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Research Article

Interaction of Oxazaphosphorines with Multidrug Resistance-Associated Protein 4 (MRP4)

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Abstract. Multidrug resistance-associated protein 4 (MRP4) is an organic anion efflux pump capable of transporting nucleoside, nucleotide analogs, and cyclic nucleotide. MRP4 could have an influence on the resistance and transport of the two oxazaphosphorines, cyclophosphamide (CP) and ifosfamide (IF). V/HepG2 (HepG2, hepatoma cells stably transfected with an empty vehicle plasmid) and MRP4/HepG2 (HepG2 cells stably expressing MRP4) were exposed to CP and IF in the absence or presence of various MRP4 inhibitors. HepG2 and HEK293 human kidney cells were also used to investigate the inducing potency of oxazaphosphorines on the MRP4 expression. In this study, insertion of MRP4 gene in HepG2 cells was found to confer significant resistance to CP and IF in the 48-h drug-exposure assays. In the presence of various MRP4 inhibitors, the resistance to CP and IF was then partially reversed. These indicate that CP and IF are highly possible substrates of MRP4. In addition, CP and clofibrate (CFB), a reported MRP4 inducer, *in vivo* significantly increased the MRP4 expression at both protein level and mRNA level in HEK293 cells at higher concentrations, while IF significantly decreased the MRP4 expression at mRNA level at lower concentration and had no effect at higher concentrations. However, all tested compounds (CP, IF, and CFB) did not change the MRP4 protein expression in HepG2 cells. CP and CFB are cell-specific and concentration-dependent MRP4 inducers. The finding may have implications in the CP- or IF-based chemotherapy.

KEY WORDS: cyclophosphamide; cytotoxicity; drug transporter; ifosfamide; multidrug resistance-associated protein 4.

INTRODUCTION

The major problem in antitumor chemotherapy is the development of multidrug resistance. Multidrug resistance is the phenomenon in which cells show simultaneous resistance to several structurally and functionally unrelated drugs that do not have the same mechanism of actions. Tumor cells may have intrinsic resistance to current chemotherapeutic agents or develop resistance after exposure. Cellular mechanisms of multidrug resistance include defective drug transport (reduced drug uptake or increased drug efflux), altered drug activation or inactivation, and/or enhanced repair or tolerance to DNA damage. P-glycoprotein (PgP) is the first drug efflux pump identified as the contributor of multidrug resistance (1). In addition to PgP, many other ATP-binding cassette transporters including breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs) confer the resistance to clinically important chemotherapeutic agents. MRP4, as a member of MRP family, was demonstrated to confer resistance to multiple chemotherapeutic agents such as methotrexate (MTX) (2), topotecan (3), and the active metabolites of CPT-11, SN-38 (4).

The oxazaphosphorines including cyclophosphamide (CP) and ifosfamide (IF) represent an important group of therapeutic agents due to their substantial antitumor and immunomodulating activity. The anticancer effect of oxazaphosphorines is generally considered to result from DNA crosslink formation through covalent bonding of highly reactive alkyl groups of the alkylating nitrogen mustards of oxazaphosphorines with specific nucleophilic groups of DNA molecules. Nooter *et al.* reported that MRP expression was associated with increased risk for failure in breast cancer patients who received adjuvant systemic chemotherapy with CMF (CP, methotrexate, and 5-fluorouracil) (5). In addition, several studies suggested that patients who were treated with CMF chemotherapy and whose tumors were MRP1 negative experienced a significant reduction in relapse rate and a decrease in mortality compared to patients with tumors of high MRP1 expression (6,7). Furthermore, Faneyte *et al.* reported no evidence linking MRP1–3 mRNA expression to response to FEC (5-fluorouracil, epirubicin, and CP) chemotherapy in breast cancer samples (8). It seems that the association of MRP expression to the efficiency of CP-based chemotherapy remains uncertain. Further studies on this association are needed.

Resistance to oxazaphosphorines, *e.g.*, CP and IF, has been demonstrated in *in vitro* studies (9) and murine models (10). Based on a number of *in vitro* and *in vivo* studies, a variety of factors has been implicated in causing resistance to oxazaphosphorines. High intracellular glutathione (GSH) diminishes the antitumor activity of 4-hydroxy-CP by limiting the conversion of 4-hydroxy-CP to the ultimate toxic metab-

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olite, phosphoramide mustard (11). In addition, overexpression of glutathione S-transferase (GST) can contribute to the acquired resistance of tumor cells to 4-hydroxy-CP, suggesting that modulators that target the GSH/GST system could be useful in overcoming CP resistance in clinics (12). Therefore, drug resistance to oxazaphosphorines has been associated to the higher intracellular concentrations of GSH and increased expression of GST enzymes. GSH has also been found to play an important role in the transport function of MRP4. MRP4 can mediate the export of GSH. Depletion of intracellular GSH adversely affects the export of cAMP by MRP4. MRP4 has been found to mediate resistance to purine analogs (13). MRP4 also mediates the cotransport of bile acids and GSH from hepatocytes into blood (14). Because of the observed effects of MRP4 on intracellular GSH and the conferred resistance to purine analogs, MRP4 could also be involved in modulating the resistance to oxazaphosphorines.

As the toxicities and resistance to oxazaphosphorines limit their clinical application, we aimed to evaluate whether MRP4 has a role in the regulation of the toxicities and resistance to oxazaphosphorines. Hence, the effect of increased MRP4 expression on the cytotoxicity of CP and IF was examined in a validated cell model. In addition, the effect of CP and IF on the expression of MRP4 was investigated *in vitro*.

MATERIALS AND METHODS

Chemicals and Reagents

Blasticidin S hydrochloride, DL-buthionine-(S,R)-sulfoximine (BSO), CP, clofibrate (CFB), Bradford reagent, 2-mercaptoethanol, albumin from bovine serum (BSA), ammonium persulfate, sodium azide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazonium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), and penicillin/streptomycin were all obtained from Sigma Chemical Co (St. Louis, MO, USA). Bis (pivaloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine (bis-POM-PMEA, also known as adefovir dipivoxil), MTX, celecoxib, diclofenac, and IF (all compounds with a purity >99%) were from SinoChem Ningbo Co. (Ningbo, China). The leukotriene antagonist, 3-([(3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl)-((3-dimethylamino-3-oxopropyl)-thio)-methyl]thio)propanoic acid (MK571) was a gift from Dr. Ford-Hutchinson (Merck Frosst Canada, Inc. Kirkland, QC, Canada). Complete Mini tablets (protease inhibitor cocktail tablets) were purchased from Roche Diagnostics (Mannheim, Germany). Triton X-100, glycine, bromophenol blue, Tween 20, acrylamide/bis 37.5:1 30% solution and protein standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Fetal bovine serum was from Life Technologies (Carlsbad, CA, USA). The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade or high-performance liquid chromatography grade obtained from commercial sources.

Cell Culture

HepG2 cells with insertion of vector or *MRP4* (V/HepG2 or MRP4/HepG2) obtained from Dr. Theresa Tan (Department of Biochemistry, National University of Singapore) were maintained in DMEM medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum with the presence of 0.25 μg/ml blasticidin S hydrochloride. HEK293 cell and HepG2 cell obtained from the American Type Culture Collection (Rockville, MD, USA) were maintained in DMEM medium containing 100 units/ml penicillin G, 100 μg/ml streptomycin, and 10% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Experiments were performed on cells within ten passages. Viable cells were counted using the trypan blue exclusion method.

Cytotoxicity Assay

Drug effects on exponentially growing tumor cells were determined using the MTT assay as described previously (15). Cells were seeded at a density of 5,000 cells per well in 96-well plates and allowed to attach for 24 h at 37°C under 5% CO_2. After the attachment period, tumor cells were exposed to drugs at different concentrations in culture medium for 4 or 48 h. CP was freshly prepared by dissolving in H_2O and diluted in culture medium. IF, MTX, and bis-POM-PMEA were freshly prepared by dissolving in DMSO before being diluted. The final concentration of DMSO was 1% (*v*/*v*), and such concentration showed little cytotoxicity to both strains of cells (<7%) when incubated for 4 or 48 h. For short drug exposure (*e.g.*, MTX exposure), the medium with drug at different concentrations was removed by aspiration 4 h after drug addition. The cells were washed two times with phosphate buffered saline (PBS), and fresh drug-free medium was added, and the cells were incubated for a further 44 h. At 48 h for both long and short drug exposures, 100-μl MTT reagents (0.5 mg/ml) were added to each well after removal of medium, and cells were incubated for a further 4 h at 37°C. Thereafter, the MTT reagent was discarded, and the purple precipitates were dissolved in 100 μl of DMSO. The absorbance of formazan, a metabolite of MTT, in the resulting solution was photometrically measured at a wavelength of 595 nm using a microplate reader (Tecan Instruments Inc., Research Triangle Park, NC, USA). The experiment was performed in eight replicate wells for each drug concentration and carried out independently at least three times. The cytotoxicity was evaluated with reference to the IC_{50} value, which was defined as the concentration needed for a 50% reduction of survival based on the survival curves. IC_{50} values were calculated from dose–response curves (*i.e.*, cell survival *vs*. drug concentration) obtained in multi-replicated experiments.

In vitro Cytotoxicity Inhibition Assay

BSO, MK571, celecoxib, and diclofenac are all known inhibitors for MRP4 (13,16,17). To check for the effects of BSO, celecoxib, diclofenac, and MK571 on drug resistance, cells were preincubated with or without BSO (200 μM) for 24 h, celecoxib (50 μM), diclofenac (200 μM), or MK571 (100 μM) for 2 h. All inhibitors were prepared by dissolving in DMSO and diluted by PBS. The final concentration of DMSO was 1% (*v*/*v*). All inhibitors at the concentrations used did not show any significant cytotoxicity (<10%) when

incubated with BSO for 24 h or with other inhibitors for 2 h. Before the cells were exposed to the oxazaphosphorines, the medium with these inhibitors was removed, and the cells were washed twice with PBS. Then oxazaphosphorines were added and MTT assay performed as herein.

Western Blot Analysis

HepG2 and HEK293 cells were cultured in the absence or presence of drug (CP, IF, or CFB in different concentration) for 6 days. Media changes were performed daily. The medium with drug at different concentrations was removed by aspiration 6 days after drug addition. The cells were washed twice with PBS and scraped off into 1 ml PBS. After centrifugation, the cell pellets were suspended in 100 μl lysis buffer (85 μl 1% triton X100/PBS and 15 μl protease inhibitor cocktail solutions) and incubated on ice for 60 min. Thereafter, the cell suspension was centrifuged at 20,000*g* for 30 min at 4°C. The resulting supernatant was retained, and the protein content of the samples used was determined by the Bradford assay. Then, 20-μg aliquots of supernatant protein were fractionated on 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were soaked in blocking buffer which contains TBST (50 mM Tris–HCl, 150 mM NaCl, and 0.1% (*v*/*v*) Tween 20, pH 7.5) containing 5% (*w*/*v*) skim milk powder at room temperature with agitation overnight. After washed with TBST, membranes were subsequently incubated with monoclonal anti-MRP4 antibody (M4I-10, Alexis Biochemicals, Lausen, Switzerland, 1:2,000 dilution in TBST containing 1% (*w*/*v*) skim milk powder) (4) and with monoclonal anti-actin antibody (Sigma Chemical Co., St. Louis, Mo, USA, dilution 1:1,000 in TBST containing 1% (*w*/*v*) skim milk powder) for 2 h at room temperature with agitation. Thereafter, membranes were incubated with the secondary antibody for 1 h at room temperature with agitation after being washed with TBST. The secondary antibody for MRP4 is goat anti-rat IgG horseradish peroxidase-conjugated antibody (Sigma Chemical Co., St. Louis, MO, USA), which was used at a dilution of 1:20,000 in TBST containing 1% (*w*/*v*) skim milk powder, whereas the secondary antibody for actin is goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Pierce, Rockford, IL, USA), which was used at a dilution of 1:1,000 in TBST containing 1% (*w*/*v*) skim milk powder. Blots were developed using the Pierce SuperSignal® West Femto Maximum Sensitivity Substrate Kit (Pierce, Rockford, IL, USA).

Quantitative Real-time Polymerase Chain Reaction (PCR)

HEK293 cells were cultured in the absence or presence of drug (CP, IF, or CFB in different concentration) for 6 days. Media changes were performed daily. The medium with drug at different concentrations was removed by aspiration 6 days after drug addition. The cells were washed twice with PBS and scraped off into 1-ml PBS. After centrifugation, the cell pellets were used for RNA extraction. Total RNA was extracted from cells using the RNeasy mini kit (QIAGEN GmbH, Germany) following the manufacturer's protocol. Pelleted cells were resuspended and counted using a hemocytometer before RNA extraction to prevent overloading of the RNeasy columns. Cells were homogenized using a QIAshredder (QIAGEN GmbH, Germany). RNA concentration of each sample was measured using the Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and equal amounts of RNA were used for reverse transcription. Total RNA was reverse-transcribed by TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) and thereafter subjected to real-time polymerase chain reaction (PCR) assays using the fluorescent dye SYBR Green methodology and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed with the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). For MRP4, the forward primer is 5′-GCTCAGGTTGCCTATGTGCT-3′, and the reverse primer is 5′-CGGTTACATTTCCTCCTCCA-3′. GAPDH was used as the housekeeping gene. The forward primer is 5′-GACCACAGTCCATGCCATCAC-3′, and the reverse primer is 5′-CATACCAGGAAATGAGCTTGA-3′. Amplification and detection of samples were performed in an ABI 7500 sequence detection system. Conditions were optimized to certify similar amplification efficiencies for all products. Melting curves for each primer pair showed one specific signal. All experiments were repeated at least three times. Relative quantification was obtained by the comparative threshold cycle (ΔΔCT) method (18) (RQ software version 1.2.3, Applied Biosystems). Expression level of MRP4 (Ct) was normalized against the level of mRNA of a housekeeping gene (GAPDH) determined in each sample ($\Delta Ct = Ct_{MRP4} - Ct_{GAPDH}$). Normalization using GAPDH was calculated as ΔCt with MRP4 levels at 0 μM tested drug (CP, IF, or CFB) serving as a calibrator, which resulted in the relative expression levels being described by $2^{-\Delta\Delta Ct}$ presuming 100% efficiency for each PCR cycle.

Statistical Analysis

Data are presented as mean ± standard deviation or mean ± standard error of the mean (for PCR results only). Statistical analysis was performed using the GraphPad Prism® program Version 3.0 (GraphPad Software, San Diego, CA, USA). Student's unpaired *t* test was conducted for comparisons between two groups. The statistical analysis to evaluate the differences of continuous variables among the different groups was performed by one-way analysis of variance followed with a post hoc test (Dunnett's multiple comparison test). $p < 0.05$ was regarded as significant.

RESULTS

Validation of Cell Models

Both MTX and bis-POM-PMEA are reported as substrates of MRP4 (2,19). To validate the MRP4 functionality in V/HepG2 and MRP4/HepG2 cells used in the present study, the cytotoxicities of both bis-POM-PMEA and MTX were assessed in these cells. As shown in Table I, overexpression of MRP4 conferred 5.15-fold resistance to bis-POM-PMEA when the cells were exposed to the test drug for 48 h. In addition, MRP4-transfected HepG2 cells exposed to MTX for

Table I. Sensitivity of $HepG_2$ Cells Expressing MRP4 or Blank Vector to Two MRP4 Substrates

Treatment	Drug exposure time (h)	IC_{50} (μM)		Fold resistance
		V/HepG2	MRP4/HepG2	
MTX	4	3.44±0.26	20.8±0.47*	6.06
Bis-POM-PMEA	48	17.3±1.30	89.3±1.53*	5.15

Data are the means ± standard deviation. Fold resistance is calculated as IC_{50} in MRP4/HepG2 cells over that in V/HepG2 cells. Number of independent experiments=3. Each experiment was performed in eight replicate wells for each drug concentration.
*$p<0.05$ by Student's *t* test, MRP4/HepG2 *vs* V/HepG2

4 h were 6.06-fold more resistant compared with V/HepG2 cells. These data are consistent with the fold resistance reported in the previous studies (2,19) and suggest that the established HepG2 cells with overexpression of MRP4 conferred resistance to both bis-POM-PMEA and MTX.

Western blot analysis on the MRP4 protein expression in V/HepG2 and MRP4/HepG2 cells with the monoclonal antibody M4I-10 revealed that MRP4/HepG2 contained a substantial amount of MRP4 (see in Fig. 1). In addition, *MRP4* gene expression in V/HepG2 cells and MRP4/HepG2 cells at mRNA level was examined by quantitative real-time PCR. MRP4 mRNA was quantified using real-time PCR analysis (SYBR Green) standardizing against the endogenous control GAPDH. Quantitative real-time PCR analysis showed that MRP4/HepG2 cells contained a substantial quantity of MRP4 mRNA compared with V/HepG2. There is a significant difference ($p<0.01$ by Student's *t* test, MRP4/HepG2 *vs* V/HepG2) on the MRP4 expression between V/HepG2 cells and MRP4/HepG2 cells. MRP4 mRNA expression in MRP4/HepG2 cells is around 623 K-fold to that in V/HepG2 cells. These data confirmed the overexpression of MRP4 in the established HepG2 cells.

Human MRP4 Conferred Resistance to Cyclophosphamide and Ifosfamide

The cytotoxicities of oxazaphosphorines including CP and IF in MRP4-transfected HepG2 cells were examined. Table II showed the results on the sensitivities of V/HepG2 and MRP4/HepG2 cells to CP and IF. Notably, MRP4/HepG2 cells showed increased IC_{50} values by 3.06- and 6.03-folds in the 48-h exposure assay for CP and IF, respectively, compared with V/HepG2 cells. These data indicate that cells overexpressing MRP4 are substantially more resistant to the cytotoxic effects of CP and IF.

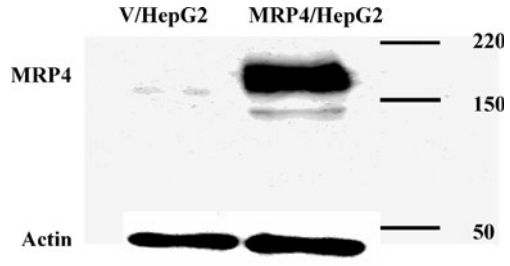

Fig. 1. Western blot analysis of MRP4 expression in V/HepG2 and MRP4/HepG2 cells. The molecular mass (kilodalton) is indicated

Inhibition of MRP4-Mediated Resistance to Cyclophosphamide and Ifosfamide by Various Inhibitors

The effects of the GSH synthesis inhibitor BSO on the cytotoxicity of CP and IF in V/HepG2 and MRP4/HepG2 cells were investigated (shown in Table II). The addition of BSO significantly reduced the IC_{50} values of CP by 61.2% and IF by 28.0% in 48-h assay in HepG2 cell overexpressing MRP4. In addition, the effects of some known MRP inhibitors such as diclofenac, celecoxib, and MK571 on the cytotoxicity of CP and IF in V/HepG2 and MRP4/HepG2 cells were investigated (shown in Table II). Preincubation with diclofenac, celecoxib, or MK571 for 2 h significantly decreased the IC_{50} values of CP and IF in MRP4/HepG2 cells. Addition of diclofenac significantly decreased IC_{50} of CP by 48.0% and of IF by 44.8% for 48-h exposure assays, respectively, in MRP4/HepG2 cells. Similarly, addition of celecoxib also decreased the IC_{50} of CP by 30.9% and the IC_{50} of IF by 45.9% for 48-h exposure assays, respectively, in MRP4/HepG2 cells. Furthermore, MK-571 reduced the IC_{50} of CP by 41.2% and of IF by 38.0% for 48-h exposure assays, respectively, in MRP4/HepG2 cells. In addition, the MRP2 and BCRP expression were checked by M2III6 antibody and BXP-21 antibody through western blot. There were no obvious MPR2 expression and BCRP expression in neither V/HepG2 nor MRP4/HepG2 cells (data not shown). Altogether, these findings indicated that MK571, celecoxib, and diclofenac sensitized MRP4/HepG2 cells to CP and IF cytotoxicity and partially reversed the MRP4-mediated resistance to CP and IF. In contrast, all inhibitors used had little effect on the cytotoxicity of CP and IF in V/HepG2 cells. It appears that the GSH synthesis inhibitor BSO and MRP4 inhibitors such as diclofenac, celecoxib, and MK571 can partially reverse the resistance to CP and IF mediated by MRP4. These findings indicate that the overexpression of MRP4 contributes to resistance to CP and IF and that CP and IF are highly likely the substrates for MRP4.

The Effect of Cyclophosphamide and Ifosfamide on the MRP4 Expression at Protein Level and mRNA Level

M4I-10, an MRP4 monoclonal antibody, has been previously used for detecting MRP4 expression in wild-type mice (3) and human HEK293 cells (4). Therefore, M4I-10 was chosen to detecting MRP4 protein expression in our test cell lines in this study. The peroxisome proliferator-activated receptor α (PPARα) agonist CFB was chosen as the positive control because it upregulated mRNA levels and enhanced protein expression of Mrp 3 and Mrp 4 in mice (20). As

Table II. Drug Sensitivity of HepG2 Cells Expressing MRP4 or Vector Only to Oxazaphosphorines Without or With the Presence of Some Known MRP4 Inhibitors

Treatment	Drug exposure time (h)	IC_{50} (μM)		Fold resistance	*N*
		V/HepG2	MRP4/HepG2		
CP	48	21.1±1.08	64.5±1.94*	3.06	7
+BSO	48	20.5±0.79	25.1±0.73***	1.25	7
+Diclofenac	48	24.4±1.26	33.5±2.68***	1.37	5
+Celecoxib	48	24.5±2.53	45.1±1.85***	1.84	5
+MK571	48	22.0±0.95	37.9±1.51***	1.73	4
IF	48	25.6±0.68	154.5±1.90*	6.03	7
+BSO	48	25.4±1.01	111.3±0.42***	4.38	7
+Diclofenac	48	25.0±0.06	85.2±4.47***	3.40	5
+Celecoxib	48	24.5±0.90	83.6±2.67***	3.42	5
+MK571	48	25.8±1.89	95.9±2.05***	3.71	5

Data are the means ± standard deviation. Fold resistance is calculated as IC_{50} in MRP4/HepG2 cells over that in V/HepG2 cells. *N*=number of independent experiments. Each experiment was performed in eight replicate wells for each drug concentration and carried out independently four to seven times

*$p<0.05$ by Student's *t* test, MRP4/HepG2 *vs* V/HepG2

**$p<0.05$, test drug *vs* test drug + inhibitor by one-way analysis of variance followed with Dunnett's multiple comparison test

shown in Fig. 2, western blot analysis using HEK293 cells revealed a significant increase in MRP4 protein expression 6 days after treatment with CP or CFB. However, IF-treated HEK293 cells showed similar MRP4 protein expression compared with the control. Interestingly, the MRP4 protein expression was evaluated in a concentration-dependent manner after 6 days after treatment with CP or CFB. Low concentration of CP (100 μM) did not change the MRP4 protein expression in HEK293 cells, but higher concentration of CP significantly increased the cellular MRP4 protein expression in HEK293 cells, especially with 1,000 μM CP (about 1.41-fold). Similarly, higher concentration of CFB (400 μM) significantly increased the cellular MRP4 protein expression about 2.28-fold. The induction factors (see the bar graph in Fig. 2) were calculated by densitometric analysis using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). However, all tested compounds (CP, IF, and CFB) did not change MRP4 protein expression in HepG2 cells (shown in Fig. 3).

The Effect of Cyclophosphamide and Ifosfamide on the MRP4 Expression at mRNA Level

In addition, the inducibility of the human *MRP4* gene expression at mRNA level was quantitatively investigated by real-time PCR using HEK293 cells. MRP4 mRNA was quantified using quantitative real-time PCR (SYBR Green method) standardizing against the endogenous control GAPDH. Data were normalized to GAPDH and expressed as percentage ratio to the controls. As shown in Fig. 4, the

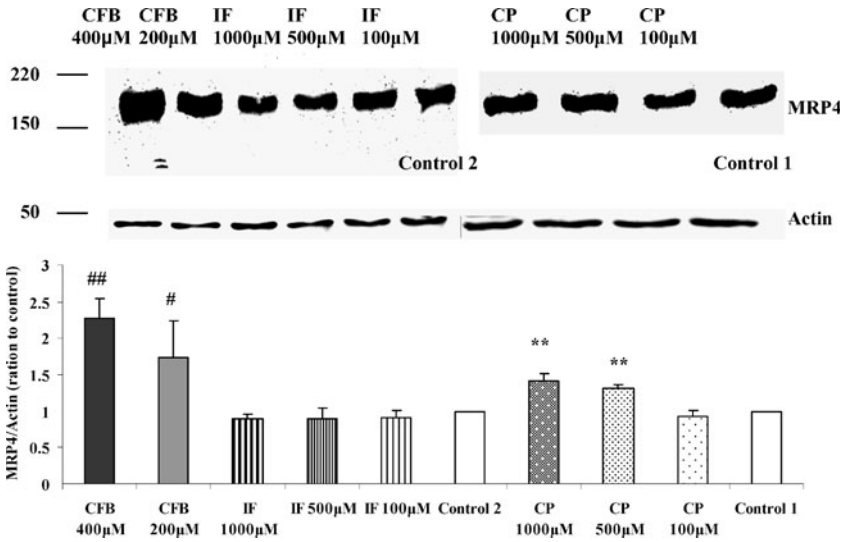

Fig. 2. MRP4 protein expression by western blot in HEK293 cells exposed to different concentration of cyclophosphamide (CP), ifosfamide (IF), or clofibrate (CFB) for 6 days. The *bar graph* shows the quantification of the band intensity. Control 1 and control 2 are the control for CP and IF or CFB, respectively. *Asterisk*, $p<0.01$ when CP-treated cells are compared with control 1 group. Significant difference: *single number sign*, $p<0.05$, *double number sign*, $p<0.01$ between CFB-treated cells and control 2 group

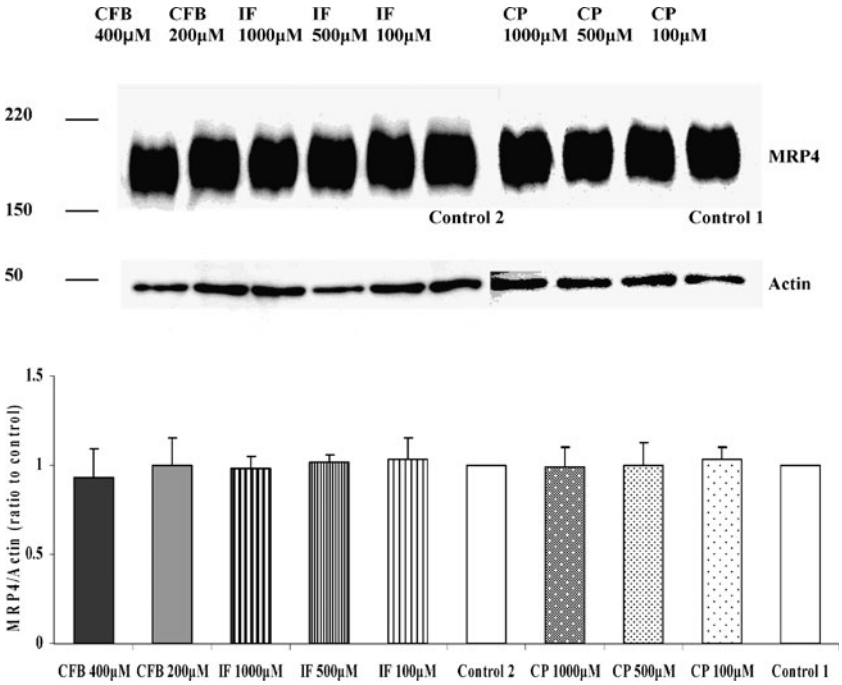

Fig. 3. MRP4 protein expression by western blot in HepG2 cells exposed to different concentration of cyclophosphamide (CP), ifosfamide (IF), or clofibrate (CFB) for 6 days. The *bar graph* shows the quantification of the band intensity. Control 1 and control 2 are the control for CP and IF or CFB, respectively

real-time PCR analysis suggested that CP and CFB also upregulated the *MRP4* expression at mRNA level in the HEK293 cells. While the induction of the gene product was again influenced by CP and CFB concentration, there were subtle differences when compared to the induction of the MRP4 protein. Significant induction of the *MRP4* expression in the HEK293 cells was seen only at higher concentrations (500 and 1,000 μM) of CP, causing the *MRP4* expression to increase to about 194% or 145% of the control. Similarly, significant induction of the MRP4 protein as evidenced by western blot analysis was seen only at higher concentrations of CP (500 and 1,000 μM), especially at 1,000 μM (1.41-fold). Additionally, similar to the results from western blot, significant induction of *MRP4* expression was seen at higher concentration (400 μM) of CFB. However, real-time PCR showed inhibition of *MRP4* expression at lower concentration of CFB (200 μM) though it was not significantly different from the control. Furthermore, real-time PCR also showed a significant inhibition of *MRP4* expression after co-incubation with lower concentration (100 μM) of IF (35.0% to the control), but no significant inhibition at higher concentration of the respective 500 and 1,000 μM.

DISCUSSION

The MRP4 protein and gene expression and the function of the cell model used in this study were examined. A substantial amount of MRP4 was found in MRP4 over-

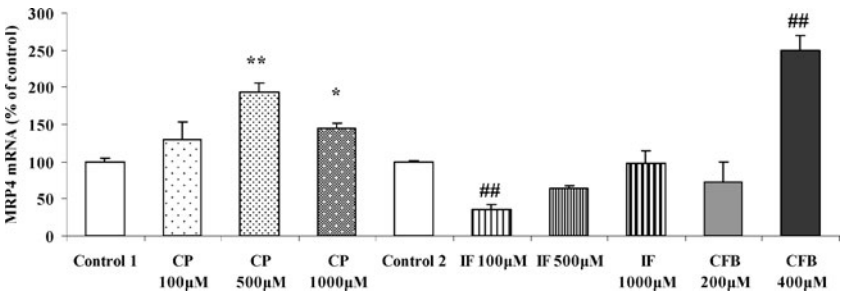

Fig. 4. Quantitative real-time polymerase chain reaction analysis of *MRP4* mRNA in HEK293 cells exposed to different concentration of cyclophosphamide (CP), ifosfamide (IF), or clofibrate (CFB) for 6 days. *MRP4* mRNA was quantified using quantitative real-time polymerase chain reaction (SYBR Green method) standardizing against the endogenous control GAPDH. Data were normalized to GAPDH and expressed as percentage ratio of control levels ($n=3$). Control 1 and control 2 are the control for CP and IF or CFB, respectively. Significant difference: *single asterisk*, $p<0.05$, *double asterisk*, $p<0.01$ between CP-treated cells and control 1 group. *Number sign*, $p<0.01$ when IF- or CFB-treated cells are compared with control 2 group

expressing HepG2 cells at protein levels and mRNA levels compared with V/HepG2 cells using western blot analysis and quantitative real-time PCR (Fig. 1). In addition, MRP4 overexpressing HepG2 cells conferred significant resistance to bis-POM-PMEA or MTX at the drug exposure time of 48 and 4 h, respectively (Table I). This was consistent with the reported results for these two compounds obtained from other cells with overexpression of MRP4 (2,19). These results indicated the validity of V/HepG2 and MRP4/HepG2 cells as a set of cell model in the study of potential role of MRP4 to oxazaphosphorines.

The oxazaphosphorines including CP and IF represent an important group of therapeutic agents because of their substantial antitumor and immunomodulating activity. Valuable insight into their mechanism of action, pharmacokinetics, toxicities, and tumor resistance was yielded by many studies. The major clinical problem is resistance to oxazaphosphorines which often results in therapeutic failure. Detailed investigations aimed at identification of resistant proteins and circumventing approaches of intrinsic drug resistance are thus needed. The present study provided solid evidence that MRP4 conferred significant resistance to CP and IF using the validated HepG2 cell model with stable overexpression of MRP4. Increased resistance to the cytotoxicity of CP and IF was observed in MRP4/HepG2 cells compared with V/HepG2 cells (Table II). The addition of BSO, a GSH synthesis inhibitor, significantly reversed MRP4-mediated resistance to CP and IF. This indicated that GSH played an important role in MRP4-mediated efflux of CP and IF. MRP4 inhibitors, including diclofenac, celecoxib, and MK571, also significantly reduced the resistance of MRP4 to CP and IF (Table II). It seemed that CP and IF could be clearly substrates for MRP4 transporter. This is supported by the following: (a) MRP4/HepG2 cells had lesser sensitivity to CP and IF than V/HepG2 cells; (b) MRP4-mediated resistance to CP and IF was inhibited by BSO; and (c) MRP4-mediated resistance to CP and IF was inhibited by diclofenac, celecoxib, and MK571. CP and IF are widely used alkylating agents in the treatment of a variety of solid tumors and hematological malignancies including breast, lung, ovarian and prostate cancer, leukemia, lymphomas, and multiple myeloma (21). Therefore, MRP4-mediated resistances to CP and IF and identification of CP and IF as MRP4 substrates have important clinical implications. MRP4 expression level in the tumor cells may serve as an important determinant for the antitumor efficacy of oxazaphosphorines. In addition, CP and IF are usually used with other type of chemotherapeutic agents in the combination chemotherapy protocols. For example, CP has been used with etoposide which is a substrate of MRP1-3 (22,23) for the treatment of liver metastases of breast cancer (24). Hence, drug–drug interactions and relationship to different multidrug resistant-related drug transporters should be considered in choosing CP- or IF-based combination chemotherapy. Furthermore, when MRP4 modulators are combined with CP and IF, drug–drug interactions should be considered for increasing efficacy in drug therapies.

Some members of the MRP family are inducible by drugs and toxins, especially MRP2 and MRP3. For example, the antibiotic rifampin treatment increased MRP2 expression in human duodenum (25) and in cultured HepG2 cells (26). Similarly, MRP3 induction after treatment with rifampin was demonstrated in HepG2 and Huh7 cells (27). Most of MRP4 inductions are founded in rats or mice. For instance, two electrophile response element activators, such as ethoxyquin and oltipraz, induced Mrp4 mRNA expression in rat liver (28) and mouse liver (29). This study provided solid evidence on the MRP4 induction in human cell lines. CFB, which was reported as an inducer of Mrp4 in CD-1 mice liver (20), was chosen as a positive control in this study. The induction effect of CFB on the MRP4 protein and gene expression was demonstrated using HEK293 cells in this study. In addition, this upregulation of MRP4 is dependent on the concentration and incubation time of CFB. Less than 6 days' incubation of CFB, no upregulation of MRP4 was found (data are not shown). Notably, both MRP4 gene and protein expression were upregulated in HEK293 cells by different concentration of CP after 6 days' co-incubation, this parallel changes suggesting a CP-mediated transcriptional modulation (Figs. 2 and 3). However, the MRP4-inducing effect of CP was less than that of CFB. On the other hand, IF did not significantly change the MRP4 protein expression in HEK293 cells, whereas IF exhibited a significant inhibition on the MRP4 gene expression at 100 μM (Fig. 4). The absence of corresponding changes in MRP4 suggests posttranscriptional mechanisms involved in the MRP4 expression with IF co-incubation in HEK293 cells. Although IF has similar chemical structure to CP, it did not show the influence on the MRP4 expression. It seems that the different intramolecular distance between the chloroethyl groups in CP or IF may have an effect on the MRP4 inducing potency. Furthermore, the fact that CP acts as an MRP4 inducer might be clinically relevant, as treatment of tumor patients with CP-based chemotherapy might promote the development of the undesired multidrug resistance in tumor cells.

Less is known about the mechanism implicated in the induction of MRP4 expression. The constitutive androstane receptor (CAR) activator 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) induced Mrp4 in mice liver (29). In addition, administration of TCPOBOP enhanced Mrp4 expression in the liver of the wild-type mice, but not in the CAR-null mice (30). It appears that CAR might be involved in the induction of mouse Mrp4 expression. Several studies suggested that the induction of mouse Mrp4 may be mediated by the nuclear factor-E2-related factor 2 (Nrf2) and PPARα. Nrf2 activators including ethoxyquin and oltipraz and the PPARα agonist CFB induced Mrp4 in mice liver (20,29). Very recently, Maher *et al.* reported that perfluorodecanoic acid, as a PPARα agonist, increased hepatic Mrp4 mRNA expression in wild-type mice. The induction of Mrp4 mRNA after perfluorodecanoic acid administration was attenuated in both PPARα- and Nrf2-null mice (31). Our study also showed that the PPARα agonist CFB induced human MRP4 expression in HEK293 cells, but not in HepG2 cells. The tissue-specific expression profile of human PPARα is very different from that of rodents'. In particular, the human liver expresses generally low levels of PPARα in contrast to the rodents (mice and rats) (32). In addition, Ammerschlaeger *et al.* reported that peroxisome proliferators did not induce human PPARα activity in human HepG2 and primary human hepatocytes (33). These suggest that PPARα might be required for CFB-mediated MRP4 induction. Recently, a study indicated that MRP4 mRNA and protein expression were elevated in liver samples from patients after toxic

acetaminophen ingestion and also in liver samples from patients with primary biliary cirrhosis (34). The study from this same group also showed that hepatic Mrp4 induction following acetaminophen exposure is dependent on Kupffer cell function (35). That may further explain why CFB induces Mrp4 in mice liver, but this has no effect on HepG2 cells. Our study also showed that CP can increase MRP4 expression in HEK293 cells, but not in HepG2 cells. It seemed that PPARα and Kupffer cell function might also be involved in the CP-mediated MRP4 induction. It warrants to further study the role of those receptors, including CAR, Nrf2, and PPARα, and Kupffer cell function on the induction of MRP4 and mechanism implicated in the CP- and CFB-mediated MRP4 induction.

In summary, the findings from this study indicated that CP and IF are highly possible substrates of MRP4, and CP is an inducer of MRP4 in a cell-specific manner. Further studies are needed to explore the role of MRP4 expression in oxazaphosphorine-based chemotherapy in patients to discern the appropriate mechanism implicated in the CP-mediated or other drugs or toxins-mediated MRP4 induction *in vivo* and *in vitro*. Elucidation of the role of MRP4 in the pharmacokinetic and pharmacodynamic behavior of oxazaphosphorines is important to optimization of oxazaphosphorine-based chemotherapy and further development of new oxazaphosphorines.

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Conflicts of interest None.

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