig. 3a). Like [^{3}H]-vinblastine accumulation experiments, [^{3}H]-vinblastine efflux from cells resulted in significant increase of [^{3}H]-vinblastine retention dose dependently (Fig. 3b). The amount of intracellular [^{3}H]-vinblastine in wildtype MCF-7 cells was not affected by exposure to THC (data not shown). As a positive control, 20 μM verapamil was tested in [^{3}H]-vinblastine accumulation and efflux experiments, and found to have a significant increase effect on the retention of intracellular [^{3}H]-vinblastine compared to the DMSO control.

THC stimulated the ATP hydrolysis mediated by P-gp in a concentration dependent manner

The profile of the drug stimulated ATPase activity is thought to reflect the nature of the interaction of P-gp with drug substrates [1]. We therefore evaluated the interaction of THC and P-gp by determining the effect of THC on the ATP hydrolysis by P-gp. As shown in Fig. 4a, THC activated P-gp mediated ATPase activity in a concentration-dependent manner with a concentration required for 50% stimulation of 1.8 μM. The result indicates the interaction of THC and P-gp.

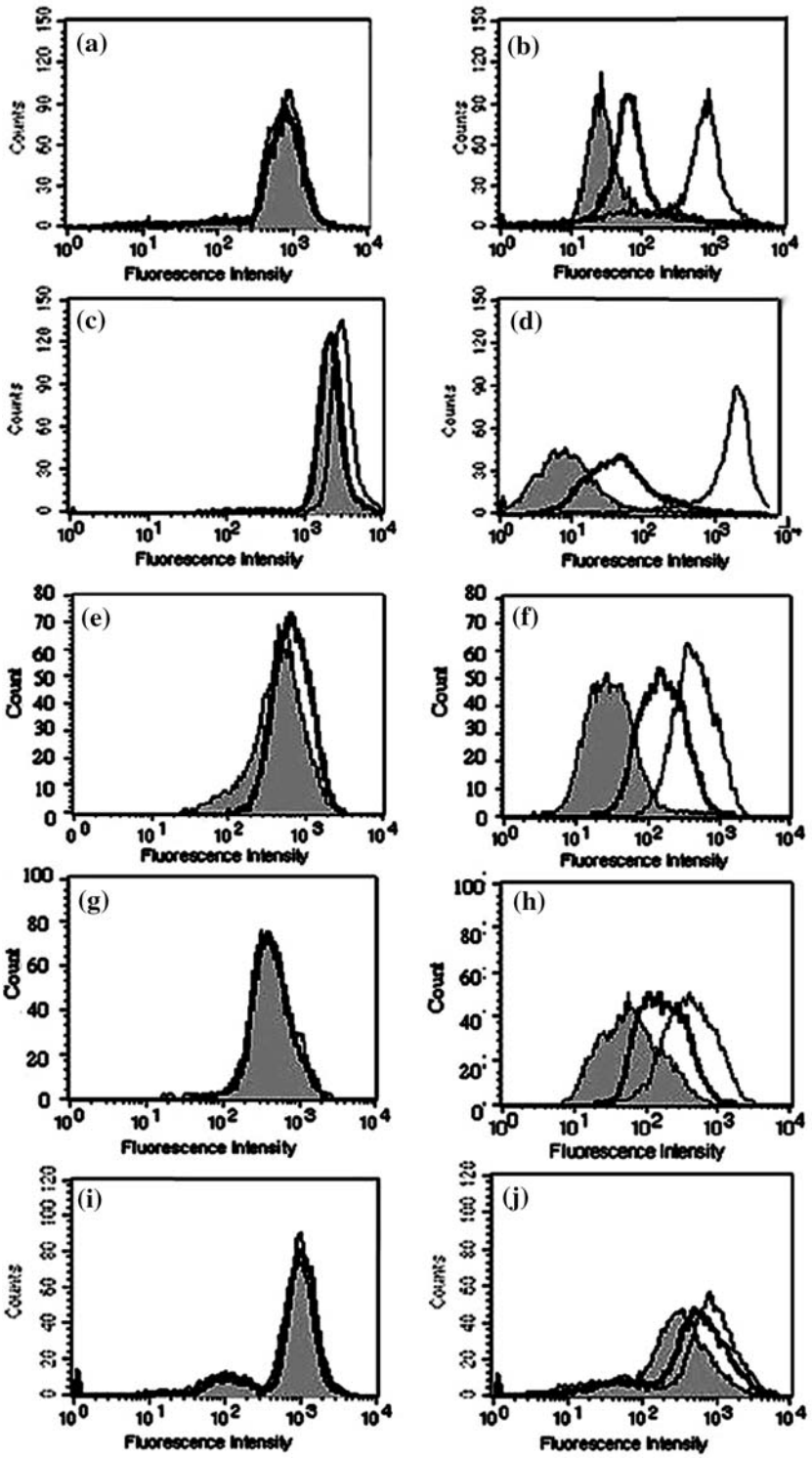

Fig. 2. Effect of tetrahydrocurcumin (THC) on the accumulation of fluorescence substrates. (a–d) KB-3-1 and KB-V-1 cells, (e–h) MCF-7 and MCF7FLV1000 cells or (i–j) pcDNA3.1 and MRP1-HEK 293 were resuspended in IMDM supplemented with 5% FBS. 0.5 μg/ml rhodamine 123 (a, b), 0.25 μM calcein-AM (c, d), 5 μM mitoxantrone (e, f), 5 μM pheophorbide (g, h) or 0.25 μM calcein-AM (i, j) was added in the presence of DMSO vehicle control (filled) or 50 μM THC (–), 10 μM cyclosporin A (a–d, –), 10 μM FTC (e–h, –), or 20 μM MK-571 (i, j, –). The cells were incubated at 37 °C in dark for 45 min (10 min 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 histogram derived from the Cell Quest software depicts fluorescence intensity and represents the fluorescence intensity of cells treated with DMSO control (gray filled) or 50 μM THC (bold line) or the specific inhibitor (thin line).

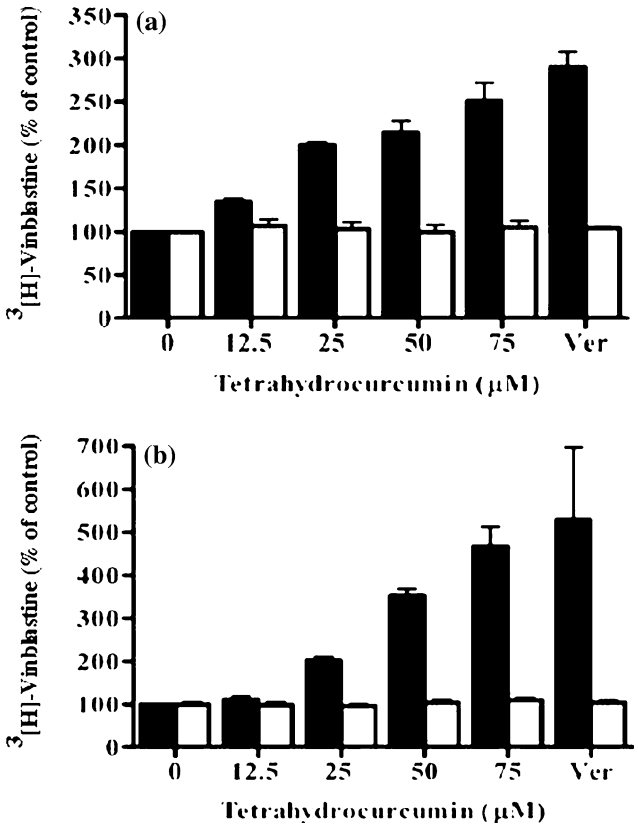

Fig. 3. Effect of tetrahydrocurcumin (THC) on ^{3}[H]-vinblastine accumulation (a) and efflux (b) in MCF-7MDR cells. (a) The cells (MCF-7, open bar and MCF-7MDR, filled bar) were cultured in completed DMEM in 6 well plate for 24 h. Cells were treated with THC (0–75 μM) or 20 μM verapamil (positive control) in the presence of 0.05 μCi ^{3}[H]-vinblastine/ml for 60 min while vehicle control is 0.5% DMSO. Cells were then harvested and the amount of intracellular radioactivity was measured using β-counter as described in Materials and methods. (b) The effect of THC on ^{3}[H]-vinblastine efflux. The cells were plated out as described for drug accumulation assay. In order to load cells with radiolabeled drug, cells were incubated for 60 min at 37 °C with 0.05 μCi ^{3}[H]-vinblastine/ml in the presence 20 μM verapamil. Cells were then washed with ice cold PBS (pH 7.4), following which medium containing THC (0–75 μM) or 20 μM verapamil was added. After incubation at 37 °C for 30 min, cells were washed with ice-cold PBS pH 7.4 and harvested. The amount of intracellular radioactivity was determined by scintillation counting. The data are represented as mean values ± SE of three-independent experiments.

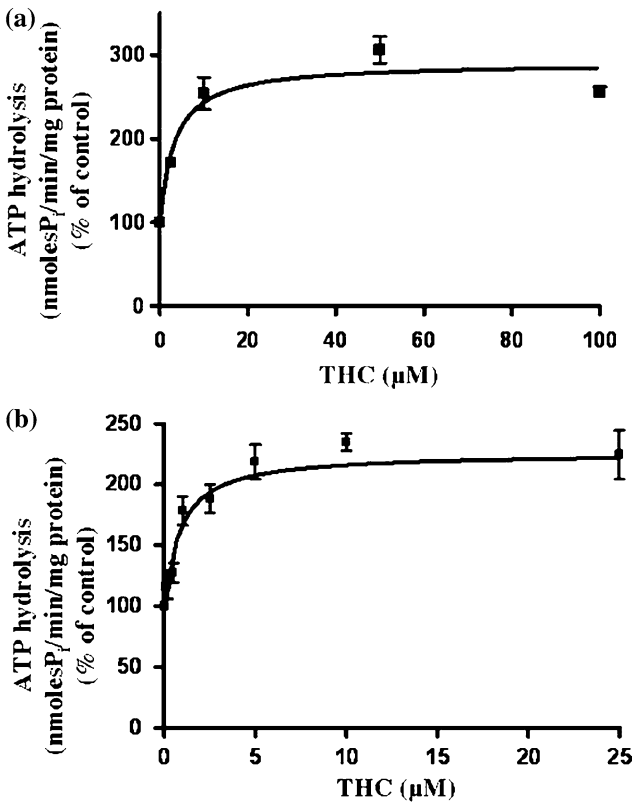

Fig. 4. Effect of tetrahydrocurcumin (THC) on P-glycoprotein (P-gp) and ABCG2 ATPase activity. Crude membranes (100 μg protein/ml) from (a) high five insect cells expressing *MDR*1 or (b) ABCG2 were incubated at 37 °C with varying concentrations with increasing concentrations of THC in the presence and absence of (a) 0.3 mM sodium orthovanadate or (b) BeFx (0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) in ATPase assay buffer for 10 min. The reaction was started by the addition of 5 mM ATP and was stopped by the addition of 0.1 ml of 5% SDS solution. P-gp and ABCG2 specific activity was recorded as the vanadate or BeFx sensitive respectively as described in 'Materials and methods'. The percent increase in ATP hydrolysis from basal level was plotted as a function of different concentrations of THC. The results are representative of at least three independent experiments ± SD.

Effect of THC on beryllium fluoride-sensitive ATPase activity of ABCG2

The BeFx-sensitive basal and the substrate-stimulated ATPase activity of ABCG2 was also determined in crude membranes isolated from high five insect cells expressing 482G-ABCG2. Crude membranes (100 μg protein/ml) were incubated with increasing concentrations of THC (0–25 μM) in presence or absence of BeFx (0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) in ATPase assay buffer. As shown in Fig. 4b, the presence of different concentrations of THC stimulated the ATPase activity of ABCG2 in a concentration-dependent manner to 2–2.5-folds of the basal level with a concentration required for 50% stimulation of 0.84 μM.

THC inhibited the [^{125}I]-IAAP incorporation into P-gp

[^{125}I]-IAAP has been used to characterize the drug binding sites of P-gp and ABCG2, and it is known that prazosin and several of its derivatives are known substrates of both ABCG2 and P-gp [34–36]. We also determined the effect of THC on [^{125}I]-IAAP photolabeling of P-gp. As shown in

Fig. 5a, THC inhibited the incorporation of [^{125}I]-IAAP into P-gp in a concentration dependent pattern with the IC_{50} values of 24.3 μM, suggesting THC binds directly to the substrate binding site of P-gp, probably at the same binding sites with prazosin.

Effect of THC on binding of [^{125}I]-IAAP to ABCG2

The results described above suggested that THC might interact with the drug substrate binding sites of ABCG2 therefore the binding of the photoaffinity substrate analogs [^{125}I]-IAAP to ABCG2 was studied. The crude membranes from 482R-ABCG2 overexpressing MCF7FLV1000 cells were used to study the photolabeling of ABCG2. The crude membranes from these cells were incubated with various concentrations of THC for 10 min at room temperature, and the samples were photolabled with 3–6 nM [^{125}I]-IAAP as described in 'Materials and methods'. It was observed that the presence of THC inhibited the binding of [^{125}I]-IAAP to ABCG2 in a dose-dependent manner with IC_{50} values of 6.9 μM (Fig. 5b).

THC increased etoposide (VP-16) sensitivity in MRP1-HEK 293 cells but not in pcDNA3.1-HEK

We first evaluated the cytotoxicity of THC in pcDNA3.1- and MRP1-transfected HEK 293 cells. Both cell lines were incubated with various concentrations of THC (0–100 μM) for 72 h. The survival cells were detected by MTT assay. Non-cytotoxic concentrations (>80% cell survival) of THC was used in combination with various concentrations of etoposide. The modulating effect of THC on etoposide cytotoxicity was examined by the MTT assay. As shown in Table 1, THC and all the three positive inhibitors of MRP1 (indomethacin, MK-571 and vinblastine) did not affect the etoposide sensitivity in pcDNA3.1-HEK 293 cells, whereas they significantly increased the sensitivity in MRP1-HEK 293 cells. The results were clearly confirmed by the effect of THC on the accumulation of MRP1-fluorescent substrate, calcein-AM. Consistent with the MDR reversing activity, THC increased the accumulation of calcein-AM in a concentration dependent manner (for clarity, data with only 50 μM THC are shown in Fig. 2j)

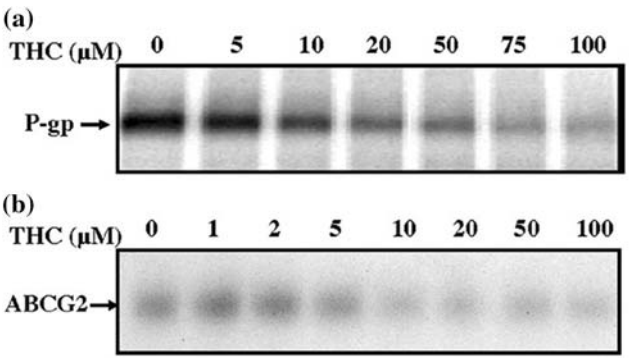

Fig. 5. Effect of various concentrations of tetrahydrocurcumin (THC) on photoaffinity labeling of P-glycoprotein (P-gp) and ABCG2 with [^{125}I]-iodoarylazidoprazosin (IAAP). The crude membranes from (a) High Five insect cells expressing *MDR1* or (b) drug selected MCF7FLV1000 cells overexpressing ABCG2 were incubated with varying concentrations of THC (0–100 μM) in the presence of IAAP. The samples were then illuminated with a UV lamp (365 nm) for 10 min and were processed as described in Materials and methods. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system. The autoradiogram shows the incorporation of IAAP into the P-gp and ABCG2 band in the presence of THC was shown in (a) and (b) respectively.

THC increased mitoxantrone sensitivity in MCF7AdrVp3000 cells but not in MCF-7

THC exhibited a very low toxicity to MCF-7 and MCF7AdrVp, the non-cytotoxic dose (>80% cell survival) of THC in these cell lines is more than 100 μM. We chose the concentrations at 25 and 30 μM for the MDR phenotype

Table 1. Reversal of resistance to selected drugs by tetrahydrocurcumin (THC)

Drug	IC_{50}[a, b] (Relative resistance)[c]	
	KB-3-1 (nM)	KB-V-1 (μM)
Vinblastine	1.9 ± 0.2	1.1 ± 0.3 (578.9)
(+) 25 μM THC	2.1 ± 0.7	0.9 ± 0.5 (428.6)*
(+) 50 μM THC	1.8 ± 0.3	0.7 ± 0.2 (388.9)*
	MCF-7 (nM)	MCF7AdrVp (μM)
Mitoxantrone	1.5 ± 0.9	>50 (>33333.3)
(+) 25 μM THC	1.3 ± 0.5	21.2 ± 5.7 (16307.7)*
(+) 30 μM THC	1.1 ± 0.7	14.6 ± 2.8 (13272.7)*
	pcDNA3.1 HEK 293(μM)	MRP1-HEK 293 (μM)
Etoposide	0.5 ± 0.1	78.2 ± 1.7 (156.4)
(+) 5 nM Vin	0.6 ± 0.3	57.5 ± 6.3 (95.8)*
(+) 40 μM Indo	0.6 ± 0.0	18.3 ± 4.8 (30.5)*
(+) 20 μM THC	0.4 ± 0.4	14.5 ± 3.7 (36.3)*
(+) 25 μM THC	0.5 ± 0.1	11.9 ± 2.8 (23.8)*

[a]The data represent the mean values from two independent experiments performed in triplicate.
[b]IC_{50} values refer to the drug concentration required for 50% inhibition of the growth.
[c]Relative resistance values were obtained by dividing the IC_{50} value of drug resistant cell lines by the IC_{50} value of the drug sensitive cell lines.
Asterisk denotes values that were significantly different from the vehicle control ($p < 0.05$).

study. As clearly indicated in Table 1. THC was able to sensitize MXR expressing cells, MCF7AdrVp, to mitoxantrone anticancer drug in a concentration dependent manner. This effect was not found in drug sensitive MCF-7, suggesting the reversal activity of THC on MXR mediated MDR phenotype.

THC increased vinblastine sensitivity in KB-V-1 cells but not in KB-3-1

THC exhibited low toxicity to KB-3-1 and KB-V-1 cells (IC_{50} value more than 75 μM), the non-cytotoxic dose of THC was used for the MDR phenotype study. As shown in Table 1, THC was able to sensitize drug resistant KB-V-1 cells to vinblastine significantly in a concentration dependent manner. This effect was not found in drug sensitive KB-3-1, suggesting the reversal activity of THC on P-gp mediated MDR phenotype.

Discussion

We reported previously that three major pure forms of curcuminoids purified from turmeric, curcumin I, curcumin II and curcumin III inhibited functional activities of P-gp and MRP1 [17, 18]. In this study, we raised the question as to whether an ultimate metabolite form of curcuminoids is able to extend their MDR reversing capacity. THC is acknowledged as an active metabolite form of curcuminoids [13, 15, 37, 38] and it has been reported widely on its potential antioxidant activity as well as some other biological properties, for instance, anti-inflammatory and anti-carcinogenesis [21, 39–43]. As indicated in Fig. 2, THC significantly increased the accumulation of rhodamine and calcein in KB-V-1, in a concentration dependent manner. This effect was not found in KB-3-1, suggesting that THC significantly inhibited the efflux function by P-gp. The inhibitory effect of THC was not cell type dependent since it was also able to increase the accumulation and inhibit the efflux of ^{3}[H]-vinblastine in MCF-7MDR, in a concentration dependent manner, and not in its parental MCF-7 (Fig. 3). MCF-7MDR cell line is the MDR1 transfected cell that had been maintained in the anticancer drug colchocine [44]. In our study, we demonstrated that THC inhibited the efflux of mitoxantrone and pheophorbide A (Fig. 2) from MXR expressing cells (MCF7FL1000).

The direct interaction of THC with the P-gp and ABCG2 was assessed by ATPase and photoaffinity labeling assays. THC stimulated several folds the ATPase activity of P-gp (Fig. 4a) and ABCG2 (Fig. 4b). The incorporation of IAAP into P-gp and ABCG2 was significantly inhibited by THC in a concentration dependent manner (Fig. 5). Thus, it can be suggested that THC exhibited the inhibitory effect on the ABC drug transporter by interacting directly with the transporter molecule, probably at the same binding site with prazosin.

The effect of THC on P-gp, MRP1 and MXR mediated MDR phenotype was evaluated by MTT assay (MDR reversing activity assay). In the first set of experiments, THC was tested for its cytotoxicity in all cell culture models used in this experiment. The IC_{50} of THC is not statistically different compared to drug resistant KB-V-1, MRP1-HEK293 and MCF7AdrVp3000 and their parental drug sensitive cells suggesting that THC most likely is not transported by these transporters (data not shown). This phenomenon is similar to our previous reports with other forms of curcuminoids as reported elsewhere. As shown in Table 1, non-cytotoxic dose of THC was able to reverse MDR phenotype in P-gp, MRP1 or MXR overexpressing cells. THC at 20–50 μM substantially enhanced the sensitivity toward vinblastine, etoposide and mitoxantrone in drug resistant KB-V-1, MRP1-HEK293 and MCF7AdrVp3000 cells respectively, while THC had no effect in wild-type drug sensitive cells. The data demonstrate that THC can be used as a potent chemosensitizer. A consistent finding was reported in MDCKII cells transfected with MRP1; THC significantly increased ^{3}[H]-EGCG in MDCKII/MRP1 overexpressing cells [45]. Our previous report demonstrated that all three pure forms of curcumin I (curcumin), curcumin II (demethoxycurcumin) and curcumin III (bisdemethoxycurcumin) inhibited the function of ABCB1, ABCC1 and ABCG2 drug transporters, and curcumin I was the most potent inhibitors. In Table 2, we compared the modulatory effect of THC on these ABC transporters with curcumin I. Although curcumin I is more

Table 2. Effect of tetrahydrocurcumin (THC) and curcumin I on various activities of ABCB1, ABCC1 and ABCG2

Parameter	Compound	ABCB1	ABCC1	ABCG2
ATPase activity	Curcumin I[a]	1.4	1.4	3.3
(fold stimulation)	THC[b]	2.65	ND[c]	2.5
Conc. required for maximal	Curcumin I[a]	0.5	1.5	0.005
ATPase stimulation (μM)	THC[b]	21.2	ND[c]	4.78
IC_{50} of curcumin for inhibition	Curcumin I[a]	5.8	ND[c]	0.54
of IAAP labeling (μM)	THC[b]	16.7	ND[c]	9.87
Fold reversal of cytotoxicity	Curcumin I[a]	5.66[d]	20[e]	3[f]–10[g]
of selected drugs	THC[b]	1.35[d]	6.6[e]	2.04[f]

[a]Data from Refs. [17–19].
[b]This study; THC: tetrahydrocurcumin.
[c]Not determined.
[d]Vinblastine.
[e]Etoposide.
[f]Mitoxantrone.
[g]topotecan.

potent modulator than THC but THC is considered to be an important active secondary metabolites *in vivo*.

Taken together, the present study clearly showed that THC inhibited the efflux function of P-gp, MRP1 and MXR by direct binding to the ABC transporters and it is able to extend the MDR reversing activity of curcuminoids to P-gp, MRP1 and MXR *in vivo*. The systemic bioavailability of curcumin is low, perhaps attributable, at least in part, to metabolism. Many evidences suggest that curcumin was first biotransformed to dihydrocurcumin and THC and that these compounds subsequently were converted to monoglucuronide conjugates. Curcumin glucuronide was identified in intestinal and hepatic microsomes, and curcumin sulfate, THC, and hexahydrocurcumin were found as curcumin metabolites in intestinal and hepatic cytosol from humans and rats [11–14]. Since curcumin undergoes extensive metabolic conjugation and reduction, pharmacological implications of curcumin metabolism in intestine and liver should be taken into account in the design of future clinical trials of curcumin and its analogues.

It is premature to explain the mechanisms of action of THC on the three ABC transporters and extensive pharmacokinetic studies of curcuminoids are still necessary. However, evidence reported in the present study reveals that THC is able to extend the MDR reversing capacity of curcuminoids *in vivo*, and these findings support the development of curcuminoids purified from turmeric as a modulator to be used in combination with conventional chemotherapy.

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