CHAPTER II
MATERIALS AND METHODS

2.1 Chemicals and materials

The names of the chemicals, reagents and instruments are shown in Appendix B-E.

2.2 Extraction and fractionation of curcuminoids [128].

Curcuminoids were prepared following the method described previously in Ref. no [128]. Turmeric rhizomes were dried and blended to a powder form then extracted with 95% ethanol at room temperature for twenty-four hours. The ethanolic extract was filtered through Whatman filter paper no.1 to remove the remaining powder, and then the ethanol was removed by using a rotary evaporator at 60°C for 1-2 h. Turmeric curcuminoids were then precipitated by adding petroleum ether then allowing the formation of orange crystals at 10°C for three days. The supernatant was removed and the crystal curcuminoids were dried. The purification of curcumin I, II and III from the crude curcuminoids was accomplished by first washing the crude curcuminoids with isopropanol to reduce the percentage of curcumin III, and then fractionating them by silica gel 60 column chromatography as follows: 3 grams of curcuminoids were dissolved in 30 ml acetone and then centrifuged at 2000 rpm for 10 minutes. The supernatant was collected and loaded onto a silica gel 60G column (44x1.6 cm) and eluted by using 100% $CHCl_3$ and increasing polarity of $CHCl_3$/methanol (98:2, 95:5). The fractions were collected and spotted onto TLC aluminum sheets coated with silica gel 60 F254 and developed by mobile phase; $CHCl_3$:ethanol: acetic acid = 94: 5: 1. Fractions that showed the same pattern on TLC were pooled and the organic solvent was removed to obtain the powder form.

The identity and purity of curcuminoids were further verified using analysis by HPLC, IR, MS, and NMR [28, 129]. For HPLC, the samples were submitted to Pharmaceutical Science Research and Service Center, Faculty of Pharmacy, Chiang Mai University. Whereas, for the IR and MS analysis, the samples were sent to Science and Technology Service Center (STSC) and Postgraduate Education and

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Research Program in Chemistry Higher Education Development Project, Ministry of Education (PERCH), Faculty of Science, Chiang Mai University, respectively. For NMR, the samples were analysed by the service of BIOTEC Central Research Unit, National Center for Genetic Engineering and Biotechnology (NSTDA).

In this study the effects of curcumin I, II and III purified from turmeric were evaluated in comparison with curcumin mixture on function and expression of three ABC transporters: ABCB1 (P-gp), ABCC1 (MRP1) and ABCG2 (MXR) as summarized in Figure 10. Finally, tetrahydrocurcumin, which is a major metabolite form of curcumin in vivo [122], was studied to obtain a brief additional information of curcumin metabolite.

2.3 Cell lines

As shown in Figure 10, the effects of three major forms of curcuminoids as well as curcumin mixture from Sigma on three types of ABC transporters – P-gp, MRP1 and MXR- were characterized. Drug sensitive and drug resistant cell lines used in this study are given in Table 11.

Table 11. Drug sensitive and drug resistant cell lines used in this study.

Type of ABC transporter	Cell lines	
	Drug sensitive	**Drug resistant**
ABCB1 (P-gp)	KB-3-1 NIH3T3 MCF-7	KB-V-1 NIH3T3-MDR-G185 MCF7-MDR
ABCC1 (MRP1)	pcDNA3.1-HEK 293	MRP1-HEK293
ABCG2 (MXR)	pcDNA3.1-HEK 293 MCF-7	MXR(482R)-HEK 293 MXR(R482T)-HEK293 MCF-7AdrVp (R482T)

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All cell lines used in this study were generous gifts from Laboratory of Cell Biology, NCI/NIH., which is headed by Dr. Michael M. Gottesman, director, and Dr. Suresh Ambudkar, the principal investigator. Details of cell lines as follow;

KB-3-1 cell lines: The parental cell line was obtained from the primary cervix carcinoma of a 31-year-old African-American woman. The drug-sensitive KB-3-1

cell line was derived from a single clone of human KB epidermoid carcinoma cell after two subclonings [130].

KB-V-1 cell lines: The multidrug resistant subline was derived from the parental line KB-3-1 by serial passage with increasing vinblastine concentrations. KB-V-1 cell line was maintained in the presence of 1 μg/ml vinblastine. These cells overexpress P-glycoprotein and possess 100-fold amplification of *MDR*1 gene [130].

NIH3T3 cell lines: The drug-sensitive NIH3T3 parental cell line is a continuous line of contact-inhibited cells derived from NIH Swiss mouse embryo cultures. NIH3T3 is the parent cell line for a number of MDR cell lines (i.e., NIH-MDR-G185, and NIH-MDR-V185) [131].

NIH3T3-MDR-G185: The NIH3T3-MDR-G185 cell line is a clone of mouse NIH3T3 cells transfected with the retroviral vector pHaMDR1/A (wild type glycine at position 185), and then selected in 60 ng/ml colchicines. This cell line is over 100-fold more resistant to colchicines than NIH3T3 cells and cross resistant to vinblastine, duanorubicin, taxol, etc [131].

MCF-7: The MCF-7 cells were derived from human breast cancer. It is the parental line for MCF-7 MDR, clone 10.3 cells. Grown as monolayer cultures at 37°C in 5% CO_2 using DMEM with 4.5 g of glucose/liter and 1 mM or 110 mg/liter sodium pyruvate plus 10% fetal bovine serum (FBS), 5 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml) [132]

MCF-7MDR: The MCF-7 MDR, clone 10.3, cells were derived from the parental cell line, human breast MCF-7. They are drug resistant clonal populations maintained in 60 ng/ml of colchicine. Grown as monolayer culture at 37 ^{o}C in 5% CO_2 using DMEM and supplemented with 1mM or 110 mg/liter sodium pyruvate, plus 10% FBS, 5 mM L-glutamine, penicillin (50units/ml) and streptomycin (50μg/ml) [132].

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pcDNA3.1-HEK 293 (drug sensitive cell lines for MRP1): The drug sensitive human embryonic kidney tranfected with empty vector pcDNA 3.1 [133].

MRP1-HEK 293: The drug resistant human embryonic kidney tranfected with pcDNA3.1 plasmid carrying MRP1 cDNA construct. Transfection was carried out using the lipofectamine TM2000 reagent kit (Life Science, Gaithersburg, MD), according to instructions. The stable transfectants were selected in geneticin (G418, 800 μg/ml), Mediatech Inc., Herndon, VA), and the selected clones were cultured in etoposide (VP-16) (5 μM) [133].

pcDNA3.1-HEK 293 (drug sensitive cell lines for MXR): HEK 293 cells were transfected with pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The stable transfectants were selected in 2000 μg/ml G418 [65].

MXR-HEK 293 (482R and R482T): The cells were transfected with pcDNA3.1 vector containing full-length MXR coding either an arginine (482R) or threonine (R482T) for amino acid 482. Expression of MXR in the transfectants was enforced by selection in G418 (Invitrogen, Carlsbad, CA, USA). Stable transfectants were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS, penicillin (50 units/ml), and streptomycin (50μg/ml) with G418 at a concentration of 2mg/ml. Clones were preliminarily screened for MXR expression by examining the ability of the cells to efflux bodipy-prazosin in a flow cytometry-based assay [65].

MCF-7: Human breast cancer cell line which is a parental cell line for MCF-7AdrVp [67].

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MCF-7AdrVp (R482T): The adriamycin (Adr)-resistant subline of MCF-7. The cells were derived from the MCF-7 cell line by a series of stepwise selections with adriamycin in 10 μg/ml of verapamil. The cells were grown as monolayer cultures at 37 ^{0}C in 5% CO_2, and maintained by regular passage in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 units/ml), and

streptomycin (50 μg/ml). Adriamycin was added to the medium at 3 μg/ml with verapamil at 5 μg/ml [67].

2.4. Cell survival measurement (MTT assay)

The MTT assay measures the conversion of the tetrazolium 3-(4, 5 dimethylthiazol-2yl)-5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan in living cells. Mitochondrial succinate dehydrogenase catalyzes the reaction (Figure 11), which requires NADH to be supplied by the living cells, thus providing an indication of cell viability. The formazan crystals are then solubilized with solvents, such as dimethyl sulfoxide (DMSO). The solubilized formazan product is spectrophotometrically measured using an ELISA plate reader.

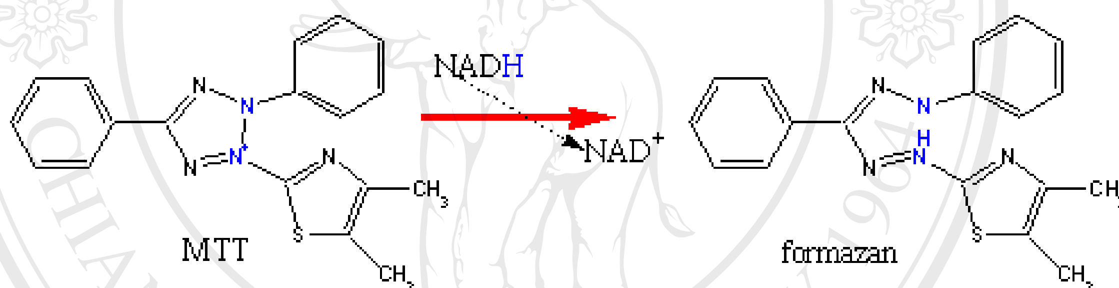

Figure 11. The reaction of MTT by dehydrogenase in cell mitochondria

Procedure

MTT assay was used to detect cytoxicity of curcuminoids and their influence on the cytotoxicity of anticancer drugs. The procedure of MTT assay was conducted by plating the cells (3.0-5.0x10^3 cells) into 96 well plates cultured overnight. Various concentrations of tested compounds were then added and incubated for an additional 72 h. After incubation, 20 μl of MTT dye (5 mg/ml in PBS) was added to each well which was incubated for another 4 h. The bulk of the medium from each well was suctioned off. DMSO (200 μl) was added to each well to dissolve the crystals and the plates were shaken for 10-20 minutes. Absorbance was measured using an ELISA plate reader at 570 nm with a reference wavelength of 650 nm. The fractional absorbance was calculated by the following formula: % Cell survival = (mean absorbance in test well)/(mean absorbance in control wells) x 100 as previously described [27].

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2.5 Cytotoxicity of curcuminoids

To determine the cytoxicity of curcuminoids, the cells (both drug sensitive and drug resistant cell lines) were plated in 96 well plates at concentration 3.0-5.0 $x10^3$ cells/well overnight. The next day, various concentrations of curcuminoids (0-100 μM) were added to each well then incubated for further 72 h at 37°C. After that, the cell viability was determined by MTT assay as described in Section 2.4. The experiment was performed in triplicate with at least three independent experiments.

2.6 Modulating effect of curcuminoids on MDR phenotype

To study whether curcuminoids were able to increase the sensitivity of anticancer drugs, the cells (3.0-5.0 $x10^3$ cells/well) were treated with various concentrations of anticancer drugs in a fixed concentration of curcuminoids or positive modulator for 72 h. The number of viable cells was determined by MTT assay (Section 2.4). The anticancer drugs and positive modulators used in this study as shown in Table 12.

Tabel 12. Anticancer drug(s) and positive modulator(s) used in this study

ABC transporter	Cell lines	Anticancer drug	Positive modulator
ABCB1 (P-gp)	KB-3-1, KB-V-1	Vinblastine Paclitaxel	Verapamil
ABCC1(MRP1)	pcDNA3.1-HEK 293 MRP1-HEK 293	Etoposide	MK571 Indomethacine Vinblastine
ABCG2 (MXR)	pcDNA3.1-HEK 293 MXR-HEK293 MCF-7 MCF-7AdrVp	Mitoxantrone Topotecan SN-38 Doxorubicin	Mitoxantrone

2.7 Cell surface expression assay: staining cell surface P-gp with human P-gp-specific monoclonal antibody, MRK-16

To detect the expression of P-gp at the cell surface, drug sensitive (KB-3-1) and drug resistant cell line (KB-V-1) were stained with a human P-gp specific monoclonal antibody, MRK-16 and analyzed by using the Fluorescence-activated Cell Sorter (FACS). The procedure was carried out following the previous described

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protocol [133] with some minor modifications. Briefly, the drug sensitive and the drug resistant cells were harvested then resuspended in IMDM supplemented with 5% FBS. The labeling was done using 5 μg of antibody per 500,000 cells at 4°C for 30 min. The cells were washed with IMDM supplemented with 5% FBS, then antimouse IgG2a FITC conjugated secondary antibody was added at 0.2 μg per 100, 000 cells (1 μg/500,000 cells, final volume 200 μl) and incubated in the dark at 4°C for 30 min. In the control experiment, the mouse IgG2a kappa was used as isotype control. After 30 min incubation, IMDM medium was added to volume ~ 3 ml then the cells were centrifuged to pellet and resuspended in 300 μl of PBS containing 0.1% BSA. Samples were analyzed immediately by using the flow cytometer.

2.8 Fluorescent drug accumulation assay by FACS (Fluorescence Activated Cell Sorter).

The fluorescence assay method was used in this study to discriminate between drug resistant and drug sensitive cell lines, as well as to determine the inhibitory effect of curcuminoids on the ABC(s) transporter mediated drug efflux function. Various types of fluorescent substrates were used as shown in Table 13.

Table13. List of fluorescent compounds and positive modulators used in this study.

ABC transporter	Fluorescent compound	Positive modulator
ABCB1 (P-gp)	Rhodamine123, calcein-AM, bodipy -FL-vinblastine	Cyclosporin A
ABCC1(MRP1)	Calcein AM, fluo-4AM	MK-571
ABCG2 (MXR)	Mitoxantrone, bodipy-FL-prazosin, rhodamine123	Fumitremorgin C (FTC)

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Procedure

A FACSort flow cytometer equipped with Cell Quest software (Becton-Dickinson, San Jose, CA) was used for FACS analysis in this study. Accumulation assay was conducted in either drug resistant or drug sensitive cell lines following protocol as previously described [65, 133, 134] with some modifications. Briefly, the drug sensitive and the drug resistant cells were harvested then re-suspended in IMDM

supplemented with 5% FBS. After that the cells were added to 4 ml of IMDM containing fluorescent substrate in the presence or absence of testing compound, then incubated in a water bath at 37°C in the dark for a specific time; rhodamine, mitoxantrone, bodipy-prazosin 45 minutes and calcein-AM, bodipy-FL-vinblastine, fluo-4-AM 10 minutes. After the incubation time, the cells were pelleted by centrifugation at 500 x g, then re-suspended in 300 µl of PBS containing 0.1% BSA and analyzed immediately by using the flow cytometer.

2.9 Plasma membrane preparation from mammalian cells

The cells maintained in T-75 flask were washed and scraped off with ice-cold PBS (w/o Ca^{2+}/Mg^{2+}) containing 1% aprotinin and 1 mM 4-(2-Aminoethyl) benzene sulphonyl fluoride (AEBSF). The lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$, 1% aprotinin and 1 mM AEBSF) was added to the cells and then quickly frozen on dry ice. After that the cells were thawed and then homogenized for 50 strokes with both pestle A and B of a hand – held homogenizer (glass). The suspension cells were diluted for 2 to 3 fold with TSNa buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 250 mM Sucrose, 1 mM AEBSF and 1% aprotinin). The unbroken cells and nuclei were removed by centrifugation at 2000 rpm (500 x g) for 10 minutes in a Sorvall A6000 rotor. The supernatant was transferred to Ti 45 rotor tubes then micrococcal nuclease (50 units/ml) and 1 mM $CaCl_2$ were added. The suspension cells were centrifuged at 120,000 x g for 1 h and then the pellet was re-suspended in 0.5-1 ml of TSNa buffer containing 10% glycerol. Finally, the membrane aliquots were stored at -70 °C until use [28, 135]. The protein content of the membrane was determined by the amido black method.

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2.10 Protein determination assay

The amido black protein assay is used to measure the protein content of samples derived from cell lines and primary cell cultures. The principle of this method is the use of trichloroacetic acid (TCA) to precipitate proteins permits removal by filtration of interfering substances in the various sample preparations, including detergents, dyes, and reducing agents. The procedure in this study is modified from Schaffner and Weissman [136]

Procedure

Along with the protein samples, protein standard (bovine serum albumin, BSA) was prepared for a standard curve by pipetting 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 25 µl of BSA, 1 mg/ml stock solution into polystyrene tubes, which were then adjusted to the total volume of 300 µl with deionized water. After that, 25 µl of 10% of SDS was added and mixed thoroughly before incubating at room temperature for 10 min. After incubation, 200 µl of 50% trichloroacetic acid (TCA) was added to precipitate the protein. The reactions were mixed thoroughly and then incubated for an additional 2 min (at least) at room temperature. The samples were spotted onto presoaked filter (s) under vacuum and rinsed with 500 µl of 6% TCA. Next, the filters were placed in Petri dish containing 0.1% napthol blue black and then washed with deionized water for one minute and destained with destaining solution (methanol/acetic acid/deionized water: 90:2:8). Finally, the filters were washed with deionized water for one minute and air- dried at room temperature. In an elution step, the stained protein on the filter was sliced (circle) carefully and transferred to the tube containing elution buffer, incubated for 20 min or longer. The protein was measured by reading the absorbance at 630 nm.

2.11. Western blot analysis and ECL detection

The procedure of Western blot analysis was carried out following the protocol as described previously [129, 137, 138] with some modifications. The membrane proteins were electrophoresed on a 7 % NuPAGE gel at 170 volts for ~ 1 h, then electroblotted onto nitrocellulose filters at 400 mA for 1 h in the transfer buffer (blotting buffer). After that the blot was incubated with 20% skim milk in PBST (0.05% PBS-tween) for 30 min, following with a primary monoclonal antibody diluted in 5% skim milk for 16-18 h. Next, the blot was washed with a washing buffer PBST for 3 times (5 min in each) and incubated with a secondary peroxidase conjugated antibody for 1 h at room temperature. Finally, the blot was detected by using the SuperSignal® protein detection kit (enhanced chemiluminescence, ECL), and then exposed an Biomax MR film.

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Enhanced chemiluminescence (ECL) system

ECL Western blotting is a light emitting nonradioactive method for detecting immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase-labeled antibodies. The principles of this method are shown in Figure 12. In the presence of hydrogen peroxide, luminol decomposes through intermediates, emitting light as it decomposes. The light produced by this enhanced chemiluminescent reaction peaks after 5-20 min and decayed slowly with a half-life of approximately 60 min. The maximum light emission at 425 nm wavelength can be detected by a short exposure to autoradiography film.

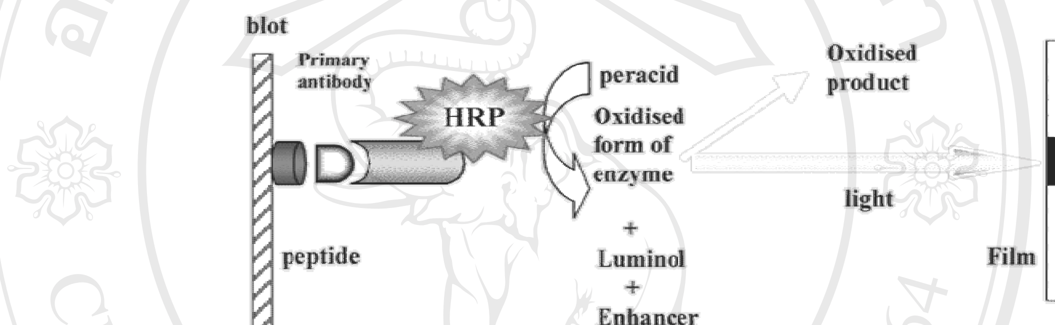

Figure 12. The principle of Enhanced chemiluminescence (ECL) system

2.12 Plasma membrane preparation from insect cells

High Five insect cells (BT1-TN-5B1-4, Invitrogen, Carlsbad, CA) derived from *Trichoplusia ni* egg cell homogenates was shown to be capable of expressing significantly higher levels of secreted recombinant proteins compared to other insect cells and other mammalian expression system. In this study, the High-Five insect cells infected with the recombinant baculovirus carrying either the human MDR1 cDNA with a six-histidine tag (BV-MDR1(H6)), or human MRP1 cDNA with ten-histidine tag (BV-MRP1(H10)) at the C-terminal end were used as a source of P-gp or MRP1 crude membrane for biochemical studies (ATPase, photoaffinity labeling and photocrosslinking assays). The crude membranes were prepared as described previously [137, 139] with some modifications. The cells were lysed and homogenized using a glass-Teflon tissue homogenizer in TMEP (50 mM Tris, pH 7.0, with HCl, containing 50 mM mannitol, 2 mM EGTA, 10 μg/ml leupeptin, 8 μg/ml aprotinin, 0.5 mM PMSF and 2 mM β-mercaptoethanol), and the disrupted

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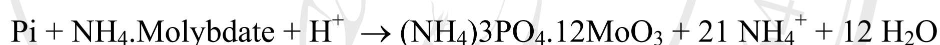
cells and nuclear debris were removed by centrifugation at 500 x g for 10 minutes. The supernatant fluid was then centrifuged for 60 minutes at 100,000 x g and the pellet containing the membrane re-suspended in TMEP. All procedures were carried out at 4°C, and the membranes were stored at -70 °C until use. The protein content of the membrane was determined by the amido black method as described in section 2.10

2.13 ATPase assay

P-gp, MRP1 and MXR pump substrates out of the cell by using energy provided by the hydrolysis of ATP, which can be described by the following reaction.

$$ATP + H_2O \rightarrow ADP + Pi + Energy$$

The ATP hydrolysis yields inorganic phosphate (Pi); thus, the amount of Pi liberated by the transporter (s) is proportional to the activity of the transporter. The Pi released can be detected by a simple colorimetric reaction. The reaction as follow:



$$Pi + NH_4.Molybdate + H^+ \rightarrow (NH_4)3PO_4.12MoO_3 + 21\ NH_4^+ + 12\ H_2O$$

The colorimetric reaction in this study based the formation of phosphomolybdate complex [140, 141]. Ammonium molybdate and antimony potassium tartrate were reacted in an acid medium with dilute solutions of phosphorus to form an antimony-phospho-molybdate complex. After that this complex is reduced to an intensely blue-colored complex by ascorbic acid, which can be measured by using UV-visible spectrophotometer.

Procedure

The crude membranes (100 µg protein/ml) prepared from MDR1 or MRP1 transfected baculovirus transfected HF cells, or MXR transfected HEK 293 cells were incubated with increasing concentrations of curcuminoids in the presence and absence of sodium orthovanadate or beryllium fluoride. The reaction was initiated by the addition of 5 mM ATP and incubated for 20 min at 37°C. SDS (2.5% final concentration) was added to terminate the reaction, and the amount of Pi released was quantified by modification of the sensitive colorimetric reaction. Briefly, the SDS-containing samples were supplemented with 0.4 ml of Pi buffer (containing 2.5 N H_2SO_4, 1% ammonium molybdic acid and 0.014% antimony potassium tartrate) and 1

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ml of distilled water. For the reduction of the complex 0.2 ml of 1%, ascorbic acid (freshly prepared) was added and the optical density was read at 880 nm. The specific activity of the transporter was recorded as the vanadate or BeF-sensitive ATPase activity.

2.14 Photoaffinity labeling of P-gp and MXR with IAAP

In this study, [^{125} I] Iodo-Aryl-Azido-Prazosin, [^{125} I] IAAP, was used to identify the binding site of curcuminoids on P-gp. It has been proposed that the putative binding sites for IAAP is at the regions from the C-terminus of TM11 to the Walker A region of the second nucleotide binding domain of P-gp [142, 143] .As shown in Figure 13, [^{125} I] IAAP is a prazosin analogue which contains a radioactive iodine [^{125}I] and a photoactive azido (-N_3) group substitution in the ring of the prazosin. The azido group is chemically inert until photoactivated by UV light. Upon photolysis it generates a highly reactive nitrene which inserts into either the peptide backbone or the amino acid side chains of the protein to which it is bound. This insertion forms a covalent linkage between the photoprobe and the protein allowing it to be permanently tagged for identification.

Procedure

The crude membranes of High-Five insect cells (50 -100 µg protein) were incubated with increasing concentrations of curcumin mixture, curcumin I, II or III at room temperature in 50 mM Tris-HCl, pH 7.5, for 3 min. IAAP (5-10 nM) was added and further incubated for additional 5 min under subdued light. The samples were then illuminated with a UV lamp (365 nm) assembly (PGC Scientifics, Gaithersburg, MD) fitted with two black light (self-filtering) UV-long wavelength – F15T8BLB tubes for 10 min at room temperature (21-23 ^{o}C). Following SDS-PAGE on an 8% Tris-glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at –70 ^{o}C for 12-24 h to obtain an autoradiogram. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and software ImageQuaNT [144, 145]. Gels were also exposed to an X-ray film (Biomax MR film) to obtain an autoradiogram.

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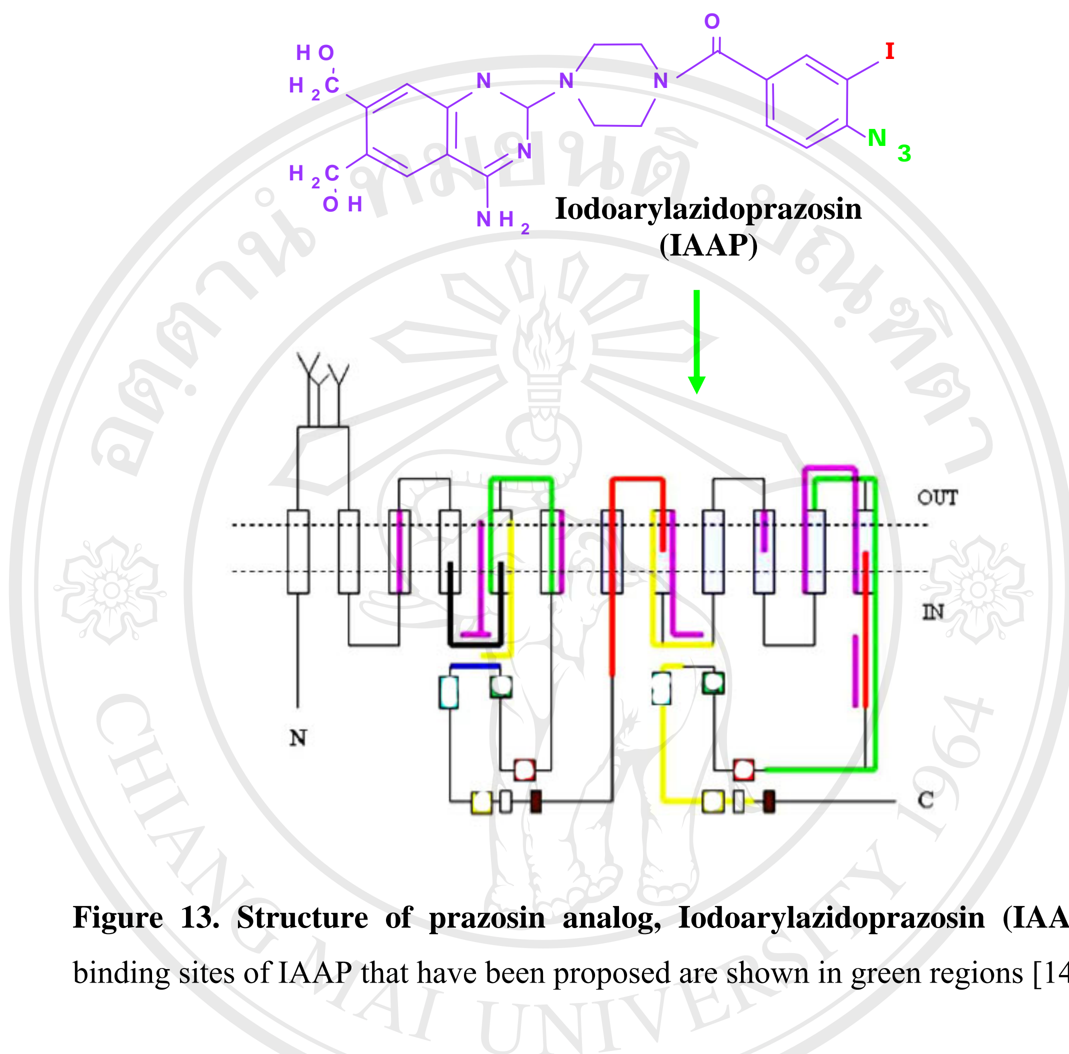

Figure 13. Structure of prazosin analog, Iodoarylazidoprazosin (IAAP). The binding sites of IAAP that have been proposed are shown in green regions [143].

2.15 Photoaffinity labeling of MRP1 with 8-azido [α-^{32}P]-ATP

P-gp, MRP1 or MXR are transporter proteins that contain nucleotide binding sites, which are putative sites for ATP binding. The binding of ATP to the nucleotide binding sites is one of a critical step for ABC transporters to exhibit their efflux function. Nucleotide photoaffinity analog, 8-azido ATP were used in this study to determine whether curcuminoids can bind to the ATP binding site of MPR1 transporter. The 8-azido ATP (8-N_3ATP) contains a photoactive azido (-N_3) group substitution in the base ring of the nucleotide (Figure 14). As described earlier in section 2.14 that once the azido group is photoactivated by UV light, it will generate reactive nitrene which will be able to form covalent bond with the peptide backbone of the protein allowing it permanently tagged for identification.

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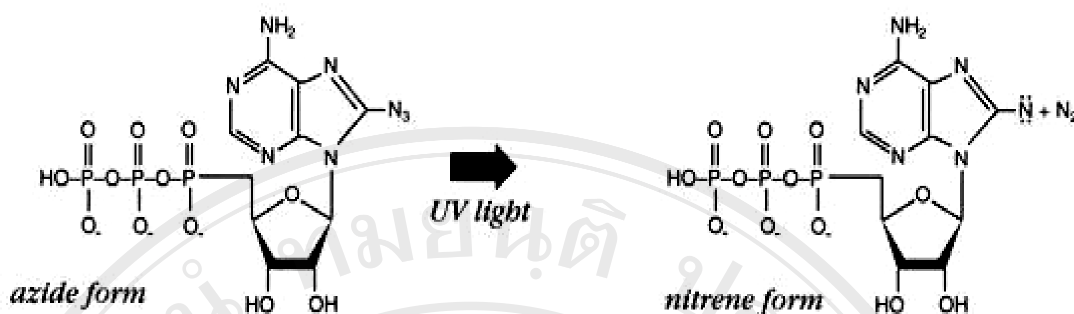

Figure 14. Structure of 8-N_3-ATP and the formation of nitrene upon proteolysis of 8-N_3-ATP (http://www.altcorp.com/AffinityLabeling/labeltheory.htm).

Procedure

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

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2.16 Statistical analysis

Data were the mean ± standard error (SE) of the mean from duplicate or triplicate samples of three independent experiments. Differences between the means were analyzed by one-way analysis of variance. Statistical significance was considered when $P < 0.05$.

2.17 Modulation of human P-gp function by curcuminoids

2.17.1. Cytotoxicity of curcuminoids in KB-3-1 and KB-V-1 cells

To determine the cytotoxicity of curcuminoids in KB-3-1, KB-V cells, the cells (3.0-5.0 x 10^3 cells) were seeded into 96 well plates and cultured overnight. Various concentrations of curcuminoids (0-100 μM) were then added and incubated for an additional 72 h in a 37 °C incubator. The number of viable cells was determined by MTT assay (Section 2.4) in triplicate, and the values represent mean ± SE from three independent experiments.

2.17.2 Effect of curcuminoids on MDR phenotype in KB-3-1 and KB-V-1 cells

To study the effect of curcuminoids on the cytotoxicity of vinblastine in KB-V-1 and KB-3-1, the cells were grown in the presence of 10 μM of curcuminoids with vinblastine (0-5 nM for KB-3-1 and 0-2.5 μM for KB-V-1) or paclitaxel (0-10 nM for KB-3-1 and 0-30 μM). Cell survival was assessed by MTT assay as described in Section 2.4

2.17.3 Effect of curcuminoids on calcein-AM, rhodamine123 and bodipy-FL-vinblastine accumulation in KB-3-1 and KBV-1 cells

Three fluorescent substrates of P-gp were used for accumulation assay in KB-3-1 and KB-V-1 cells. Briefly, the cells (5 x 10^5 cells) were harvested and re-suspended in IMDM supplemented with 5% FBS. After that rhodamine123 (0.5 μg/mL) or bodipy-FL-vinblastine (0.5 μM) or calcein-AM (0.25 μM) was added in 4 ml of IMDM in the presence or absence of reversing agent cyclosporin A (10 μM) or curcuminoid(s) (0-25 μM). The cells were incubated at 37°C in dark. After 45 min for rhodamine123 and bodipy-vinblastine or 10 min incubation time for calcein-AM, the cells were pelleted by centrifugation at 500 x g and re-suspended in 300 μl of PBS containing 0.1% BSA. Samples were analyzed immediately using the Flow cytometer (Section 2.8).

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2.17.4 Effect of curcuminoids on ATPase activity of P-gp in crude membrane of High Five insect cells expressing *MDR1*.

To determine the effect of curcuminoids on basal and verapamil-stimulation of P-gp ATPase activity, the crude membranes of High Five (HF) insect cells expressing *MDR*1 (100 μg protein/ml) were incubated with increasing concentrations of curcumin I, II, III or the curcumin mixture (0-30 μM) in the presence and absence of 5 μM verapamil. The details of this assay are described in Section 2.13. Control values show the basal activity measured in the absence of added compounds.

2.17.5 Effect of curcuminoids on photoaffinity labeling of P-gp with [^{125}I]-iodoarylazidoprazosin

To assess whether curcuminoids interact directly with the subtrate binding sites(s) of P-gp, their effect on photoaffinity labeling of P-gp by IAAP was determined. The principle as described in Section 2.13. The crude membranes of High Five insect cells (0.5 to 1 mg/ml protein) were incubated with increasing concentrations (0-25 μM) of curcumin mixture, curcumin I, II or III at room temperature in 50 mM Tris-HCl, pH 7.5, for 3 min. IAAP (5-10 nM) was added and further incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp (365 nm) for 10 min at room temperature (21-23 ^{o}C). Following SDS-PAGE on an 8% Tris-glycine gel, gels were dried and exposed to Bio-Max MR film at –70 ^{o}C for 12-24 h. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 Phosphorimager system.

2.18 Modulation of multidrug resistance related protein 1 (MRP1, ABCC1) function by curcuminoids

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2.18.1 Cytotoxicity of curcuminoids in pcDNA3.1- and MRP1-HEK 293 cells

Standard 72 h incubation MTT assay was used to investigate how curcuminoids affect the viability of the pcDNA3.1 control vector- and MRP1-transfected cells. Various concentrations of the curcuminoids (0-50 μM) were exposed to the cells for 72 h and cytotoxicity was determined by MTT assay as described in Section 2.4. The IC_{50} values were calculated from dose response curves

obtained from at least three independent experiments. The mean values from three independent experiments performed in triplicate are plotted and the error bars represent standard error (SE).

2.18.2 Effect of curcuminoids on MDR phenotype mediated by MRP1

The curcuminoids were tested for their ability to reverse the sensitivity of pcDNA 3.1 - and MRP1-HEK 293 cells to etoposide. The cells were grown in 5 μM and 10 μM of curcuminoids or 10 nM vinblastine in the presence of indicated concentration of etoposide (0-4 μM for pcDNA3.1, and 0-100 μM for MRP1). The number of viable cells after 72 h incubation was determined by MTT assay as described in Section 2.4.

2.18.3 Effect of curcuminoids on MRP1 expression in MRP1-HEK 293 cells

To investigate the effect of curcuminoids on MRP1 expression, MRP1-HEK 293 cells (3 x 10^6 cells) were treated for three days with DMSO (vehicle control), and 10 μM of the curcuminoids at 37°C then the SDS-PAGE samples were prepared as described in section WB. The samples were separated by electrophoresis on NuPAGE gel (7 % w/v) and the proteins were electroblotted on nitrocellulose membrane. Immunoblotting was performed using MRPm6 (1:4000) as first antibody and anti-mouse horseradish peroxidase (HRP) conjugate (1:10000) as the secondary antibody. HRP-dependent luminescence was developed using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Science) and detected with a Lumino Imagine Analyzer FAS-1000 (Toyobo).

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2.18.4 Effect of curcuminoids on calcein-AM and fluo-4AM accumulation in pcDNA- and MRP1-HEK 293 cells.

To study effect of the curcuminoids on MRP1 transport function, the activity of MRP1 was assessed by measuring the intracellular accumulation of two fluorescent MRP1 substrates, fluo-4AM and calcein-AM. Cells were incubated at 37°C in the dark for 45 min with 0.5 μM Fluo-4AM or 10 min with 0.5 μM calcein-AM in the presence or absence of 20 μM MK571 or various concentrations of the

curcuminoids (0-20 μM). The reaction was stopped by centrifugation and pelleted cells were re-suspended in 300 μl of ice-cold PBS containing 0.1% BSA. The accumulation of the fluorescent substrates was analyzed immediately by FACS.

2.18.5 Effect of curcuminoids on ATPase activity of MRP1 in crude membrane of High Five insect cells expressing MRP1.

To study the effect of curcuminoids on the ATP hydrolysis of MRP1, the crude membranes (100 μg protein/ml) of MRP1 baculovirus infected HF insect cells were incubated at 37°C for 5 min with curcuminoids in the presence or absence of BeFx. The reaction was initiated by the addition of 5 mM ATP and terminated with SDS (2.5% final concentration) after 20 min incubation at 37°C. The amount of P_i released was quantified using a colorimetric method as described in Section.2.13. MRP1-specific activity was recorded as the BeFx-sensitive ATPase activity. Values represent mean $\pm$ SE from at least three independent experiments. Control values show the basal activity measured in the absence of added compounds.

2.18.6 Effect of curcuminoids on quercetin stimulated ATPase activity of MRP1

To evaluate whether curcuminoids interact with MRP1 at the substrate binding sites maybe at the same binding sites with flavonoid quercetin. The crude membranes (100 μg protein/ml) of HF insect cells expressing MRP1 were incubated at 37°C for 5 min with various concentrations of curcuminoids (0-50 μM) in the presence of 10 μM flavonoid quercetin, which is known to produce 2-fold increase in MRP1-mediated ATP hydrolysis at 10 μM. The mean values from three independent experiments are given and the error bars represent standard error (SE).

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2.18.7. Effect of curcuminoids on photoaffinity labeling of MRP1 with 8-azido [^{32}P] ATP

To study whether curcuminoids bind to ATP binding sites of MRP1, the crude membranes (50-75 μg protein) of MRP1 baculovirus infected HF insect cells were incubated at 4°C for 5 min with 10 μM [α-^{32}P]-8-azidoATP (10 μCi/nano mole) in the presence and absence of 20 μM curcuminoids. The photocross linking with 366 nm UV light was carried out on ice for 10 min as described in Section 2.15. The

incorporation of 8-azido [α-^{32}P]-ATP was detected by phosphorimaging after gel electrophoresis.

2.19 Modulation of ABCG2 (MXR) function by curcuminoids

2.19.1 Cytotoxicity of curcuminoids in pcDNA3.1-HEK 293, MXR-HEK 293, MCF-7 and MCF-7AdrVp

Cytotoxicity assays of curcuminoids were conducted in pcDNA (drug sensitive) -and MXR-HEK 293 cells (drug resistance).Various concentrations of the curcuminoids (0-50 μM) were exposed to the cells for 72 h and cytotoxicity was determined by MTT assay (Section 2.4).

2.19.2 Effect of curcuminoids on MDR phenotype mediated by MXR

Cytotoxicity assays of mitoxantrone, topotecan and SN-38 were conducted in the presence and absence of curcuminoids. The cells were incubated for three days with various concentrations of drug in the presence of curcuminoids at concentrations 3, 5 and 10 μM, then the cell survival was determined by the MTT assay as described in Section 2.4

2.19.3 Effect of curcuminoids on the accumulation of bodipy prazosin, mitoxantrone and rhodamine in MXR-overexpressing cells.

To determine the effect of curcuminoids on bodipy-prazosin and rhodamine accumulation in MXR expressing cells, the cells were incubated with either 250 nM bodipy-prazosin or 20 μM mitoxantrone or 0.5 μg/ml rhodamine123 in the presence of various concentrations of curcuminoids 0-20 μM) or 10 μM FTC. After 45 min incubation in dark, the cells were washed and analyzed by using flow cytometer as described in Section 2.8.

2.19.4 Effect of curcuminoids on ATPase activity of MXR

The effect of curcuminoids on ATPase activity of isolated membrane from MXR-HEK 293 cells wild type (482R) and mutant (R482T) was determined by ATPase assay as described in Section 2.13

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2.19.5 Effect of curcuminoids on MXR expression

To investigate the effect of curcuminoids on MXR expression, MCF-7AdrVp cells were treated with 3 and 10 μM curcuminoids for three days. Membrane fraction was prepared and 15 μg of membrane protein was separated by SDS-PAGE, and analyzed by Western blot. Immunoblotting was performed using BXP-21 (1:500) as first antibody and anti-mouse horseradish peroxidase (HRP) conjugate (1:10000) as the secondary antibody (see details of Western blot analysis in Section 2.9).

2.20 Modulation of P-gp, MRP1 and MXR by tetrahydrocurcumin

2.20.1 Cytotoxicity of tetrahydrocurcumin

The cells were exposed to various concentrations of tetrahydrocurcumin for 72 h and cytotoxicty was determined by MTT assay (as described in Section 2.4)

2.20.2 Effect of tetrahydrocurcumin on calcein-AM accumulation in KB-3-1 and KBV-1 cells.

The cells were re-suspended in IMDM supplemented with 5% FBS then incubated with 0.5 μM of calcein AM in the presence of tetrahydrocurcumin (0-100 μM), and then the calcein accumulation was analyzed by using flow cytometer as described in Section 2.8

2.20.3. Effect of tetrahydrocurcumin on the accumulation and efflux of ^{3}H-vinblastine

The effect of tetrahydrocurcumin on P-gp mediated drug transport was confirmed by monitoring the intracellular radiolabeled drug accumulation. The method was modified from Plouzek et al [146]. MCF-7MDR 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: 8.10 mCi/mmol) in the presence of tetrahydrocurcumin (12.5, 25, 50, 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 40 ^{o}C for 2 min. Cells were dissolved with 200 μl of 3 N NaOH, then neutralized with 100 μl of

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6 N HCl. Cell lysate, 250 µl was pipetted into the scintillation via and 3 ml of scintillation fluid was added to each via. 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 [147], 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 (12.5, 25, 50, 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.

2.20.4 Effect of tetrahydrocurcumin on photoaffinity labeling of P-gp with IAAP.

The crude membranes of HF insect cells expressing MDR1 (0.5 to 1 mg/ml protein) were incubated with increasing concentrations of tetrahydrocurcumin (0-100 µM) in the presence of IAAP (5-10 nM), then the IAAP incorporation was determined as described in Section 2.14

2.20.5 Effect of tetrahydrocurcumin on MDR mediated phenotype by MRP1 and MXR.

Cytotoxicity assays of etoposide in pcDNA3.1-and MRP1-HEK 293 cells for MRP1 study or mitoxantrone in MCF-7 and MCF-7AdrVp for MXR study were conducted in the presence of DMSO or tetrahydrocurcumin. The cells were incubated for 72 h then the cell viability was determined by MTT assay as described in Section 2.4

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