

# Modulation of the function of the multidrug resistance-linked ATP-binding cassette transporter ABCG2 by the cancer chemopreventive agent curcumin

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## Abstract

Curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III) are the major forms of curcuminoids found in the turmeric powder, which exhibit anticancer, antioxidant, and anti-inflammatory activities. In this study, we evaluated the ability of purified curcuminoids to modulate the function of either the wild-type 482R or the mutant 482T ABCG2 transporter stably expressed in HEK293 cells and drug-selected MCF-7 FLV1000 and MCF-7 AdVp3000 cells. Curcuminoids inhibited the transport of mitoxantrone and pheophorbide a from ABCG2-expressing cells. However, both cytotoxicity and [<sup>3</sup>H]curcumin I accumulation assays showed that curcuminoids are not transported by ABCG2. Nontoxic concentration of curcumin I, II, and III sensitized the ABCG2-expressing cells to mitoxantrone, topotecan, SN-38, and doxorubicin. This reversal was not due to reduced expression because ABCG2 protein levels were unaltered by treatment with 10 μmol/L curcuminoids for 72 hours. Curcumin I, II, and III stimulated (2.4- to 3.3-fold) ABCG2-mediated ATP hydrolysis and the IC<sub>50</sub>s were in the range of 7.5 to 18 nmol/L, suggesting a high affinity of curcuminoids for ABCG2. Curcuminoids also inhibited the photolabeling of ABCG2 with [<sup>125</sup>I]iodoarylazidoprazosin

and [<sup>3</sup>H]azidopine as well as the transport of these two substrates in ABCG2-expressing cells. Curcuminoids did not inhibit the binding of [α-<sup>32</sup>P]8-azidoATP to ABCG2, suggesting that they do not interact with the ATP-binding site of the transporter. Collectively, these data show that, among curcuminoids, curcumin I is the most potent modulator of ABCG2 and thus should be considered as a treatment to increase the efficacy of conventional chemotherapeutic drugs. [Mol Cancer Ther 2006;5(8):1995–2006]

## Introduction

Development of multidrug resistance (MDR) is a problem in cancer chemotherapy that limits the effectiveness of anticancer drugs (1, 2). The overexpression of ATP-binding cassette (ABC) transporters, such as ABCB1 (P-glycoprotein) and ABCC1 (MRP1), have been shown to confer resistance to chemotherapeutic agents by exporting drugs from cells in an ATP-dependent manner. ABCG2 (also called mitoxantrone resistance-associated protein, breast cancer resistance protein, and placental ABC transporter) is a half-transporter of the ABCG subfamily of ABC transporters, the overexpression of which also plays a major role in the development of the MDR phenotype of malignant cells (3–6). It confers resistance to various anticancer agents, such as doxorubicin, mitoxantrone, topotecan, and SN-38 (7–10). One of the possible mechanisms by which the development of MDR can be circumvented during chemotherapy is by blocking the functions of these ABC transporters. Tsuruo et al. (11) showed the ability of the calcium channel blocker, verapamil, to reverse ABCB1-mediated MDR. Since then, many other reversal agents, including trifluoperazine, amiodarone, reserpine, phenothiazines, cyclosporine A, FK506, and a cyclosporine derivative (PSC-833; reviewed in refs. 1, 2), have been introduced and explored. A mycotoxin fumitremorgin C from the fungus *Aspergillus fumigatus* was found to be a potent and specific inhibitor of ABCG2 and *in vitro* reversed MDR mediated by this transporter (12). However, the neurotoxic effects of fumitremorgin C and its structural analogues precluded its use as a modulator for *in vivo* studies (13–15).

Turmeric powder has been used for centuries as a spice, coloring, and therapeutic agent (16–22). The active constituents of turmeric are three major curcuminoids: curcumin or diferuloylmethane (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III; refs. 23, 24). We reported earlier that curcumin mixture and purified curcumin I, II, and III could reverse the drug resistance in cells expressing ABCB1 and ABCC1 by inhibiting the functions of these transporters

Received 2/16/06; revised 5/5/06; accepted 6/14/06.

**Grant support:** Intramural Research Program, Center for Cancer Research, National Cancer Institute/NIH and Thailand National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency.

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**Note:** W. Chearwae and S. Shukla contributed equally to this work.

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doi:10.1158/1535-7163.MCT-06-0087

(25–27). It was also shown that curcumin I, which is a major constituent (70–75%) of curcumin mixture, among the purified curcuminoids, was the most potent inhibitor of the activity of both the transporters (25–27).

It has been reported that the amino acid at position 482 has a crucial role in the substrate and inhibitor specificity of ABCG2 and that mutants R482-T/G exhibit altered drug resistance profiles and substrate specificity of ABCG2 (28–30). Therefore, we investigated the modulating effects of curcuminoids on ABCG2 activity in both wild-type R482-expressing HEK293 cells and mutant 482T-expressing MCF-7 AdVp3000 cells. The drug-selected MCF-7 FLV1000 and MCF-7 AdVp3000, which overexpressed the wild-type R482 and mutant 482T ABCG2, respectively, were chosen because the protein was overexpressed in these breast cancer cell lines under its own promoter at higher levels (31) for biochemical characterization. Curcuminoids inhibited the efflux of ABCG2 substrates, such as mitoxantrone and pheophorbide a, and the presence of nontoxic concentrations of curcuminoids increased the sensitivity of ABCG2-expressing cells to anticancer drugs. In addition, [<sup>3</sup>H]curcumin I transport assays show that the curcuminoids are not transported by ABCG2. At biochemical levels, curcuminoids stimulated ATPase activity of ABCG2 at very low concentrations (7–18 nmol/L), and fumitremorgin C, which is an inhibitor of ABCG2, abolished the stimulatory effect of curcumin, indicating that curcuminoids have an effect on ABCG2-specific ATP hydrolysis. Curcuminoids also inhibited both the photolabeling of ABCG2 with two photoaffinity analogs, [<sup>125</sup>I]iodoarylazidoprazosin (IAAP) and [<sup>3</sup>H]azidopine and the transport of these agents.

Curcuminoids are natural compounds that are consumed daily in Southeast Asian countries. They interact with the transporter with very high affinity and inhibit ABCG2-mediated drug resistance. Taken together, our previous work with ABCB1 and ABCC1 (25–27) and this study with ABCG2 suggest that curcumin I is a very effective modulator, which should be considered as a potential compound for development of reversal agents designed to overcome MDR-mediated by these three major ABC drug transporters.

## Materials and Methods

### Chemicals

DMEM, RPMI, fetal bovine serum, penicillin, streptomycin, trypsin/EDTA, and PBS were purchased from Life Technologies (Grand Island, NY). G418 was procured from the Research Products, Inc. (Mt. Prospect, IL). Curcumin (curcumin mixture; Sigma, St. Louis, MO), mitoxantrone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, and ouabain were purchased from Sigma. Pheophorbide a was obtained from Frontier Scientific (Logan, UT). Radiolabeled [<sup>125</sup>I]IAAP (2,200 Ci/mmol) was from Perkin-Elmer Life Sciences (Wellesley, MA), [ $\alpha$ -<sup>32</sup>P]8-azidoATP (15–20 Ci/mmol) was from Affinity Labeling Technologies, Inc. (Lexington,

KY), and [<sup>3</sup>H]azidopine (60 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ) were procured. Purified curcumin I (10 Ci/mmol) was custom labeled with tritium by American Radiolabeled Chemicals (St. Louis, MO). The BXP-21 monoclonal antibody was procured from Kamiya Biomedical Co. (Seattle, WA). Silica Gel 60F<sub>254</sub> plates were obtained from EMD Chemicals (Gibbstown, NJ).

### Cell Lines and Culture Conditions

HEK293 cells stably transfected with either empty pcDNA3.1 vector (pcDNA3.1-HEK293) or pcDNA3.1 containing ABCG2 coding either arginine 482 (482R-HEK293) or threonine 482 (482T-HEK293) were maintained in Eagle's MEM supplemented with 10% FCS, penicillin, streptomycin, and 2 mg/mL G418 (29). MCF-7 FLV1000 (482R) and MCF-7 AdVp3000 (482T) cells overexpressing ABCG2 were cultured in RPMI with 10% fetal bovine serum with 1  $\mu$ g/mL flavopiridol or 3  $\mu$ g/mL doxorubicin and 5  $\mu$ g/mL verapamil, respectively (28, 32). These cell lines were provided by Dr. Susan Bates (National Cancer Institute/NIH, Bethesda, MD). The cells were maintained in drug-free medium for 3 to 6 days before conducting the experiments to prevent possible effects or interactions of these drugs with curcuminoids. In crude membranes of control HEK293 and MCF-7 and ABCG2-expressing HEK293, MCF-7 FLV1000, and MCF-7 AdVp3000 cells, both ABCB1 and ABCC1 were not detected by Western blot analysis (data not shown), consistent with previous reports (29, 32, 33).

### Purification of Curcuminoids from Turmeric Powder

Turmeric rhizomes were dried and blended to a powder form. The powder was extracted with 95% ethanol for 24 hours. The ethanolic extract was filtered through Whatman filter paper and ethanol was removed by a rotary evaporator. Curcumin I, II, and III were further purified from the crude ethanolic extract as described earlier (26).

### Isolation of Crude Membranes from ABCG2-Expressing HEK293 or MCF-7 Cells

Crude membranes from ABCG2-transfected HEK293 or MCF-7 cells were prepared as described elsewhere (26, 34). The protein content was estimated using the Amido Black B dye-binding assay as described earlier (35).

### Fluorescent Drug Accumulation Assay by Fluorescence-Activated Cell Sorting

Accumulation assays with mitoxantrone or pheophorbide a (5  $\mu$ mol/L) in ABCG2-transfected HEK293 cells or MCF-7, MCF-7 FLV1000, and MCF-7 AdVp3000 cells were done using fluorescence-activated cell sorting as described previously (31). Fumitremorgin C (10  $\mu$ mol/L) was used as an inhibitor of ABCG2 in flow cytometry assays. For all samples, 10,000 events were counted and the analysis was done with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). The mean fluorescence intensity was calculated using the histogram stat program in CellQuest software.

### Cytotoxicity Assay

Cytotoxicity of curcuminoids was determined by MTT assay as described previously (26). The IC<sub>50</sub> was calculated from linear regression analysis of the linear portion of

the growth curves (26). The cytotoxicity assays were also carried out using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (Dojindo Molecular Technologies, Gaithersburg, MD), which produces a water-soluble formazan dye on bioreduction to monitor the viability of cells, and similar results were obtained; for this reason, only data obtained with MTT dye are given. In both assays, curcuminoids were incubated with CCK8 or MTT dye in the absence of cells and these values were subtracted from samples with cells.

#### ATPase Assay

Crude membranes prepared from 482R-HEK293 and pcDNA3.1-HEK293 cells (100 µg protein/mL) were incubated with varying concentrations of curcuminoids, prazosin, 10 µmol/L fumitremorgin C, or indicated concentration of curcumin I plus 10 µmol/L fumitremorgin C in the presence and absence of beryllium fluoride (BeF<sub>x</sub>; 0.2 mmol/L BeSO<sub>4</sub>, 2.5 mmol/L NaF) in ATPase assay buffer [0.05 mmol/L KCl, 5 mmol/L NaN<sub>3</sub>, 2 mmol/L EGTA, 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L DTT, 50 mmol/L MOPS (pH 7.5)] for 5 minutes at 37°C. The reaction was started by the addition of 5 mmol/L ATP and incubated for 20 minutes at 37°C. SDS solution (0.1 mL of 5% SDS) was added to terminate the reaction and the amount of inorganic phosphate released was quantified by modification of the sensitive colorimetric reaction as described previously (34). The specific activity of the transporter was recorded as BeF<sub>x</sub>-sensitive ATPase activity (34).

#### Photoaffinity Labeling of ABCG2 with [<sup>125</sup>I]IAAP and [<sup>3</sup>H]Azidopine

Crude membranes (1 mg protein/mL) from MCF-7 AdVp3000 cells were incubated with various concentrations of curcuminoids for 10 minutes at 21°C to 23°C in 50 mmol/L Tris-HCl (pH 7.5). [<sup>125</sup>I]IAAP (2,200 Ci/mmol) or 0.5 µmol/L [<sup>3</sup>H]azidopine (60 Ci/mmol) at 3 to 5 nmol/L was added and incubated for an additional 5 minutes under subdued light. The samples were illuminated with a UV lamp (365 nm) for 10 minutes at room temperature. The labeled ABCG2 was immunoprecipitated by adding 800 µL radioimmunoprecipitation assay buffer containing 1% aprotinin followed by addition of 10 µg BXP-21 antibody. Protein A-Sepharose beads (100 µL) were added and further incubated at 4°C for 16 hours. The protein A-Sepharose beads were pelleted at 4°C and washed with radioimmunoprecipitation assay buffer containing 1% aprotinin. SDS-PAGE sample buffer (50 µL) was added and incubated at 37°C for 1 hour. The samples were separated on a 7% Tris-acetate gel at constant voltage. The [<sup>3</sup>H]azidopine gel was incubated with Fluoro-Hance (Research Products) for 30 to 45 minutes and both gels were dried under vacuum and exposed to X-ray film for 12 to 24 hours at -80°C. The incorporation of [<sup>125</sup>I]IAAP or [<sup>3</sup>H]azidopine into the ABCG2 band was quantified by estimating the radioactivity incorporated into the band using the STORM 860 PhosphorImager System (Molecular Dynamics, Sunnyvale, CA) and the software ImageQuant as described (36).

#### [<sup>125</sup>I]IAAP, [<sup>3</sup>H]Azidopine, and [<sup>3</sup>H]Curcumin I Accumulation Assays

MCF-7, MCF-7 AdVp3000, or MCF-7 FLV1000 cells (0.25 × 10<sup>6</sup> per well in 24-well plates) were grown in monolayer at 37°C. The assay was initiated by incubating cells with 3 to 5 nmol/L [<sup>125</sup>I]IAAP (2,200 Ci/mmol) or 25 nmol/L [<sup>3</sup>H]azidopine (60 Ci/mmol) or 200 nmol/L [<sup>3</sup>H]curcumin I (10 Ci/mmol). Curcumin mixture or fumitremorgin C at 10 µmol/L was added to [<sup>125</sup>I]IAAP or [<sup>3</sup>H]azidopine wells and incubated at 32°C for 5 to 60 minutes under subdued light (to avoid photo-cross-linking). After incubation, cells were washed with PBS and lysed by incubation with 0.3 mL/well trypsin/EDTA at 37°C for 30 minutes. The cell lysates were transferred to scintillation vials containing 15 mL Bio-Safe II scintillation fluid and the radioactivity was measured in a scintillation counter. Cells washed with PBS immediately after addition of the assay mix were used as the 0-minute time point. The value for accumulated [<sup>125</sup>I]IAAP, [<sup>3</sup>H]azidopine, or [<sup>3</sup>H]curcumin I at 0 minute (within 3–5 seconds) was subtracted from a given time as nonspecific binding of these compounds to the cells. The accumulation of labeled compounds was expressed as pmol/10<sup>6</sup> cells. These assays were done at 32°C to slow the rate of efflux from these cells for studying the accumulation/efflux in a time-dependent manner.

#### Curcumin I and Mitoxantrone Accumulation Assay

MCF-7 (control), MCF-7 AdVp3000, and MCF-7 FLV1000 cells (2.5 × 10<sup>6</sup> per well in 24-well plates) were grown in monolayer. The assay was initiated by incubating the cells with medium containing 10 µmol/L curcumin I or 5 µmol/L mitoxantrone at 32°C in a CO<sub>2</sub> incubator for 60 minutes. After incubation, the cells were washed with cold PBS. The accumulated curcumin I or mitoxantrone in the cells was extracted by adding 95% ethanol. The extract was centrifuged at 3,000 rpm to pellet the cell debris and the supernatant was dried and then resuspended in 500 µL ethanol for the mitoxantrone assay or 25 µL for the curcumin I assay. The ethanolic curcumin extract (10 µL) was spotted on Silica Gel 60 F<sub>254</sub>-coated TLC plates and separated using the solvent system (CHCl<sub>3</sub>/ethanol/acetic acid, 94:5:1; ref. 26). Alternatively, the fluorescence intensity of 200 µL mitoxantrone or 5 µL curcumin ethanolic extract diluted with 195 µL ethanol was read at 695 and 540 nm, respectively (excitation wavelength, 620 nm for mitoxantrone and 420 nm for curcumin I).

#### Photo-Cross-Linking of ABCG2 with [α-<sup>32</sup>P]8-AzidoATP

The crude membranes from MCF-7 AdVp3000 cells were incubated with 25 µmol/L curcumin mixture, I, II, and III or 10 mmol/L ATP for 10 minutes at 4°C in ATPase assay buffer (without DTT) and 10 µmol/L [α-<sup>32</sup>P]8-azidoATP (10 µCi/nmol) was added under subdued light and incubated for an additional 5 minutes at 4°C. The samples were then illuminated with a UV lamp (365 nm) on ice for 10 minutes and separated on SDS-7% Tris-acetate polyacrylamide gel at constant voltage. The gel was dried and exposed to X-ray film for 12 to 24 hours at -80°C.

### Western Blot Analysis

Crude membrane protein (0.2–2 µg/lane) was subjected to electrophoresis and transferred to nitrocellulose membranes as described previously (28). Blots were probed with mouse monoclonal ABCG2-specific BXP-21 antibodies at 1:500 dilution. Horseradish peroxidase-conjugated goat anti-mouse IgG at 1:10,000 dilution was used as the secondary antibody and the signals were detected using enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). For detection of ABCB1 and ABCC1, C219 and MRP1 antibodies, respectively, were used as described previously (33, 37).

### Statistical Analysis

Data are the mean  $\pm$  SD from at least three independent experiments. Differences between the means were analyzed by one-way ANOVA. Results were considered to be statistically significant when  $P < 0.05$ .

## Results

### Curcuminoids Inhibit the Efflux of Mitoxantrone and Pheophorbide a from Both Wild-type and R482T Mutant ABCG2-Expressing Cells

To investigate the effect of curcuminoids on the accumulation of ABCG2 substrates, mitoxantrone and pheophorbide a accumulation assays (31) were done in wild-type 482R-HEK293 cells and mutant 482T ABCG2-expressing MCF-7 AdVp3000 cells. The pcDNA3.1-HEK293, 482R-HEK293, MCF-7 and MCF-7 AdVp3000 cells were incubated with 5 µmol/L mitoxantrone or pheophorbide a in combination with increasing concentrations of curcumin mixture, curcumin I, II, and III, or 10 µmol/L fumitremorgin C, a specific inhibitor of ABCG2, and incubated at 37°C in the dark for 45 minutes. The accumulation of the fluorescent substrate in the cells was measured as described in Materials and Methods by flow cytometry. The presence of curcuminoids increased the accumulation of mitoxantrone and pheophorbide a in wild-type (R482) ABCG2-expressing cells in a concentration-dependent manner. Representative results at 5 µmol/L curcuminoids are shown in histograms (Fig. 1). Control (pcDNA3.1-transfected) cells showed higher accumulation of both mitoxantrone and pheophorbide a and curcuminoids (up to 20 µmol/L) did not have any effect (data not shown). It should be noted that the curcumin mixture and curcumin I (at 5 µmol/L) were more potent in inhibiting the accumulation of both fluorescent substrates than curcumin II and III (compare *brown, blue, purple, and light blue lines* in Fig. 1). Curcuminoids also inhibited the efflux of mitoxantrone and pheophorbide a from the mutant 482T ABCG2-expressing MCF-7 AdVp3000 cells in a concentration-dependent manner. Fig. 1 (*top two right and bottom two right*) shows representative histograms at 5 µmol/L curcuminoids. Curcumin I and the curcumin mixture were also more effective in inhibiting the transport in mutant 482T ABCG2-expressing cells than curcumin II and III, whereas the highest concentration of curcuminoids tested (20 µmol/L) had no effect on the control MCF-7 cells

(data not shown). The effect of curcuminoids on efflux of these two fluorescent substrates was determined in terms of mean fluorescence intensity and these results are given in Supplementary Fig. S1.<sup>3</sup> The IC<sub>50</sub>s derived from data in Supplementary Fig. S1 are given in Table 1. These data clearly show that curcumin I and curcumin mixture were more efficient in inhibiting the ABCG2-mediated efflux of the substrates than curcumin II and III. These findings suggest that curcumin I, which is the major constituent of the curcumin mixture, is the form that most effectively inhibits this transporter.

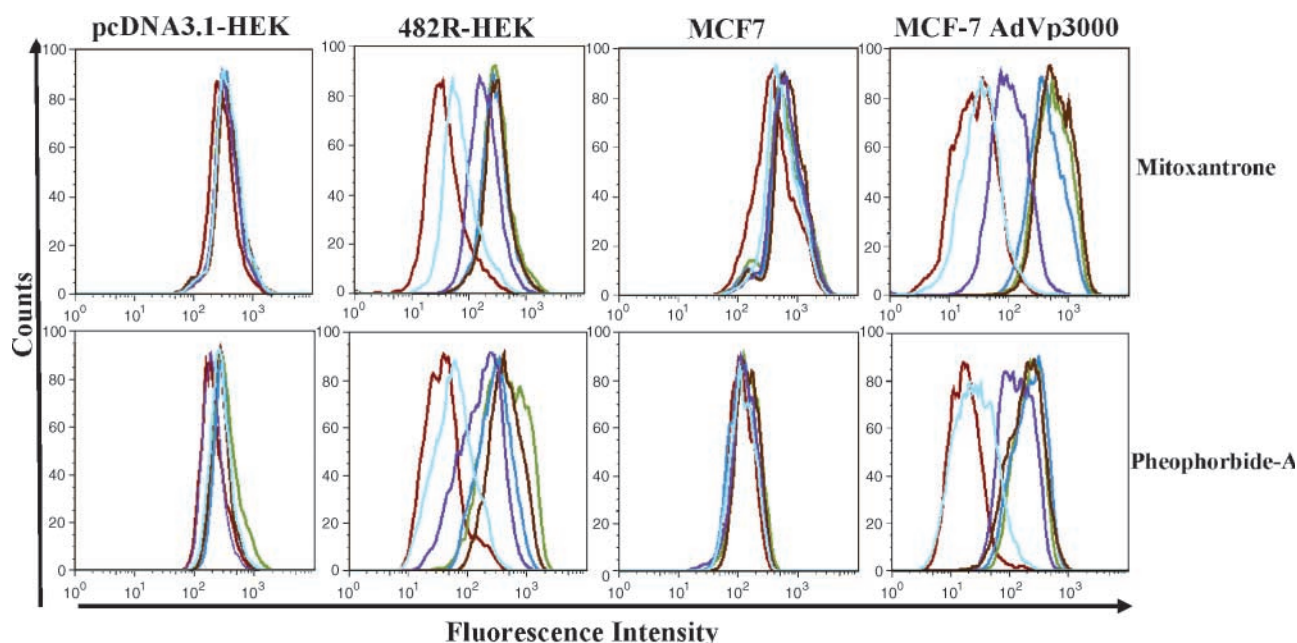
### Cytotoxicity of Curcuminoids in ABCG2-Expressing Cells

The fact that curcuminoids inhibit ABCG2-mediated efflux suggests that the curcuminoids could be acting as competing substrates for ABCG2. Therefore, cytotoxicity of curcuminoids in the control (pcDNA3.1-HEK293 and MCF-7) and ABCG2-expressing cells [ABCG2 (482R or 482T)-HEK293 and MCF-7 AdVp3000] was determined. As shown in Table 2, the IC<sub>50</sub>s of curcuminoids for pcDNA3.1, 482R-HEK293, 482T-HEK293, MCF-7, and MCF-7 AdVp3000 cells were 21 to 28, 19 to 29, 26 to 29, 30 to 52, and 25 to 51 µmol/L, respectively. The relative resistance factor was ~1- to 1.2-fold (Table 2), indicating that ABCG2 does not confer resistance to curcuminoids; thus, these compounds may not be transported by both the wild-type and the mutant 482T transporter.

### Curcumin I and [<sup>3</sup>H]Curcumin I Accumulation in ABCG2-Expressing Cells

To confirm whether curcumin is transported by ABCG2, curcumin I accumulation was measured in ABCG2-overexpressing cells. The accumulation of [<sup>3</sup>H]curcumin I or unlabeled curcumin I was studied in the MCF-7 (control), 482R ABCG2-overexpressing MCF-7 FLV1000, and 482T ABCG2-overexpressing MCF-7 AdVp3000 cells. The cells were incubated with 200 nmol/L [<sup>3</sup>H]curcumin I or 3 to 5 nmol/L [<sup>125</sup>I]IAAP (a transport substrate of ABCG2; ref. 38) in the presence of 10 µmol/L fumitremorgin C at 32°C for 5 to 60 minutes. As shown in Fig. 2A, whereas the MCF-7 cells showed an increased accumulation of [<sup>125</sup>I]IAAP over 60 minutes, the MCF-7 FLV1000 cells accumulated minimal [<sup>125</sup>I]IAAP. However, in the presence of 10 µmol/L fumitremorgin C due to inhibition of ABCG2 activity, [<sup>125</sup>I]IAAP accumulated in MCF-7 FLV1000 cells to the same level as control MCF-7 cells. At the same time, [<sup>3</sup>H]curcumin I accumulated to similar levels in MCF-7 FLV1000 cells and control MCF-7 cells (Fig. 2A). Similar results were also observed in MCF-7 AdVp3000 cells (data not shown). These data suggest that curcumin I is not transported by ABCG2. An accumulation assay with unlabeled 10 µmol/L curcumin I (concentration used for inhibition of substrate accumulation) was also carried out to make sure that curcumin I was not a transport substrate

<sup>3</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).



**Figure 1.** Effect of curcuminoids on the accumulation of fluorescent drug substrates in wild-type 482R and mutant 482T ABCG2-expressing cells. pcDNA3.1-HEK293, 482R-HEK293, MCF-7, and MCF-7 AdVp3000 (482T) cells (300,000–500,000 per tube) were incubated with 5  $\mu\text{mol/L}$  mitoxantrone (*top*) or pheophorbide a (*bottom*) for 45 min at 37°C in the presence of 5  $\mu\text{mol/L}$  curcuminoids or 10  $\mu\text{mol/L}$  fumitremorgin C. The cells were then washed and analyzed by flow cytometry as described in Materials and Methods. The histogram derived from the CellQuest software depicts fluorescence intensity (*X axis*) of either untreated (*red*) or fumitremorgin C–treated (*green*), curcumin mixture-treated (*blue*), curcumin I–treated (*brown*), curcumin II–treated (*purple*), and curcumin III–treated (*light blue*) cells, which is plotted as a function of cell number (*Y axis*). Representative experiment from three independent experiments and there was 5% to 10% variation in each data set.

of ABCG2 at higher concentrations. The accumulated curcumin I in MCF-7, MCF-7 FLV1000, and MCF-7 AdVp3000 cells after 60 minutes was extracted with ethanol and the concentration of accumulated curcumin I was determined by measuring the fluorescence of the samples. When mitoxantrone, a known substrate (29) was used, as shown in Fig. 2B, the MCF-7 cells showed 4- to 4.5-fold higher accumulation of mitoxantrone than the MCF-7 AdVp3000 or MCF-7 FLV1000 cells. On the other hand, there was no significant difference in the fluorescence of the curcumin I extracted from the MCF-7 control, MCF-7 AdVp3000, and MCF-7 FLV1000 cells (Fig. 2B). The curcumin samples were also separated on TLC plates and the

spot corresponding to curcumin I was visualized (Fig. 2C), which showed no significant differences in the intensity of the yellow spot corresponding to curcumin I between the drug-sensitive MCF-7 and the drug-resistant MCF-7 FLV1000 or MCFAdrVp3000 cells. These results show that curcumin I is not transported by ABCG2-expressing cells; furthermore, it is clear that curcumin I is not metabolized 'to a detectable level during 1-hour incubation in control or ABCG2-expressing MCF-7 cells.

#### Reversal of Drug Resistance by Curcuminoids

As it is known that mitoxantrone (29), topotecan, SN-38, and doxorubicin (39, 40) are substrates of ABCG2, the ability of curcuminoids to augment the cytotoxic effect of

**Table 1.** Effect of curcuminoids on the accumulation of mitoxantrone and pheophorbide a in 482R-HEK293 and MCF-7 AdVp3000 cells

Compound	Cell line	Mitoxantrone, IC <sub>50</sub> ( $\mu\text{mol/L}$ )	Pheophorbide a, IC <sub>50</sub> ( $\mu\text{mol/L}$ )
Curcumin M	R482-HEK	2.35 $\pm$ 0.32	3.21 $\pm$ 0.28
	MCF-7 AdVp3000	2.25 $\pm$ 0.19	2.48 $\pm$ 0.39
Curcumin I	R482-HEK	1.77 $\pm$ 0.17	1.73 $\pm$ 0.31
	MCF-7 AdVp3000	1.79 $\pm$ 0.13	1.63 $\pm$ 0.23
Curcumin II	R482-HEK	5.50 $\pm$ 0.43	6.23 $\pm$ 0.30
	MCF-7 AdVp3000	4.56 $\pm$ 0.22	6.13 $\pm$ 0.76
Curcumin III	R482-HEK	8.82 $\pm$ 0.82	9.32 $\pm$ 0.62
	MCF-7 AdVp3000	11.15 $\pm$ 1.31	9.95 $\pm$ 0.73

NOTE: The difference in the mean fluorescence intensity in the presence of 10  $\mu\text{mol/L}$  fumitremorgin C or curcuminoids and in the absence of any addition was taken as control (100%). Mean  $\pm$  SD (n = 3) derived from data given in Supplementary Fig. S1 (available at <http://mct.aacrjournals.org/>).

**Table 2. Cytotoxicity of curcuminoids in ABCG2-expressing cells**

Compound	Cytotoxicity of curcuminoids, IC <sub>50</sub> * (μmol/L)				
	pcDNA3.1-HEK293	ABCG2-HEK293 (482R)	ABCG2-HEK293 (482T)	MCF-7	MCF-7 AdVp3000 (482T)
Curcumin M	21.5 ± 0.8	19.7 ± 0.6 (0.91) <sup>†</sup>	25.7 ± 2.6 (1.19)	30.5 ± 1.3	25.0 ± 3.6 (0.82)
Curcumin I	22.2 ± 0.5	19.8 ± 0.4 (0.89)	24.7 ± 2.7 (1.11)	28.1 ± 5.5	25.0 ± 3.6 (0.89)
Curcumin II	26.3 ± 0.9	27.2 ± 0.1 (1.01)	26.5 ± 1.4 (1.01)	30.0 ± 4.1	37.0 ± 10.6 (1.23)
Curcumin III	28.7 ± 1.2	29.5 ± 1.9 (1.03)	28.7 ± 1.3 (1.00)	52.5 ± 6.4	50.7 ± 8.8 (0.96)

\*Mean ± SE of three independent experiments done in triplicates.

<sup>†</sup>Values in parentheses represent relative resistance, which was calculated by the IC<sub>50</sub> of resistant cells divided by that of the parental cells.

these drugs in ABCG2-expressing drug-resistant cells was evaluated. A nontoxic concentration of curcuminoids (5 μmol/L; <5% cell killing at this concentration) was used along with varying concentrations of mitoxantrone, topotecan, and SN-38 in cytotoxicity assays. The relative resistance was calculated based on IC<sub>50</sub>s in the absence and presence of the curcuminoids. The results shown in Table 3 show that curcuminoids were able to increase the sensitivity of the drug-resistant cells to these drugs, ranging from 3- to 8-fold. A nontoxic concentrations of fumitremorgin C (5 μmol/L) and mitoxantrone (0.5 nmol/L) of with ~95% cell survival was used as a positive control in the reversal of mitoxantrone and doxorubicin cytotoxicity, respectively, in the mutant 482T ABCG2-expressing cells.

#### Expression of ABCG2 Is Not Affected by 3-Day Treatment with Curcuminoids in Culture

To confirm that the reversal of drug resistance by curcuminoids was a direct effect on the function of the transporter and not an effect due to reduced expression of ABCG2, the MCF-7 AdVp3000 cells were grown in the presence of nontoxic concentrations (10 μmol/L) of the curcumin mixture and curcumin I, II, and III for 3 days. Crude membrane proteins (10 μg) from these cells were separated on a 7.5% SDS-PAGE gel and the protein level of ABCG2 was analyzed by Western blotting using BXP-21, the anti-ABCG2 antibody (41). No difference in ABCG2 protein level was observed in the control (DMSO treated) and curcumin-treated cells (Supplementary Fig. S2A).<sup>3</sup> Similarly, the curcumin mixture-treated (10 μmol/L) MCF-7 AdVp3000 cells did not show any significant difference in their ability to efflux mitoxantrone from the control untreated cells (Supplementary Figure S2B–E).<sup>3</sup>

#### Effect of Curcuminoids on ATP Hydrolysis by ABCG2

The ATPase activity of ABCG2 is inhibited by orthovanadate (42) as well as by BeF<sub>x</sub>.<sup>4</sup> The BeF<sub>x</sub>-sensitive basal and the substrate-stimulated ATPase activity was determined in crude membranes isolated from wild-type (482R-HEK293) cells. As shown in Fig. 3A, prazosin stimulated the ATPase activity of ABCG2 in a concentration-dependent manner to 3- to 3.2-fold of the basal level with a concentration of 2.4 μmol/L required for 50% stimulation

(Supplementary Table S1),<sup>3</sup> which was consistent with previous reports (42, 43). The presence of the curcumin mixture and curcumin I, II, and III stimulated BeF<sub>x</sub>-sensitive ABCG2 ATPase activity, with a concentration of 7.52, 7.47, 15.06, and 18.05 nmol/L required for 50% stimulation, respectively ([Fig. 3B]; Supplementary Table S1),<sup>3</sup> whereas there was no effect of curcuminoids on ATP hydrolysis in membranes prepared from pcDNA3.1-HEK293 cells (data not shown). It is important to note that basal ABCG2 ATPase activity is in the range of 20 to 25 nmol P<sub>i</sub>/min/mg protein in HEK293 membranes and the curcuminoids stimulate this activity at significantly lower (7.4–18 nmol/L) concentration compared with prazosin or mitoxantrone (Supplementary Table S1).<sup>3</sup> The ABCG2-specific inhibitor fumitremorgin C inhibited the ATPase activity in a concentration-dependent manner and the IC<sub>50</sub> for inhibition was 1.2 to 1.4 μmol/L (Fig. 3C). Furthermore, fumitremorgin C at 10 μmol/L inhibited curcumin I-stimulated ATPase activity in crude membranes of R482 wild-type ABCG2-expressing cells (Fig. 3D). These results show that curcuminoids stimulate ABCG2-specific ATPase activity.

#### Photoaffinity Labeling of ABCG2 with [<sup>125</sup>I]IAAP and [<sup>3</sup>H]Azidopine

The results described above suggest that curcuminoids might interact with the drug substrate-binding sites of ABCG2. Therefore, the binding of the two photoaffinity substrate analogues [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine to ABCG2 was studied. The crude membranes from MCF-7 AdVp3000 (482T) cells were used to study the photolabeling of ABCG2, as there was no difference in the binding of these agents to the wild-type and R482-G/T mutant ABCG2 expressed in HEK293 cells (data not shown). The crude membranes from MCF-7 (Fig. 4A and B, lane 1) and MCF-7 AdVp3000 (Fig. 4A and B, lanes 2–6) cells were incubated with 10 μmol/L of the indicated curcuminoids for 10 minutes at 21°C to 23°C and 3 to 6 nmol/L [<sup>125</sup>I]IAAP (Fig. 4A) or 0.5 μmol/L [<sup>3</sup>H]azidopine (Fig. 4B) was added and incubated for an additional 5 minutes under subdued light. The samples were then illuminated with a UV lamp (365 nm) for 10 minutes and immunoprecipitated as described in Materials and Methods. It was observed that the presence of curcuminoids inhibited the incorporation of [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine into ABCG2 and curcumin I

<sup>4</sup>S. Shukla, W. Chearwae, and S.V. Ambudkar, unpublished data.

was most effective in inhibiting this labeling (Fig. 4A and B, lane 4). Additional experiments showed that the inhibition of [ $^{125}$ I]IAAP binding to ABCG2 was concentration dependent, with  $IC_{50}$ s of 0.81, 0.54, 3.2, and  $>25$   $\mu$ mol/L for the curcumin mixture and curcumin I, II, and III, respectively (data not shown).

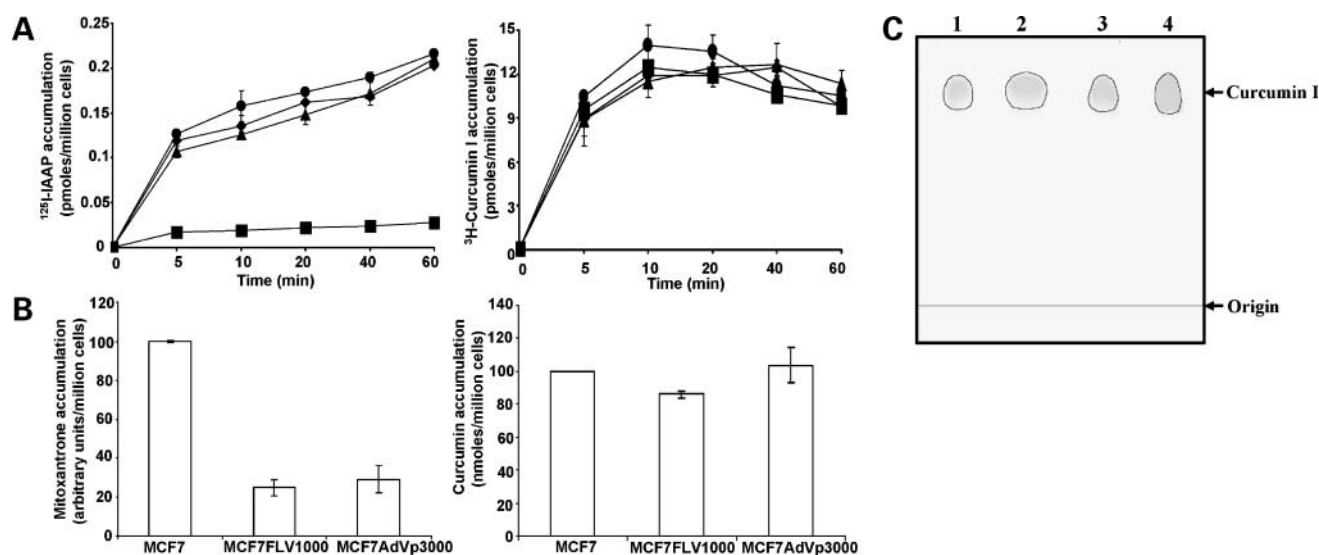
### Curcuminoids Inhibit the Transport of [ $^{125}$ I]IAAP and [ $^3$ H]Azidopine

An accumulation assay in intact cells was done to test whether the transport of [ $^{125}$ I]IAAP and [ $^3$ H]azidopine was also inhibited by curcuminoids. The MCF-7 or MCF-7 AdVp3000 cells were incubated with 3 to 5 nmol/L [ $^{125}$ I]IAAP or 25 nmol/L [ $^3$ H]azidopine in the absence (control) or presence of 10  $\mu$ mol/L curcumin mixture or fumitremorgin C at 32°C for 60 minutes. The radioactivity (drug accumulation) associated with the cells was determined as described in Materials and Methods. As shown in Fig. 4C, after 60 minutes, the MCF-7 cells accumulated 4-fold more [ $^{125}$ I]IAAP than MCF-7 AdVp3000 cells. The presence of 10  $\mu$ mol/L curcumin mixture or fumitremorgin C completely inhibited the efflux mediated by ABCG2 and [ $^{125}$ I]IAAP accumulation reached the same level in MCF-7 AdVp3000 cells as MCF-7 cells. Similar results were also observed with [ $^3$ H]azidopine accumulation. Whereas the control MCF-7 cells accumulated 14 times more [ $^3$ H]azidopine than the MCF-7 AdVp3000 cells, the presence of 10  $\mu$ mol/L curcumin mixture or fumitremorgin C completely inhibited the efflux mediated by ABCG2

in MCF-7 AdVp3000 cells (Fig. 4D). As shown in Fig. 2C, during 60-minute incubation, curcumin I is not metabolized, indicating that the curcumin I itself interferes with the binding of the transport substrate. Taken together, the transport and the photolabeling assay clearly showed that curcuminoids inhibit the binding of the substrates to the transporter and prevent efflux of [ $^{125}$ I]IAAP and [ $^3$ H]azidopine, thus increasing their concentrations inside the cells.

### Effect of Curcuminoids on the Binding of [ $\alpha$ - $^{32}$ P]8-AzidoATP to ABCG2

We reported earlier that that compounds, such as disulfiram, interact with both the ATP-binding site and the substrate-binding site of ABCB1 (36). Therefore, the effect of curcuminoids on the binding of [ $\alpha$ - $^{32}$ P]8-azidoATP to ABCG2 was investigated. The crude membranes from MCF-7 AdVp3000 cells were incubated with 25  $\mu$ mol/L of the curcuminoids for 10 minutes at 4°C in ATPase assay buffer (without DTT), and 10  $\mu$ mol/L [ $\alpha$ - $^{32}$ P]8-azidoATP (10  $\mu$ Ci/nmol) was added. The samples were then photo-cross-linked on ice and processed as described in Materials and Methods. We observed that the presence of curcuminoids did not have any detectable effect on the binding of the [ $\alpha$ - $^{32}$ P]8-azidoATP to ABCG2 (Supplementary Fig. S3),<sup>3</sup> which suggests that they interact with the substrate-binding site(s) and do not interfere with binding of ATP to the nucleotide-binding domain. Therefore, the stimulation of ATPase activity of



**Figure 2.** Accumulation of [ $^3$ H]curcumin I, [ $^{125}$ I]IAAP, or mitoxantrone in MCF-7, MCF-7 FLV1000, and MCF-7 AdVp3000 cells. **A**, MCF-7 (control; ◆ and ▲) and MCF-7 FLV1000 (■ and ●) cells were incubated with 3 to 5 nmol/L [ $^{125}$ I]IAAP or 200 nmol/L [ $^3$ H]curcumin I in the presence (▲ and ●) and absence (◆ and ■) of 10  $\mu$ mol/L fumitremorgin C at 32°C for 5, 10, 20, 40, and 60 min. The accumulated [ $^{125}$ I]IAAP or [ $^3$ H]curcumin I in the cells was estimated as described in Materials and Methods. **B**, MCF-7 (control), MCF-7 FLV1000, and MCF-7 AdVp3000 cells were incubated with 5  $\mu$ mol/L mitoxantrone or 10  $\mu$ mol/L curcumin I at 32°C for 60 min in RPMI. The cells were washed and the accumulated mitoxantrone or curcumin I in the cells was extracted by ethanol and processed as described in Materials and Methods. Histograms depict the fluorescence intensity of mitoxantrone (left) or curcumin I (right) ethanolic extracts, which was read at 695 and 540 nm for mitoxantrone and curcumin I, respectively. Bars, SE ( $n = 3$ ). **C**, extracted curcumin I resuspended in ethanol was spotted on TLC aluminum sheets coated with Silica Gel 60 F<sub>254</sub>. The curcumin I extracts from MCF-7 (lane 1), MCF-7 AdVp3000 (lane 2), and MCF-7 FLV1000 (lane 3) cells and 2.5  $\mu$ L of 10  $\mu$ mol/L standard curcumin I (lane 4) were separated using the solvent system (CHCl<sub>3</sub>/ethanol/acetic acid, 94:5:1; ref. 26). Representative plate from three individual experiments. Arrows, origin and curcumin I spot on the plate.

ABCG2 (Fig. 3B) by curcuminoids is most likely due to their interaction at the transport substrate site(s) or at allosteric site(s), which affect interaction of substrates with transport substrate-binding site(s).

## Discussion

We have shown previously that curcuminoids are inhibitors of the ABC transporters, ABCB1 (25, 26) and ABCC1 (27), which are known to play a major role in the development of MDR in cancer cells. In this study, purified curcuminoids were tested for their interaction with ABCG2. The curcuminoids inhibited the transport of the substrates from ABCG2-expressing cells (Fig. 1; Table 1) and the maximal inhibition was comparable with 10  $\mu\text{mol/L}$  fumitremorgin C, a potent inhibitor of ABCG2 (12). The cytotoxicity data suggested that the curcuminoids themselves may not be transported by ABCG2 (Table 2). This was further confirmed by curcumin I and [ $^3\text{H}$ ]curcumin I accumulation assays, which showed no difference in the accumulation of this curcuminoid in the control and ABCG2-expressing cells (Fig. 2).

The regulation of ABCG2 at the gene level is being studied and recent work has shown functional hormone (estrogen) and hypoxia response elements in the promoter (44–47) and curcuminoids have been shown to affect the function of several transcription factors (reviewed in ref. 20). For this reason, we tested the effect of curcuminoids on the expression of ABCG2. No detectable difference in the expression of ABCG2 was observed in MCF-7 AdVp3000 cells grown in the presence of 10  $\mu\text{mol/L}$  curcuminoids for 72 hours compared with the untreated cells (Supplementary Fig. S2),<sup>3</sup> indicating that curcuminoids do not affect the protein levels of the transporter and the reversal of resistance is due to their effect on the function of ABCG2.

A possible mechanism of ABCG2 inhibition, based on the above findings, was the direct interaction of curcumin with ABCG2. Therefore, it was investigated by two different approaches, an ATPase assay and photoaffinity labeling with transport substrates. It was observed that the curcuminoids stimulate ATP hydrolysis mediated by

**Table 3. Reversal of resistance to selected drugs by 10  $\mu\text{mol/L}$  curcuminoids**

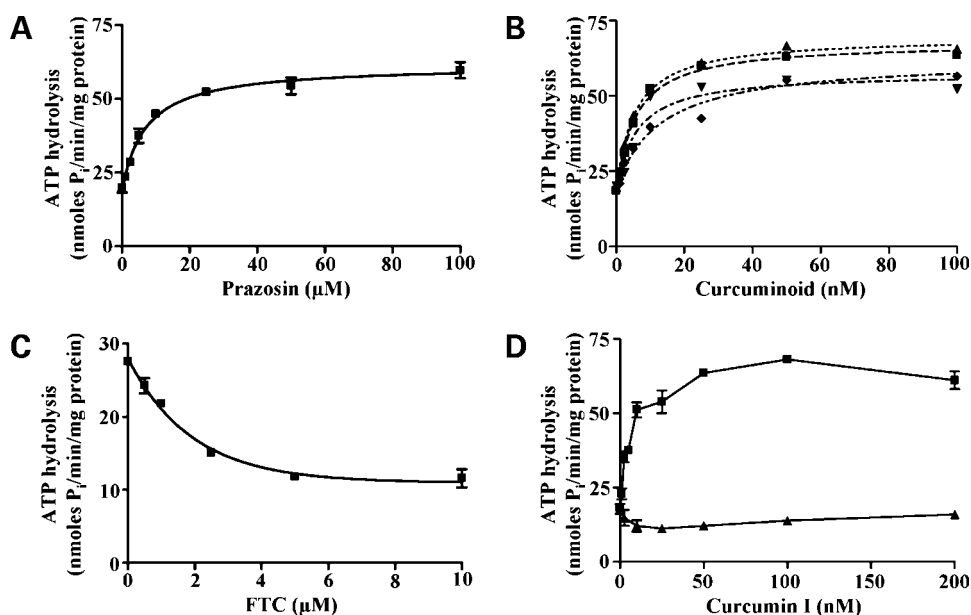
Drug	IC <sub>50</sub> * (relative resistance) <sup>†</sup>			
	pcDNA3.1-HEK-293 (nmol/L)	ABCG2 (482R-HEK293; nmol/L)	MCF-7 (nmol/L)	MCF-7 AdVp3000 (482T; nmol/L)
Mitoxantrone alone	4.80	192.40 (40)	2.4	3,400.0 (1,416)
Mitoxantrone + curcumin M	4.60	22.92 (5.0) <sup>‡</sup>	1.9	77.5 (40.7) <sup>‡</sup>
Mitoxantrone + curcumin I	4.50	21.51 (4.7) <sup>‡</sup>	2.2	46.3 (21.0) <sup>‡</sup>
Mitoxantrone + curcumin II	4.80	26.10 (5.4) <sup>‡</sup>	2.5	132.5 (53.0) <sup>‡</sup>
Mitoxantrone + curcumin III	4.50	29.60 (6.6) <sup>‡</sup>	1.9	280.0 (147.4) <sup>‡</sup>
Mitoxantrone + fumitremorgin C (5 $\mu\text{mol/L}$ ) <sup>§</sup>			2.1	210.0 (100.0) <sup>‡</sup>
Topotecan alone	14.20	327.40 (23)		
Topotecan + curcumin M	14.10	38.46 (2.7) <sup>‡</sup>		
Topotecan + curcumin I	12.00	35.38 (3.0) <sup>‡</sup>		
Topotecan + curcumin II	14.20	39.60 (2.8) <sup>‡</sup>		
Topotecan + curcumin III	14.70	38.50 (2.6) <sup>‡</sup>		
SN-38 alone	5.60	204.40 (36.5)		
SN-38 + curcumin M	5.60	44.09 (7.8) <sup>‡</sup>		
SN-38 + curcumin I	5.80	42.86 (7.4) <sup>‡</sup>		
SN-38 + curcumin II	5.80	45.25 (7.8) <sup>‡</sup>		
SN-38 + curcumin III	5.70	71.17 (12.4) <sup>‡</sup>		
			MCF-7 (nmol/L)	MCF-7 AdVp3000 (482T; $\mu\text{mol/L}$ )
Doxorubicin alone			101.0	24.10 (238)
Doxorubicin + mitoxantrone (0.5 nmol/L) <sup>§</sup>			130.0	0.50 (3.8) <sup>‡</sup>
Doxorubicin + curcumin M			105.0	6.70 (63.8) <sup>‡</sup>
Doxorubicin + curcumin I			75.30	6.20 (82.3) <sup>‡</sup>
Doxorubicin + curcumin II			126.70	6.20 (48.9) <sup>‡</sup>
Doxorubicin + curcumin III			131.70	10.2 (77.4) <sup>‡</sup>

\*Mean from three independent experiments done in triplicates. Curcuminoids (curcumin M, I, II, and III) were used at 10  $\mu\text{mol/L}$ .

<sup>†</sup>Relative resistance is calculated by dividing the IC<sub>50</sub> of anticancer drug in the presence of DMSO control divided by the IC<sub>50</sub> of the drug in the presence of a modulator.

<sup>‡</sup> $P < 0.05$ , significantly different from the control untreated cells.

<sup>§</sup>Fumitremorgin C (5  $\mu\text{mol/L}$ ) or mitoxantrone (0.5 nmol/L) alone had minimal (<5% cell killing) cytotoxic effect (data not shown).



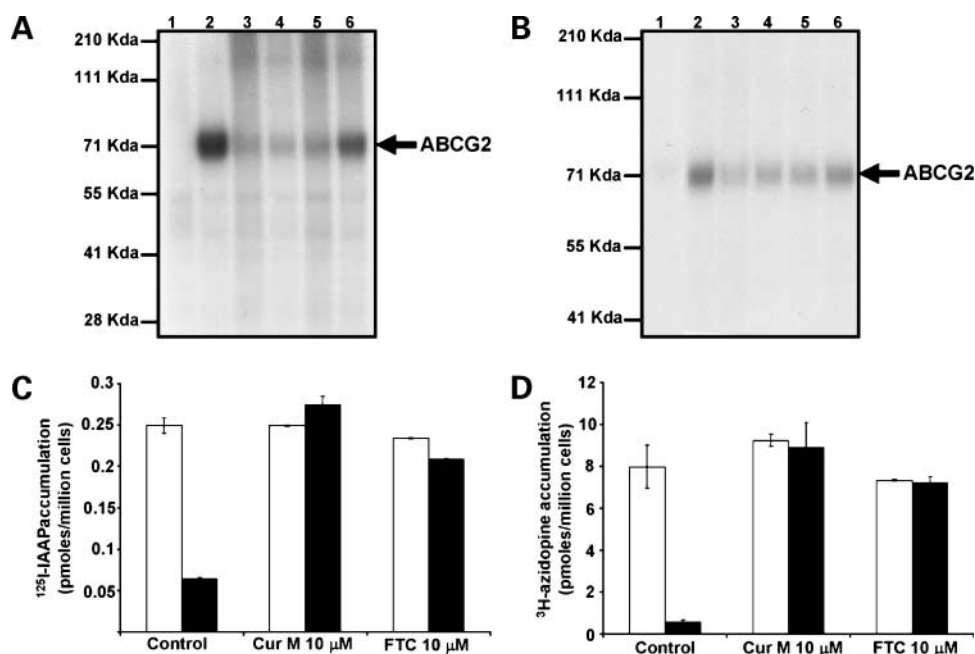
**Figure 3.** Effect of curcuminoids and fumitremorgin C on BeF<sub>x</sub>-sensitive ATPase activity in crude membranes from 482R-HEK293 cells: Crude membrane protein (100 μg protein/mL) from 482R-HEK293 cells was incubated at 37°C with varying concentrations of (A) prazosin; (B) curcumin mixture (■), curcumin I (▲), curcumin II (▼), or curcumin III (◆); (C) fumitremorgin C (■); and (D) curcumin I (■) alone or 10 μmol/L fumitremorgin C + curcumin I (▲) in the presence and absence of BeF<sub>x</sub> (0.2 mmol/L beryllium sulfate, 2.5 mmol/L NaF) in ATPase assay buffer for 10 min. The reaction was started by the addition of 5 mmol/L ATP and was stopped by the addition of 0.1 mL of 5% SDS solution. The amount of inorganic phosphate released and the BeF<sub>x</sub>-sensitive ATPase activity of ABCG2 was determined as described in Materials and Methods. Points, mean of three independent experiments done in duplicates; bars, SD.

ABCG2 at very low concentrations (Fig. 3B), and fumitremorgin C, which is an inhibitor of ABCG2 ATPase (Fig. 3C), abolished the stimulatory effect of curcumin I (Fig. 3D). However, the transport data (Fig. 2) with [<sup>3</sup>H]curcumin I or unlabeled curcumin I suggest that curcumin I may not be transported by ABCG2 but can still stimulate the ATPase activity of the transporter. These findings are consistent with our previously reported observations concerning ABCB1 and ABCC1, where we showed that curcuminoids, which are not transport substrates, do stimulate ATP hydrolysis by these transporters (26, 27). Additionally, *cis*-flupentixol has been shown to stimulate ATP hydrolysis by ABCB1 possibly by allosteric modulation, although it is not a transport substrate (47). The two photoaffinity analogues [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine have been used to study the drug-substrate interactions of ABCB1. We were also able to label ABCG2 with [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine (Fig. 4A and B) and observed that the curcuminoids inhibited this photolabeling. Additionally, we used an intact cell assay also to determine the effect of curcuminoids on the transport of [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine and found that similar to its effect on the photolabeling with these two analogues in the crude membranes it also completely blocked the transport of these two substrates (Fig. 4C and D). At present, it is not clear whether curcuminoids bind to same transport substrate site(s) or at allosteric site(s), which affect interaction of substrates with transport substrate-binding site(s). Supplementary Table S2<sup>3</sup> compares various variables

assessing the interaction of curcumin I with the three ABC transporters (ABCB1, ABCC1, and ABCG2). The degree of stimulation of substrate-stimulated ATP hydrolysis by this curcuminoid is maximal for ABCG2 (3.3-fold) compared with ABCB1 and ABCC1. In addition, the concentration of curcumin I required to obtain maximum stimulation was 5 nmol/L for ABCG2, whereas it was 500 nmol/L for ABCB1, suggesting that curcumin I exhibits higher affinity for ABCG2 than the other two ABC transporters. Similarly, the lower IC<sub>50</sub> for ABCG2 (0.54 μmol/L) compared with ABCB1 (5.8 μmol/L) for inhibition of IAAP labeling suggests that curcumin I is a potent inhibitor of the IAAP binding to ABCG2 than ABCB1.

Allen et al. reported that Ko143, a synthetic compound, is a potent inhibitor of ABCG2 (13). Both Ko143 and GF120918 compounds represent a new generation of promising inhibitors (13). The IC<sub>50</sub> of Ko143 and GF120918 was reported to be 19 and 18 μmol/L for MCF-7 cells (13), respectively, whereas curcuminoids have a cytotoxicity value ranging from 25 to 50 μmol/L for the same cells (Table 2). This indicates that curcuminoids are less toxic than these compounds and may safely be used as ABCG2 inhibitors.

Several experimental and clinical studies indicate that curcumin exhibits antioxidant, anti-inflammatory, and anticancer properties (20, 23, 49). It has also been reported that the use of commercially available curcumin supplements (2–4 g/d) or recommended therapeutic oral dose of 6 to 8 g/d results in micromolar concentrations of



**Figure 4.** Effect of curcuminoids on photoaffinity labeling of ABCG2 with [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine and accumulation of these photolabels in ABCG2-expressing cells. **A** and **B**, photoaffinity labeling with [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine. Crude membranes (500 μg/mL) from MCF-7 (control; lane 1) and MCF-7 AdVp3000 cells (lanes 2–6) were incubated with 10 μmol/L of the indicated curcuminoid (lane 2, control, DMSO alone; lane 3, curcumin mixture; lane 4, curcumin I; lane 5, curcumin II; lane 6, curcumin III) for 10 min at 21 °C to 23 °C in 50 mmol/L Tris-HCl (pH 7.5). Three to six nmol/L [<sup>125</sup>I]IAAP (2,200 Ci/mmol; **A**) or 0.5 μmol/L [<sup>3</sup>H]azidopine (60 Ci/mmol; **B**) was added and incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp (365 nm) for 10 min and processed as described in Materials and Methods. Arrows, position of the ABCG2 band. **C** and **D**, accumulation of [<sup>125</sup>I]IAAP and [<sup>3</sup>H]azidopine in ABCG2-expressing cells. The control MCF-7 (□) and MCF-7 AdVp3000 (■) cells were incubated with 3 to 5 nmol/L [<sup>125</sup>I]IAAP (**C**) or 25 nmol/L [<sup>3</sup>H]azidopine (**D**) in the absence (control) or presence of 10 μmol/L curcumin mixture or fumitremorgin C at 32 °C for 60 min in RPMI supplemented with 5% fetal bovine serum. The cells were immediately washed with cold PBS and lysed by trypsinization and the amount of radioactive drug accumulated in the cells was measured as described in Materials and Methods. Histograms show the amount of [<sup>125</sup>I]IAAP (**C**) or [<sup>3</sup>H]azidopine (**D**) accumulated in the cells in the presence or absence of 10 μmol/L curcumin mixture (*Cur M*) or fumitremorgin C (*FTC*). Columns, mean (*n* = 3); bars, SD.

curcuminoids in the gastrointestinal tract (50–55). In addition, oral intake of 8 g/d curcumin resulted in ~2 μmol/L concentration in serum (56). A recent report detected low levels of curcumin after 8 g of oral dose, but it could be because of the type of formulation used, which had 66% less curcumin than the pure curcumin powder (57). Therefore, curcuminoid intake can be expected to result in concentrations high enough to increase the levels of the anticancer drugs in the cells through its inhibitory effect on ABCG2. A similar approach has been reported to increase the oral bioavailability of topotecan by coadministration of the ABCG2 and ABCB1 inhibitor GF120918 (58). Additionally, simultaneous administration or intake of curcuminoids with ABCG2 substrates and/or antitumor agents might result in the alteration of their pharmacokinetics. This also might increase the toxicity or bioavailability of the different drugs and nutrients.

This work and our previous studies have shown that curcumin I modulates effectively the function of ABCG2, ABCB1, and ABCC1 (25–27). This is not surprising, as these transporters exhibit overlapping substrate specificity (6, 59). In summary, these findings suggest that curcuminoids may play an important role in clinical

therapy and the simultaneous administration of recommended therapeutic doses of curcuminoids with anticancer drugs would probably result in an increased bioavailability of the drugs inside the cells. Thus, these curcuminoids (or curcumin mixture) might be used as broad-spectrum modulators offering clinical benefits to improve the effectiveness of chemotherapy in cancer patients.

#### Acknowledgments

We thank Dr. Susan Bates for providing the cell lines, Dr. Michael M. Gottesman for encouragement, Drs. Zuben Sauna, Krishnamachary Nandigama, and In-Wha Kim for comments on the article, and George Leiman for assistance in the preparation of the article.

#### References

- Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* 1999;39: 361–98.
- Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48–58.
- Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* 1998;95:15665–70.

4. Maliepaard M, van Gastelen MA, de Jong LA, et al. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* 1999;59:4559–63.
5. Yang CH, Schneider E, Kuo ML, Volk EL, Rocchi E, Chen YC. BCRP/MXR/ABCP expression in topotecan-resistant human breast carcinoma cells. *Biochem Pharmacol* 2000;60:831–7.
6. Haimeur A, Conseil G, Deeley RG, Cole SP. The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. *Curr Drug Metab* 2004;5:21–53.
7. Miyake K, Mickley L, Litman T, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* 1999;59:8–13.
8. Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* 2003;22:7340–58.
9. Bates SE, Medina-Perez WY, Kohlhagen G, et al. ABCG2 mediates differential resistance to SN-38 (7-ethyl-10-hydroxycamptothecin) and homocamptothecins. *J Pharmacol Exp Ther* 2004;310:836–42.
10. Yoshikawa M, Ikegami Y, Sano K, et al. Transport of SN-38 by the wild type of human ABC transporter ABCG2 and its inhibition by quercetin, a natural flavonoid. *J Exp Ther Oncol* 2004;4:25–35.
11. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981;41:1967–72.
12. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 2000;60:47–50.
13. Allen JD, van Loevezijn A, Lakhai JM, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 2002;1:417–25.
14. Garimella TS, Ross DD, Bauer KS. Liquid chromatography method for the quantitation of the breast cancer resistance protein ABCG2 inhibitor fumitremorgin C and its chemical analogues in mouse plasma and tissues. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;807:203–8.
15. Garimella TS, Ross DD, Eiseman JL, et al. Plasma pharmacokinetics and tissue distribution of the breast cancer resistance protein (BCRP/ABCG2) inhibitor fumitremorgin C in SCID mice bearing T8 tumors. *Cancer Chemother Pharmacol* 2005;55:101–9.
16. Ammon HP, Wahl MA. Pharmacology of *Curcuma longa*. *Planta Med* 1991;57:1–7.
17. Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet R, Sigman CC. Chemopreventive drug development: perspectives and progress. *Cancer Epidemiol Biomarkers Prev* 1994;3:85–98.
18. Kelloff GJ, Crowell JA, Steele VE, et al. Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* 2000;130:467–715.
19. Ramsewak RS, DeWitt DL, Nair MG. Cytotoxicity, antioxidant and anti-inflammatory activities of curcumins I-III from *Curcuma longa*. *Phytomedicine* 2000;7:303–8.
20. Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* 2003;23:363–98.
21. Hong J, Bose M, Ju J, et al. Modulation of arachidonic acid metabolism by curcumin and related  $\beta$ -diketone derivatives: effects on cytosolic phospholipase A(2), cyclooxygenases and 5-lipoxygenase. *Carcinogenesis* 2004;25:1671–8.
22. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci* 2004;74:2157–84.
23. Kawamori T, Lubet R, Steele VE, et al. Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res* 1999;59:597–601.
24. Lechtenberg M, Quandt B, Nahrstedt A. Quantitative determination of curcuminoids in *Curcuma rhizomes* and rapid differentiation of *Curcuma domestica* Val. and *Curcuma xanthorrhiza* Roxb. by capillary electrophoresis. *Phytochem Anal* 2004;15:152–8.
25. Anuchapreeda S, Leechanachai P, Smith MM, Ambudkar SV, Limtrakul PN. Modulation of P-glycoprotein expression and function by curcumin in multidrug-resistant human KB cells. *Biochem Pharmacol* 2002;64:573–82.
26. Chearwae W, Anuchapreeda S, Nandigama K, Ambudkar SV, Limtrakul P. Biochemical mechanism of modulation of human P-glycoprotein (ABCB1) by curcumin I, II, and III purified from turmeric powder. *Biochem Pharmacol* 2004;68:2043–52.
27. Chearwae W, Wu CP, Chu HY, Lee TR, Ambudkar SV, Limtrakul P. Curcuminoids purified from turmeric powder modulate the function of human multidrug resistance protein 1 (ABCC1). *Cancer Chemother Pharmacol* 2006;57:376–88.
28. Robey RW, Honjo Y, van de Laar A, et al. A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). *Biochim Biophys Acta* 2001;1512:171–82.
29. Robey RW, Honjo Y, Morisaki K, et al. Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. *Br J Cancer* 2003;89:1971–8.
30. Honjo Y, Hrycyna CA, Yan QW, et al. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res* 2001;61:6635–9.
31. Robey RW, Steadman K, Polgar O, et al. Pheophorbide a is a specific probe for ABCG2 function and inhibition. *Cancer Res* 2004;64:1242–6.
32. Robey RW, Medina-Perez WY, Nishiyama K, et al. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (MXR/BCRP/ABCP1), in flavopiridol-resistant human breast cancer cells. *Clin Cancer Res* 2001;7:145–52.
33. Muller M, Yong M, Peng XH, Petre B, Arora S, Ambudkar SV. Evidence for the role of glycosylation in accessibility of the extracellular domains of human MRP1 (ABCC1). *Biochemistry* 2002;41:10123–32.
34. Ambudkar SV. Drug-stimulatable ATPase activity in crude membranes of human MDR1-transfected mammalian cells. *Methods Enzymol* 1998;292:504–14.
35. Schaffner W, Weissmann C. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal Biochem* 1973;56:502–14.
36. Sauna ZE, Peng XH, Nandigama K, Tekle S, Ambudkar SV. The molecular basis of the action of disulfiram as a modulator of the multidrug resistance-linked ATP binding cassette transporters MDR1 (ABCB1) and MRP1 (ABCC1). *Mol Pharmacol* 2004;65:675–84.
37. Gribar JJ, Ramachandra M, Hrycyna CA, Dey S, Ambudkar SV. Functional characterization of glycosylation-deficient human P-glycoprotein using a vaccinia virus expression system. *J Membr Biol* 2000;173:203–14.
38. Shukla S, Robey RW, Bates SE, Ambudkar SV. The calcium channel blockers, 1,4-dihydropyridines, are substrates of the multidrug resistance-linked ABC drug transporter, ABCG2. *Biochemistry* 2006;45:8940–51.
39. Chen YN, Mickley LA, Schwartz AM, Acton EM, Hwang JL, Fojo AT. Characterization of Adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. *J Biol Chem* 1990;265:10073–80.
40. Allen JD, Jackson SC, Schinkel AH. A mutation hot spot in the Bcrp1 (Abcg2) multidrug transporter in mouse cell lines selected for doxorubicin resistance. *Cancer Res* 2002;62:2294–9.
41. Maliepaard M, Scheffer GL, Faneyte IF, et al. Sub cellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res* 2001;61:3458–64.
42. Ozvegy C, Litman T, Szakacs G, et al. Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. *Biochem Biophys Res Commun* 2001;285:111–7.
43. Polgar O, Robey RW, Morisaki K, et al. Mutational analysis of ABCG2: role of the GXXXG motif. *Biochemistry* 2004;43:9448–56.
44. Bailey-Dell KJ, Hassel B, Doyle LA, Ross DD. Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. *Biochim Biophys Acta* 2001;1520:234–41.
45. Ee PL, Kamalakaran S, Tonetti D, He X, Ross DD, Beck WT. Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res* 2004;64:1247–51.
46. Krishnamurthy P, Ross DD, Nakanishi T, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heregulin. *J Biol Chem* 2004;279:24218–25.
47. Imai Y, Ishikawa E, Asada S, Sugimoto Y. Estrogen-mediated post transcriptional down-regulation of breast cancer resistance protein/ABCG2. *Cancer Res* 2005;65:596–604.

48. Maki N, Hafkemeyer P, Dey S. Allosteric modulation of human P-glycoprotein. Inhibition of transport by preventing substrate translocation and dissociation. *J Biol Chem* 2003;278:18132–9.
49. Antunes LM, Araujo MC, Darin JD, Bianchi ML. Effects of the antioxidants curcumin and vitamin C on cisplatin-induced clastogenesis in Wistar rat bone marrow cells. *Mutat Res* 2000;465:131–7.
50. Sharma RA, Euden SA, Platton SL, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res* 2004;10:6847–54.
51. Zhou S, Lim LY, Chowbay B. Herbal modulation of P-glycoprotein. *Drug Metab Rev* 2004;36:57–104.
52. Arbiser JL, Klauber N, Rohan R, et al. Curcumin is an *in vivo* inhibitor of angiogenesis. *Mol Med* 1998;4:376–83.
53. Krishnaswamy K, Raghuramulu N. Bioactive phytochemicals with emphasis on dietary practices. *Indian J Med Res* 1998;108:167–81.
54. Rao CV, Rivenson A, Simi B, Reddy BS. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res* 1995;55:259–66.
55. Kuttan R, Bhanumathy P, Nirmala K, George MC. Potential anticancer activity of turmeric (*Curcuma longa*). *Cancer Lett* 1985;29:197–202.
56. Cheng AL, Hsu CH, Lin JK, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* 2001;21:2895–900.
57. Lao CD, Ruffin MT, Normolle D, et al. Dose escalation of a curcuminoid formulation. *BMC Complement Altern Med* 2006;6:10; doi:10.1186/1472-6882-6-10.
58. Kruijtzter CM, Beijnen JH, Rosing H, et al. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* 2002;20:2943–50.
59. Litman T, Druley TE, Stein WD, Bates SE. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* 2001;58:931–59.