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Research Paper

Human Multidrug Resistance Associated Protein 4 Confers Resistance to Camptothecins

Quan Tian,[1] Jing Zhang,[1] Theresa May Chin Tan,[2] Eli Chan,[1] Wei Duan,[2] Sui Yung Chan,[1] Urs Alex Boelsterli,[1,3] Paul Chi-Lui Ho,[1] Hongyuan Yang,[2] Jin-Song Bian,[3] Min Huang,[4] Yi-Zhun Zhu,[3] Weiping Xiong,[5] Xiaotian Li,[6] and Shufeng Zhou[1,7]

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Purpose. The multidrug resistance associated protein (MRP) 4 is a member of the adenosine triphosphate (ATP)-binding cassette transporter family. Camptothecins (CPTs) have shown substantial anticancer activity against a broad spectrum of tumors by inhibiting DNA topoisomerase I, but tumor resistance is one of the major reasons for therapeutic failure. P-glycoprotein, breast cancer resistance protein, MRP1, and MRP2 have been implicated in resistance to various CPTs including CPT-11 (irinotecan), SN-38 (the active metabolite of CPT-11), and topotecan. In this study, we explored the resistance profiles and intracellular accumulation of a panel of CPTs including CPT, CPT-11, SN-38, rubitecan, and 10-hydroxy-CPT (10-OH-CPT) in HepG2 cells with stably overexpressed human MRP4. Other anticancer agents such as paclitaxel, cyclophosphamide, and carboplatin were also included.

Methods. HepG2 cells were transfected with an empty vehicle plasmid (V/HepG2) or human MRP4 (MRP4/HepG2). The resistance profiles of test drugs in exponentially growing V/HepG2 and MRP4/HepG2 cells were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide (MTT) assay with 4 or 48 h exposure time of the test drug in the absence or presence of various MRP4 inhibitors. The accumulation of CPT-11, SN-38, and paclitaxel by V/HepG2 and MRP4/HepG2 cells was determined by validated high-performance liquid chromatography methods.

Results. Based on the resistance folds from the MTT assay with 48 h exposure time of the test drug, MRP4 conferred resistance to CPTs tested in the order 10-OH-CPT (14.21) > SN-38 carboxylate (9.70) > rubitecan (9.06) > SN-38 lactone (8.91) > CPT lactone (7.33) > CPT-11 lactone (5.64) > CPT carboxylate (4.30) > CPT-11 carboxylate (2.68). Overall, overexpression of MRP4 increased the IC_{50} values 1.78- to 14.21-fold for various CPTs in lactone or carboxylate form. The resistance of MRP4 to various CPTs tested was significantly reversed in the presence of DL-buthionine-(*S*,*R*)-sulfoximine (BSO, a γ-glutamylcysteine synthetase inhibitor), MK571, celecoxib, or diclofenac (all MRP4 inhibitors). In addition, the accumulation of CPT-11 and SN-38 over 120 min in MRP4/HepG2 cells was significantly reduced compared to V/HepG2 cells, whereas the addition of celecoxib, MK571, or BSO significantly increased their accumulation in MRP4/HepG2 cells. There was no significant difference in the intracellular accumulation of paclitaxel in V/HepG2 and MRP4/HepG2 cells, indicating that P-glycoprotein was not involved in the observed resistance to CPTs in this study. MRP4 also conferred resistance to cyclophosphamide and this was partially reversed by BSO. However, MRP4 did not increase resistance to paclitaxel, carboplatin, etoposide (VP-16), 5-fluorouracil, and cyclosporine.

Conclusions. Human MRP4 rendered significant resistance to cyclophosphamide, CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT. CPT-11 and SN-38 are substrates for MRP4. Further studies are needed to explore the role of MRP4 in resistance, toxicity, and pharmacokinetics of CPTs and cyclophosphamide.

KEY WORDS: camptothecin; cyclophosphamide; drug transporter; multidrug resistance.

[1] Department of Pharmacy, Faculty of Science, National University of Singapore, Science Drive 4, Singapore 117543, Singapore.
[2] Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.
[3] Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.
[4] Department of Clinical Pharmacology, School of Pharmaceutical Science, Sun Yat-sen University, Guangzhou, China.
[5] ICU Unit, Guangdong Cardiovascular Institute, Guangdong Provincial People's Hospital, Guangzhou, China.
[6] Department of Maternal Medicine, Affiliated Hospital of Obstetrics and Gynaecology, Fudan University, Shanghai, China.
[7] To whom correspondence should be addressed. (e-mail: phazsf@nus.edu.sg)

ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BSO, DL-buthionine-(*S*,*R*)-sulfoximine; CPT, camptothecin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; $E_2$17βG, 17-β-D-estradiol-glucuronide; GSH, glutathione; HPLC, high-performance liquid chromatography; MRP, multidrug resistance associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide; MTX, methotrexate; OATP, organic anion transporting polypeptide; PgP, P-glycoprotein; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; UGT, uridine diphosphate glucuronosyltransferase.

INTRODUCTION

DNA topoisomerases are a group of enzymes that alter the topology of DNA and are present in all organisms including bacteria, viruses, yeast, and humans (1). There are two general types of topoisomerases, type I and type II. Type I cleaves and separates a single strand of DNA and alters the linkage quantity of DNA, whereas type II cleaves both strands of DNA and changes the linking number of DNA by two (1). Mammalian topoisomerase I (Top1) is particularly important for supporting replication fork movement during DNA replication and for relaxing supercoils formed during transcription (1). There is an increased interest in topoisomerases because they were found to be targets for naturally occurring anticancer drugs. Human Top2 isozymes are targets in tumor cells for anthracyclines (e.g., doxorubicin) and epipodophyllotoxins (e.g., etoposide) (2).

A relatively new group of compounds, the camptothecins (CPTs), are potent Top1 inhibitors (3–6). These compounds can induce tumor cell death due to the stabilization of Top1 complex and the generation of permanent DNA strand breaks (7). The parent compound, CPT, is an anticancer alkaloid isolated from the Chinese tree, *Camptotheca acuminata*, during a screen of plant extracts for finding anticancer agents (8). CPTs have a closed α-hydroxy-δ-lactone ring, which can undergo reversible, pH-dependent hydrolysis to yield the corresponding open-ring hydroxyl acid, namely, the more soluble carboxylate form under neutral or alkaline conditions (9). The lactone ring structure is essential for Top1 binding, antitumor activity, and the toxicity of CPTs (4,5,10,11), whereas carboxylate is only weakly cytotoxic (12). The poor aqueous solubility and unacceptable toxicity is a major obstacle for the clinical use of CPT. In the past 20 years, more effort has been made to synthesize new derivatives of CPT having improved water solubility and potent antitumor activity. This has led to the discovery of a series of CPT analogs including CPT-11 (irinotecan), topotecan, lurtotecan, 9-amino-CPT, rubitecan (9-nitro-CPT, RFS2000), 10-hydroxy-CPT (10-OH-CPT), silatecan (DB-67, 7-*tert*-butyldimethylsilyl-10-hydroxy-CPT), and exatecan (DX-8951f, a hexacyclic analog of CPT) (3). CPT-11 and topotecan have gained approval by the U.S. Food and Drug Administration for clinical use based on their ~30% response rates (13,14). CPT-11 has a broad spectrum of antitumor activity, but it is mainly used as a first-line treatment for advanced colorectal cancer in combination with 5-fluorouracil. As a prodrug, CPT-11 is transformed by carboxylesterases to its active metabolite (15), SN-38 (7-ethyl-10-OH-CPT), which exhibits a 100- to 1000-fold higher cytotoxicity than the parent drug (16). SN-38 is further converted to SN-38 glucuronide by uridine diphosphate glucuronosyltransferase (UGT) 1A1, 1A3, 1A6, and 1A9 (17). SN-38 is not suitable for direct administration to patients due to its poor aqueous solubility and unacceptable toxicity. Topotecan has modest activity as second-line therapy in patients with metastatic ovarian and small cell lung cancer, as well as myeloid malignancies (3). Other CPT analogs are being evaluated in clinical trials. Water-insoluble CPT analogs such as 9-amino-CPT and rubitecan have also been reformulated and introduced into clinical trials. Rubitecan is an oral CPT analog for the treatment of pancreatic cancer and other solid tumors (18,19).

Both severe and unpredictable dose-limiting toxicity and tumor resistance are a major hindrance for the success of CPT-based chemotherapy (20). For example, CPT-11 can only give an objective response in about 20% of treated patients with advanced colorectal or lung cancer (21,22). The mechanisms for tumor resistance to CPTs are complicated and a number of tumor-, drug- and host-related factors have been implicated (20). These include sanctuary sites for tumors, lack of or low activity of bioactivating enzymes, increased inactivation of active species, acquired DNA-repairing capacity, increased Top1 activity, and increased efflux of drugs from tumor cells resulting in reduced accumulation of drugs in tumor cells (20). In particular, increased efflux of CPTs by several drug transporters has been implicated in tumor resistance. *In vitro* studies have indicated that both CPT-11 and SN-38 are actively transported by P-glycoprotein (PgP/ABCB1), multidrug resistance associated protein 1 (MRP1/ABCC1), and MRP2 (ABCC2/cMOAT) (23,24). In rat and human bile canalicular membrane vesicles, PgP and MRP2 have been demonstrated to mediate the efflux of CPT-11 and SN-38 (25). The involvement of PgP and MRP2 in the efflux of CPT-11 and SN-38 has been further demonstrated in wild-type rats (26,27) and rats defective in MRP2 *in vivo*. Recently, Norris *et al.* (28) revealed that MRP4 also increased 5.9- and 6.0-fold resistance to CPT-11 and SN-38, respectively, using the human embryo kidney cell line HEK293 overexpressing MRP4. The breast cancer resistance protein (BCRP/MXR/ABCG2) mediates the efflux of CPT-11, SN-38 (29), 9-amino-CPT, and rubitecan (30) from tumor cells. Moreover, the organic anion transporting polypeptide (OATP)1B1 can transport SN-38, but not CPT-11 and SN-38 glucuronide *in vitro* (31).

MRPs are important members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters (32,33). MRP1 and MRP2 are lipophilic anion transporters with similar substrate specificity compared with PgP, conferring significant resistance to a panel of natural product type anticancer drugs including paclitaxel, *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and camptothecins (e.g., CPT-11 and SN-38) (32,33). However, MRP3 (ABCC3) confers resistance to a narrower spectrum of natural product type anticancer drugs compared to MRP1 and MRP2, with resistance to *Vinca* alkaloids and epipodophyllotins only (34,35). Notably, BCRP also has a narrower anticancer drug spectrum compared with PgP, MRP1, and MRP2. It renders tumor cells resistant to anthracyclines, mitoxantrone, and camptothecins, but not to *Vinca* alkaloids, paclitaxel, epipodophyllotoxins, and cisplatin (36). Like PgP, BCRP is independent on glutathione (GSH) for transport of its anticancer drug substrates (37). MRP4 (ABCC4), MRP5 (ABCC5), and MRP8 (ABCC11) are cyclic nucleotide transporters (32,33). MRP4 (ABCC4) has specific tissue expression profile, drug resistance selectivity, and substrate and inhibitor specificity, in comparison with other MRPs. In addition to cAMP/cGMP, MRP4 can transport GSH and folate (32,33). Like MRP1 and MRP2, MRP4 cotransports many of its substrates with GSH and depletion of intracellular GSH by the γ-glutamylcysteine synthetase inhibitor, DL-buthionine-(*S*,*R*)-sulfoximine (BSO), blocks the MRP4-mediated export of cAMP and abolishes resistance to nucleoside analogues (38). Moreover, MRP4 transports the anticancer agents leucovorin and methotrexate

(MTX) (32,33). In *Mrp4* knockout mice, topotecan brain concentration was significantly increased compared to the wild type, indicating that topotecan is a potential substrate for mouse *Mrp4*, which may serve as a protective barrier in the brain for cytotoxic drugs (39). This aim of this study was to investigate whether overexpression of human MRP4 in HepG2 cells modulated the resistance and intracellular accumulation to a panel of CPTs including CPT, CPT-11, SN-38, 10-OH-CPT, and rubitecan (Fig. 1). Thus, the effect of increased human MRP4 expression on the cytotoxicity and intracellular accumulation of these CPTs were examined. HepG2 cells derived from human hepatoma have an undetectable or very low level expression of MRP4, which is not inducible by rifampicin (40), but moderate levels of PgP, MRP1-3, and MRP5-6 are detected (40,41). Thus, HepG2 cells were suitable for MRP4 transfection and further functional transport studies.

MATERIALS AND METHODS

Chemicals and Reagents

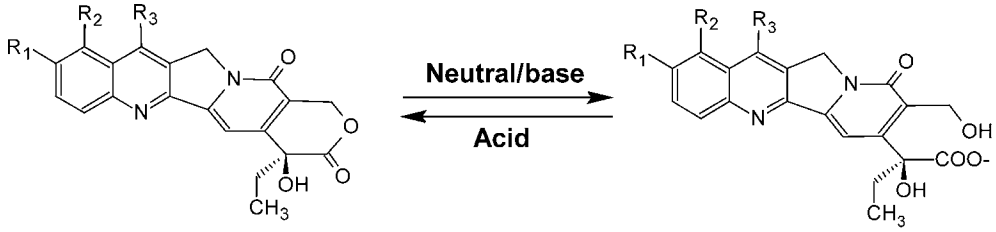
CPT analogs (all in lactone form), including CPT-11, SN-38, CPT, rubitecan, and 10-OH-CPT, were all purchased from SinoChem Ningbo Co. (Ningbo, China). All CPT analogs have a purity of >99.5% as determined by high-performance liquid chromatography (HPLC) and their structures were confirmed by mass spectrometry and [^{1}H] nuclear magnetic resonance. CPT, CPT-11, and SN-38 carboxylate forms were prepared from their lactone forms, respectively, by dilution with acetonitrile–0.02 M borate buffer at pH 9.0 (50:50, v/v) and incubation overnight. These CPTs were 99% lactone form at pH 3.0 and 98% carboxylate form at pH 9.0. Vincristine, vinblastine, MTX, adefovir dipivoxil (bis-POM-PMEA), celecoxib, and diclofenac (all compounds with a purity >99%) were also from SinoChem Ningbo Co. 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, Quebec, Canada) (42). Etoposide (VP-16), 5-fluorouracil, cyclosporine, dimethyl sulfoxide (DMSO), carboplatin, paclitaxel, BSO, Dulbecco's modified Eagle's medium (DMEM), and penicillin/streptomycin were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). Norcantharidin (with a purity of 99.5%), a protein phosphatase I inhibitor for cancer therapy, was a gift from Professor Andy Lee (Department of Oncology, Putuo District People's Hospital, Shanghai University of Traditional Chinese Medicine, China). Blasticidin was purchased from Invitrogen (Carlsbad, 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 obtained from commercial sources were of analytical or HPLC grade.

Cell Culture

HepG2 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were maintained in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. HepG2 cells with insertion of vector or *MRP4* were cultured in the same culture medium for HepG2 cells with the presence of 0.25 μg/ml blasticidin. All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Transfection of Human *MRP4* cDNA to HepG2 Cells

The transfection of human *MRP4* cDNA to HepG2 cells and the functional confirmation was described previously (38). Briefly, full-length *MRP4* cDNA was cloned into the pcDNA6/V5-His vector and then transfected into HepG2 cells using LipofectAMINE reagent. Blasticidin (0.25 μg/ml) was added to the medium for selection 48 h after the start of transfection. Parent cells and cells with stably transfected MRP4 (MRP4/HepG2) or insertion of vector alone (V/HepG2) were maintained in DMEM in the presence of



Lactone (active) Carboxylate (inactive)

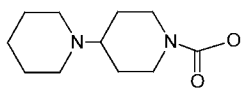
Camptothecin	R_1	R_2	R_3
CPT	H	H	H
CPT-11		H	C_2H_5
SN-38	OH	H	C_2H_5
Rubitecan	H	NO_2	H
10-OH-CPT	OH	H	H

Fig. 1. Chemical structures of CPT, CPT-11 (irinotecan), SN-38, rubitecan, and 10-OH-CPT.

0.25 μg/ml blasticidin. The sequence of human *MRP4* was analyzed and confirmed, its mRNA determined by RT-PCR assay, and the protein expression analyzed using Western blotting assay (38). The established HepG2 cells with stable expression of MRP4 demonstrated significant resistance to 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and 6-thioguanine (38). These cells are capable of transporting GSH (38) and bimane–GSH conjugate (43).

Cytotoxicity Assay

Drug effects on exponentially growing tumor cells were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide (MTT) assay as described previously (44). Cells were seeded at a density of 5000 cells/100 μl 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. All CPTs were freshly prepared by dissolving in DMSO and diluted by PBS. 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, the medium with drug at different concentrations was removed by aspiration 4 h after drug addition. The cells were washed twice with PBS, fresh drug-free medium was added, and the cells incubated for a further 44 h. At 48 h for both long and short drug exposures, 100 μl MTT reagent (0.5 mg/ml) was 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 precipitate 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 four times. The cytotoxicity was evaluated with reference to the IC_{50} value, which is 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 multireplicated experiments.

In Vitro Cytotoxicity Inhibition Assay

BSO, MK571, celecoxib, and diclofenac are all known inhibitors for MRP4 (32,33). 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 diluting 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 CPT analogs, the medium with these inhibitors was removed, and the cells washed twice with PBS. Then CPT analogs were added and MTT assay performed as described herein.

Accumulation of CPT-11 and SN-38

The accumulation of CPT-11 and SN-38 in V/HepG2 and MRP4/HepG2 cells was examined in confluent cell cultures grown on 60-mm plastic culture dishes (Corning Costar Corp., Acton, MA, USA) as previously described (45). Briefly, exponentially growing cells were exposed to 5 μM CPT-11 lactone or 1 μM SN-38 lactone for 120 min at 37°C. The medium was aspirated off at the indicated times, and the dishes were rapidly rinsed five times with 5 ml of ice-cold PBS. HPLC analysis of final washes ensured that they contained no residual CPT-11 or SN-38. After washing by ice-cold PBS, the cells were harvested and each cell pellet was suspended in 200 μl extraction solution [acetonitrile/methanol (1:1 v/v) with 0.01 N HCl] with the addition of 10 μl CPT (2 μg, used as internal standard). The acidity of the extraction solution ensures the conversion of the compound to lactone form. Subsequently, the mixture was sonicated, vortexed, and centrifuged. The supernatant was then injected into HPLC for concentration determination. Viable cells were monitored using the trypan blue exclusion method and the accumulation of CPT-11 and SN-38 was expressed as nanograms per 10^6 cells. In addition, the effect of celecoxib (50 μM), BSO (200 μM), and MK571 (100 μM) on CPT-11 and SN-38 accumulation was investigated. Both celecoxib and MK571 were prepared by dissolving in DMSO and diluting by PBS, whereas BSO was dissolved in sterile Milli-Q water. The final concentration of DMSO was 1% (v/v). The three inhibitors at indicated concentrations showed little cytotoxicity (<5%) when incubated for 2 h for 50 μM celecoxib and 100 μM MK571 or 24 h for 200 μM BSO. Celecoxib or MK571 was preincubated with cells for 2 h, whereas BSO was preincubated for 24 h. Thereafter, cells were washed with warm PBS buffer for four times. After continued incubation for 2 min for SN-38-treated cells and 30 min for CPT-11-treated cells, cells were washed five times with warm PBS. The cells were then harvested, lysed by sonication, and extracted using an ice-cold acetonitrile/methanol mixture (1:1, v/v, with 0.01 N HCl). The supernatant was injected into HPLC for the determination of CPT-11 and SN-38. The accumulation of CPT-11 and SN-38 in the absence and presence of celecoxib, BSO, or MK571 was studied in at least three independent experiments.

Determination of CPT-11 and SN-38 by HPLC

The HPLC system (Shimadzu, Nakagyo-ku, Kyoto, Japan) consisted of a LC-10AT pump, a FCV-10AL low-pressure-gradient flow-control valve, a DGU-14A on-line solvent degasser, a RF-10AXL fluorescence detector, and a SIL-10AD sample injector. Shimadzu Class-LC10 Workstation was used for system control and data were monitored and analyzed using Shimadzu CLASS VP software. Quantitative HPLC analysis of samples was performed at room temperature using a C_{18} reverse-phase column (Phenomenex, 5 μm, 4.6 × 200 mm) preceded by a C_{18} guard column (4 × 3.0 mm; Phenomenex Co., Torrance, CA, USA). The mobile phase was a mixture of 27% (v/v) acetonitrile and 73% (v/v) aqueous buffer containing 50 mM sodium hydrogen phosphate and 10 mM sodium heptane sulfonate (pH = 3.0). The flow rate was maintained at 1 ml/min. CPT-11 and SN-38

Table I. Drug Sensitivity of MRP4-Transfected HepG2 Cells to Bis-POM-PMEA and MTX

Drug	Drug-exposure time (h)	IC_{50} (μM)		Fold resistance	*N*
		V/HepG2	MRP4/HepG2		
bis-POM-PMEA	48	0.372 ± 0.081	3.863 ± 0.457[a]	10.38	11
+ BSO	48	0.983 ± 0.16[b]	0.963 ± 0.188[b]	0.98	10
bis-POM-PMEA	4	2.403 ± 0.369	9.399 ± 0.329[a]	3.91	10
+ BSO	4	3.047 ± 0.923[b]	2.597 ± 0.01[a,b]	0.95	10
Methotrexate	48	0.110 ± 0.016	0.130 ± 0.024	1.18	6
+ BSO	48	0.105 ± 0.029	0.128 ± 0.029	1.22	4
Methotrexate	4	2.592 ± 0.504	12.103 ± 2.618[a]	4.67	6
+ BSO	4	3.824 ± 0.735[b]	5.060 ± 0.962[b]	1.32	4

Data are the means ± SD. 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 4–11 times.
[a] $P < 0.05$ by Student's *t* test, MRP4/HepG2 *vs.* V/HepG2.
[b] $P < 0.05$ by Student's *t* test, test drug vs. test drug + BSO.

were detected by a fluorescence detector (Shimadzu Scientific Instruments, Columbia, MD, USA) with λ_{ex} at 380 nm and λ_{em} at 540 nm, respectively. All HPLC methods had acceptable accuracy (85–115% of true values) and precision (intra- and interassay coefficient variations <15%) over the respective concentration ranges (CPT-11: 0.20–2000 ng; SN-38: 0.01−500 ng). The limit of quantitation (the minimum concentration that could be determined with acceptable accuracy (i.e., recovery between 80 and 120%) and precision (coefficient of variation <20%) for CPT-11 and SN-38 were 0.20, and 0.01 ng, respectively, when the injection volume was 100 μl. Assay specificity was indicated by the absence of interfering chromatographic peaks in PBS buffer or cellular homogenate samples and by incubating

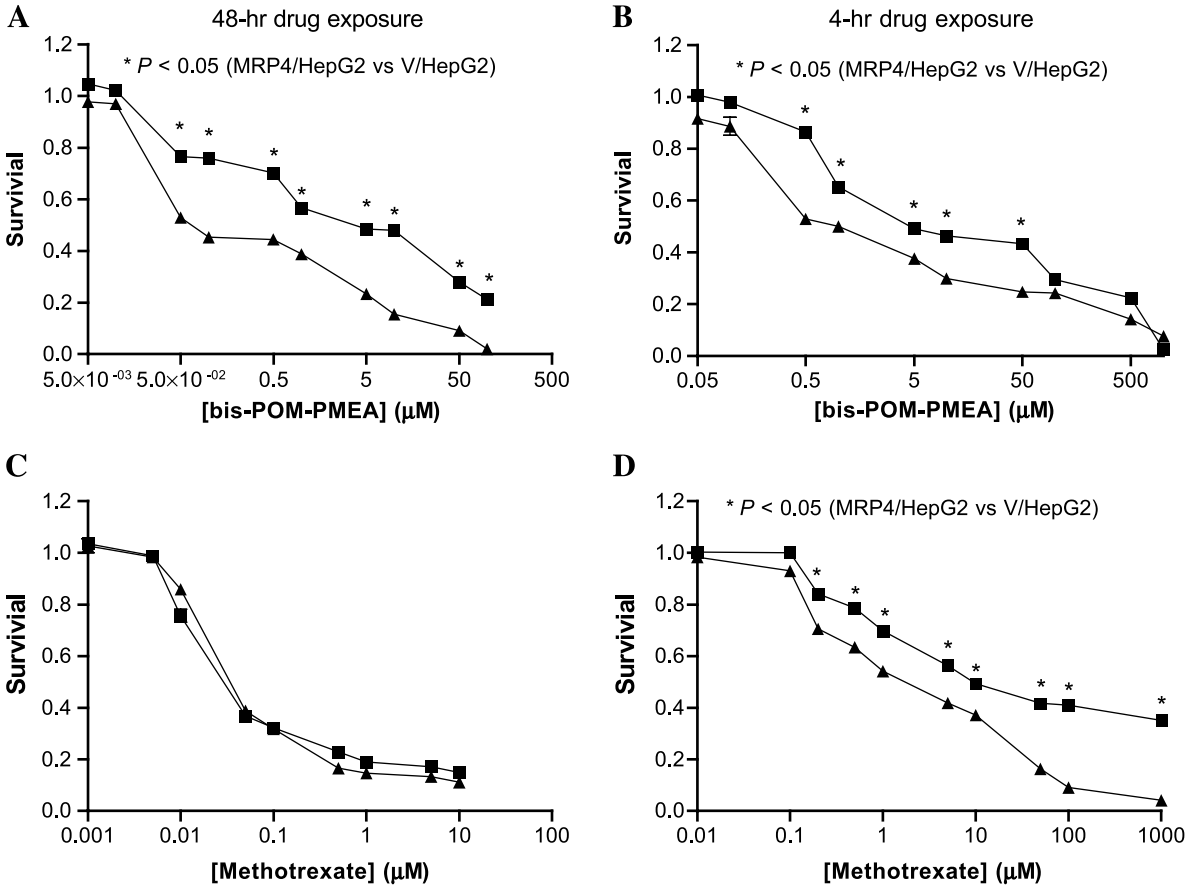


Fig. 2. Representative cytotoxicity profiles of adefovir dipivoxil (bis-POM-PMEA) and methotrexate when the cells were treated with the drug for 48 h (A and C) or 4 h (B and D) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Two-way ANOVA test was used. *$P < 0.05$.

with potential inhibitors such as diclofenac, celecoxib, and MK571.

Accumulation of Paclitaxel

Accumulation of paclitaxel was measured using a validated HPLC method as described previously (46). Exponentially growing cells were exposed to 50 nM paclitaxel at 37°C over 120 min. Cells were washed five times with ice-cold PBS and scraped using a rubber policeman at the indicated time. The harvested cells were lysed, extracted with methanol, and centrifuged at 6000 × *g* for 15 min at room temperature. The supernatant was collected and injected into a Shimadzu HPLC system equipped with C_{18} reverse-phase column (Phenomenex, 5 μm, 4.6 × 200 mm) preceded by a C_{18} guard column (Phenomenex, 4 × 3.0 mm). The analytical column was eluted with mobile-phase water/methanol (65:35, v/v) at a flow rate of 1.0 ml/min. The absorbance was determined by a UV detector (Shimadzu, SPD-M10A) at a wavelength of 227 nm. The HPLC was also validated with acceptable accuracy, precision, and specificity. The sensitivity was 5.0 ng at a 100-μl injection volume. The accumulation of paclitaxel was studied in three independent determinations.

Table II. Resistance Profiles of CPT Analogs in HepG2 Cells Overexpressing MRP4 or with Insertion of Empty Vector

Drug	Drug-exposure time (h)	IC_{50} (μM)		Fold resistance	*N*
		V/HepG2	MRP4/HepG2		
CPT-11 (lactone)	48	2.420 ± 0.071	13.644 ± 2.051[a]	5.64	12
+ Celecoxib	48	2.834 ± 0.072	3.149 ± 0.126[b]	1.11	4
+ Diclofenac	48	4.419 ± 1.551[b]	8.097 ± 1.156[a,b]	1.83	4
+ MK571	48	3.087 ± 0478	6.392 ± 0.518[a,b]	2.07	4
+ BSO	48	5.392 ± 1.213[b]	4.905 ± 0.047[b]	0.91	8
CPT-11 (lactone)	4	12.943 ± 1.418	44.907 ± 10.184[a]	3.47	12
+ Celecoxib	4	10.591 ± 1.142	9.881 ± 3.459[b]	0.93	4
+ Diclofenac	4	10.817 ± 1.036	12.687 ± 0.666[b]	1.17	4
+ MK571	4	12.924 ± 1.660	11.880 ± 3.254[b]	0.92	4
+ BSO	4	11.750 ± 3.521	14.367 ± 1.130[a,b]	1.22	7
CPT-11 (carboxylate)	48	3.910 ± 0.488	10.485 ± 0.634[b]	2.68	4
	4	20.287 ± 1.926[b]	36.085 ± 7.575[a,b]	1.78	4
SN-38 (lactone)	48	0.083 ± 0.007	0.741 ± 0.007[a]	8.91	12
+ Celecoxib	48	0.086 ± 0.003	0.349 ± 0.017[a,b]	4.06	4
+ Diclofenac	48	0.092 ± 0.014	0.366 ± 0.002[a,b]	4.00	4
+ MK571	48	0.091 ± 0.005	0.367 ± 0.010[a,b]	4.02	4
+ BSO	48	0.114 ± 0.037[b]	0.207 ± 0.006[a,b]	1.07	7
SN-38 (lactone)	4	0.948 ± 0.015	7.703 ± 0.545[a]	8.12	12
+ Celecoxib	4	0.970 ± 0.016	3.165 ± 0.159[a,b]	3.26	4
+ Diclofenac	4	0.969 ± 0.021	3.371 ± 0.020[a,b]	3.48	4
+ MK571	4	0.938 ± 0.026	3.190 ± 0.218[a,b]	3.40	4
+ BSO	4	0.877 ± 0.217	1.737 ± 0.017[a,b]	1.98	7
SN-38 (carboxylate)	48	0.079 ± 0.008	0.766 ± 0.137[b]	9.70	4
	4	0.674 0.065[b]	5.540 ± 1.170[a,b]	8.21	4
CPT (lactone)	48	0.032 ± 0.006	0.231 ± 0.062[a]	7.33	10
+ BSO	48	0.083 ± 0.012[b]	0.110 ± 0.026[a,b]	1.33	6
CPT (lactone)	4	0.808 ± 0.137	4.052 ± 0.485[a]	5.02	7
+ BSO	4	1.874 ± 0.053[b]	1.512 ± 0.024[b]	0.81	5
CPT (carboxylate)	48	0.072 ± 0.008	0.308 ± 0.060[a]	4.30	4
	4	0.841 ± 0.075[b]	2.452 ± 0.113[a,b]	2.92	4
Rubitecan (lactone)	48	0.085 ± 0.013	0.768 ± 0.212[a]	9.06	4
+ Celecoxib	48	0.088 ± 0.020	0.192 ± 0.054[a,b]	2.18	4
+ Diclofenac	48	0.062 ± 0.023	0.225 ± 0.045[a,b]	3.63	4
+ MK571	48	0.107 ± 0.042	0.299 ± 0.088[a,b]	2.79	4
+ BSO	48	0.072 ± 0.004	0.153 ± 0.012[a,b]	2.12	4
Rubitecan (lactone)	4	0.904 ± 0.147	6.681 ± 0.300[a]	7.39	4
+ BSO	4	1.036 ± 0.101	1.908 ± 0.147[a,b]	1.84	4
10-OH-CPT (lactone)	48	0.136 ± 0.008	1.931 ± 0.448[a]	14.21	5
+ BSO	48	0.154 ± 0.030	0.809 ± 0.094[a,b]	5.26	4
10-OH-CPT (lactone)	4	0.651 ± 0.019	7.730 ± 1.166[b]	11.87	4
+ BSO	4	0.717 ± 0.013	1.821 ± 0.088[a,b]	2.54	4

Data are the means ± SD. 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 4–12 times.
[a] $P < 0.05$ by Student's *t* test, MRP4/HepG2 *vs.* V/HepG2.
[b] $P < 0.05$, test drug *vs.* test drug + inhibitor (by Student's *t* test for CPT, rubitecan, and 10-OH-CPT and by one-way ANOVA for CPT-11 and SN-38).

Statistical Analysis

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

RESULTS

Human MRP4 Conferred Resistance to bis-POM-PMEA and MTX

Both bis-POM-PMEA and MTX are reported as substrates for MPR4 (47,48). To validate the MRP4 functionality in vector- and MRP4-transfected HepG2 cells used in the present study, we assessed the cytotoxicity of both bis-POM-PMEA and MTX to these cells. bis-POM-PMEA, as a prodrug, is rapidly converted to PMEA *in vitro* and *in vivo*

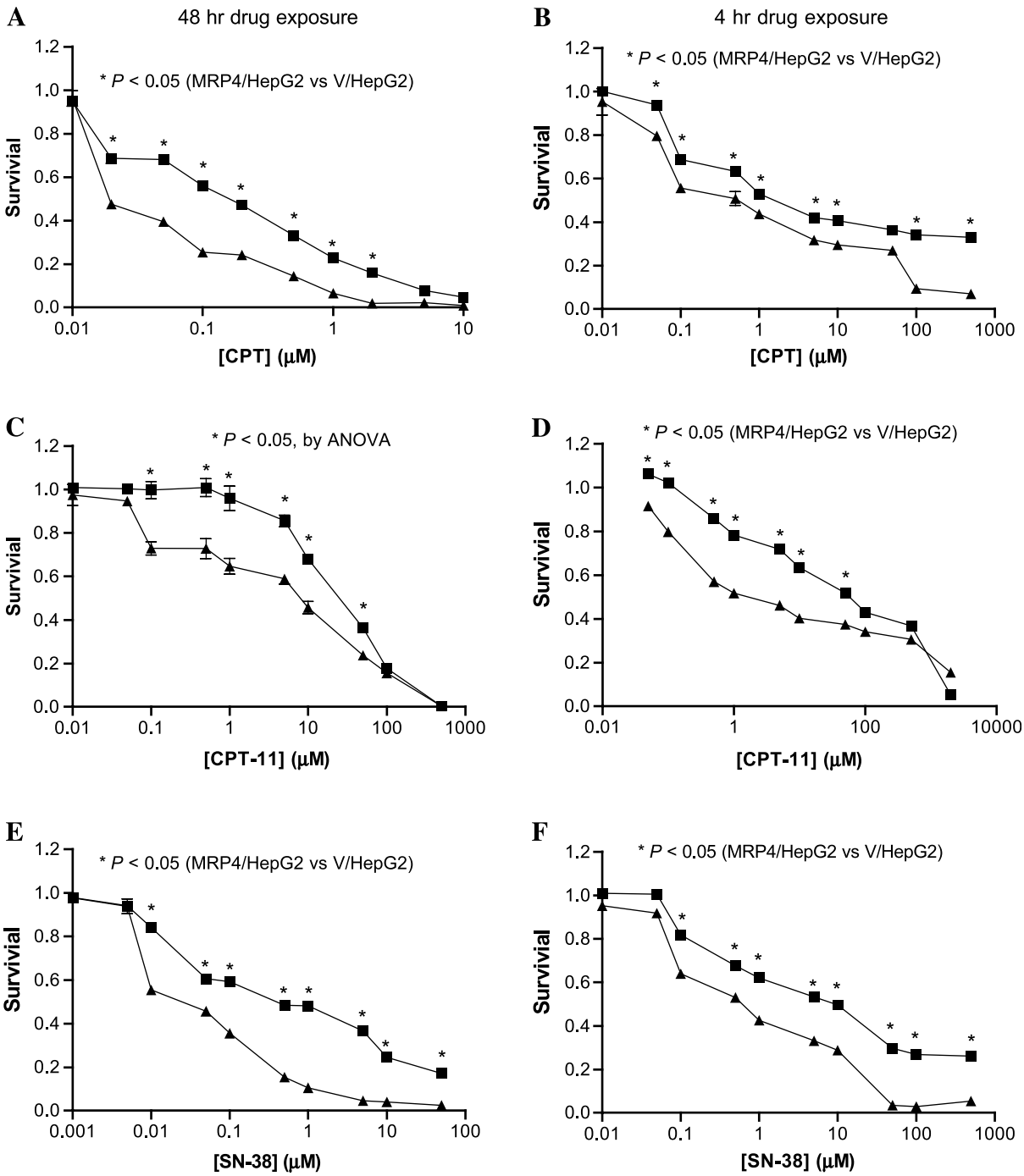


Fig. 3. Representative cytotoxicity profiles of CPT, CPT-11, and SN-38 lactone when the cells were treated with the drug for 48 h (A, C, and E) or 4 h (B, D, and F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Two-way ANOVA test was used. *$P < 0.05$.

(48). MTX was 3-fold more potent than bis-POM-PMEA in V/HepG2 cells (IC_{50} when cells were exposed to the test drug for 48 h: 0.372 ± 0.081 *vs.* 0.110 ± 0.016 μM).

As shown in Table I and Fig. 2, overexpression of MRP4 conferred 10.38- and 3.91-fold resistance to bis-POM-PMEA when the cells were exposed to the test drug for 4 and 48 h, respectively. MRP4-transfected HepG2 cells exposed to MTX for 4 h were 4.67-fold more resistant compared with V/HepG2 cells, whereas there was no significantly increased resistance when cells were exposed to MTX for 48 h. The latter may be due to the masking effect of multiple other transporters on MRP4 when the exposure time for MTX was prolonged. In addition, V/HepG2 (with insertion of empty vector) and parental HepG2 cells exhibited similar sensitivity to both bis-POM-PMEA and MTX (data not shown).

We analyzed the cytotoxic effects of bis-POM-PMEA and MTX in both V/HepG2 and MRP4/HepG2 cells by conducting two-way ANOVA using "drug concentration" and "cell line" as within- and between-sample factors, respectively. Generally, the drug concentration effect was significant with variation ($P < 0.05$) for both bis-POM-PMEA and MTX in V/HepG2 and MRP4/HepG2 cells, indicating that the cytotoxicity of these two compounds to V/HepG2 and MRP4/HepG2 cells was drug concentration dependent. Moreover, the cell line effect was significant with variation ($P < 0.05$) for both bis-POM-PMEA and MTX except for MTX with 48 h drug exposure time, indicating that MRP4/HepG2 cells had different cytotoxic profiles when incubated with bis-POM-PMEA or MTX compared to V/HepG2 cells.

The effects of BSO on the cytotoxicity of bis-POM-PMEA in vector- and MRP4-transfected HepG2 cells were also examined. In MRP4/HepG2 cells, the addition of BSO significantly increased the cytotoxicity of bis-POM-PMEA by 4.01- and 3.62-fold when the cells were exposed to bis-POM-PMEA for 48 and 4 h, respectively (Table I). BSO also significantly reversed the MRP4-mediated resistance to MTX with drug exposure time of 4 h, with the IC_{50} reduced from 12.103 ± 2.618 to 5.060 ± 0.962 μM ($P < 0.05$). Interestingly, the presence of BSO significantly decreased the cytotoxicity of bis-POM-PMEA in V/HepG2 cells by 2.64- and 1.27-fold when the cells were exposed to bis-POM-PMEA for 48 and 4 h, respectively. BSO also decreased the cytotoxicity of MTX by 1.48-fold in V/HepG2 cells. As background expression of MRP4 is very low in the control cells (V/HepG2) (38), transporters other than MRP4 (e.g., MRP1, MRP2, and MRP5) may play a dominant role in the transport of bis-POM-PMEA, PMEA and MTX. Like MRP4, these transporters can cotransport GSH with their corresponding substrates (49,50). BSO might affect their function by inhibiting GSH synthesis, resulting in different uptake and accumulation and cytotoxicity of PMEA and MTX.

Overall, these results indicate that the established HepG2 cells with overexpression of MRP4 conferred significant resistance to both bis-POM-PMEA and MTX. When cells were preincubated with 200 μM BSO, the resistance

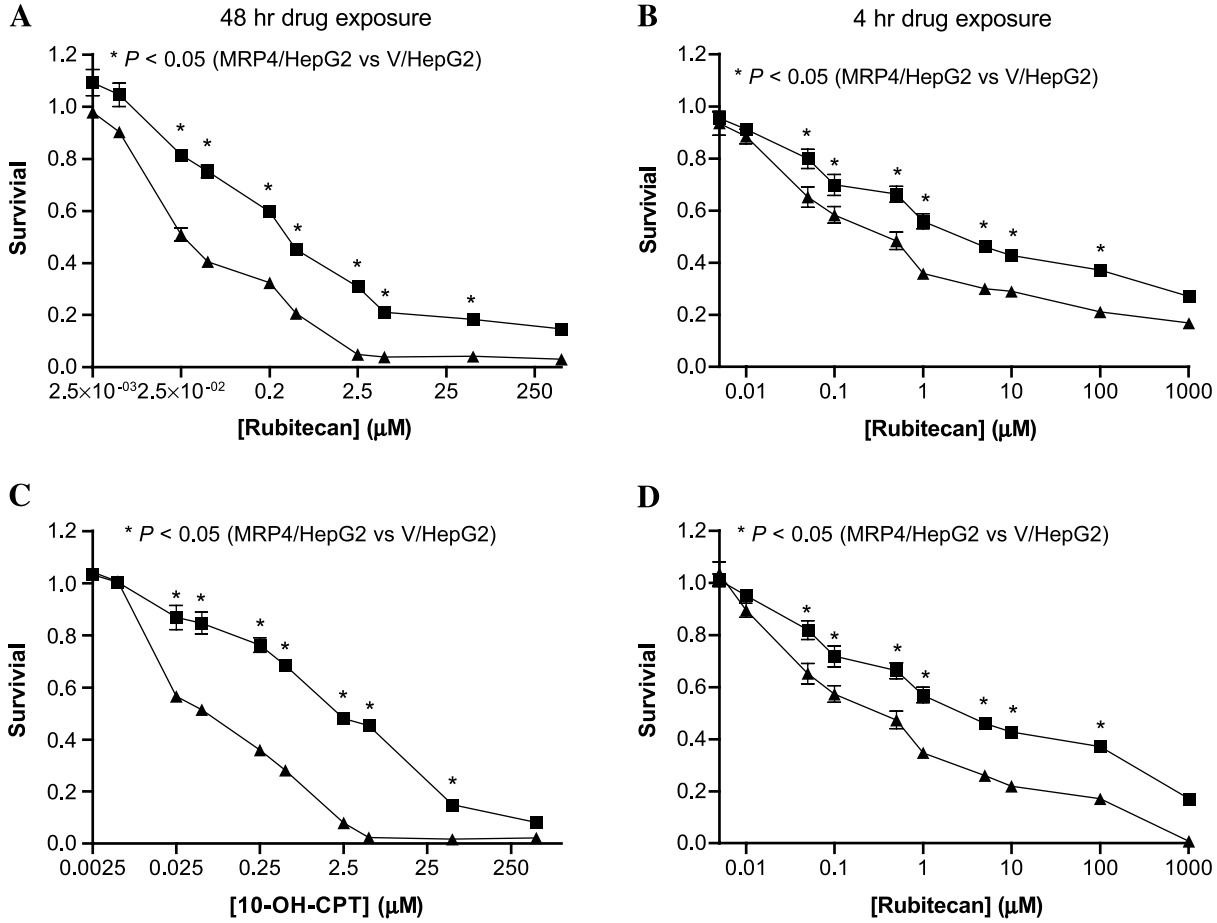


Fig. 4. Representative cytotoxicity profiles of rubitecan and 10-OH-CPT lactone when the cells were treated with the drug for 48 h (A and C) or 4 h (B and D) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Two-way ANOVA test was used. *$P < 0.05$.

capability of MRP4 to bis-POM-PMEA and MTX was significantly inhibited. All these results indicate that there is proper functionality of MRP4 in MRP4/HepG2 cells used in this study.

Human MRP4 Conferred Resistance to CPT Analogs

The cytotoxicity of CPT-11, SN-38, CPT, rubitecan, and 10-OH-CPT in lactone and carboxylate forms to both MRP4/HepG2 and V/HepG2 cells is shown in Table II. CPT lactone showed 23- and 42-fold higher cytotoxicity to V/HepG2 cells when the cells were exposed to CPT lactone for 4 and 48 h, respectively, compared with CPT-11 lactone. CPT and SN-38 lactone displayed similar cytotoxicity to V/HepG2 cells. In addition, SN-38 lactone exhibited 21- and 75-fold greater toxicity to V/HepG2 cells when the cells were exposed to SN-38 lactone for 4 and 48 h, respectively, compared with CPT-11 lactone.

CPT-11, CPT, and SN-38 in carboxylate form exhibited lesser but comparable cytotoxicity to both MRP4/HepG2 and V/HepG2 cells compared with their respective lactone (Table II). This indicated that CPTs in carboxylate form at pH 7.4 medium were rapidly converted to active lactone form, whereas conversion to carboxylate occurred when CPTs in lactone were loaded, resulting in a stable lactone percentage. In medium or buffer at pH 7.4, we found that the lactone form was ~30% when equilibrium was achieved. It is noted that as the active metabolite of CPT-11, SN-38 in either lactone or carboxylate form showed much higher toxicity to HepG2 cells than its parent drug.

MRP4 overexpression conferred significant resistance to CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT in lactone form (7.33-, 5.64-, 8.91-, 9.06-, and 14.21-fold, respectively) when the exposure time of the cells for the test drug was 48 h (Figs. 3 and 4). Cells overexpressing human MRP4 showed increased IC_{50} values by 5.06-, 3.43-, 8.12-, 7.39, and 11.87-fold when the exposure time of the cells for the test drug was 4 h for CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT in lactone form, respectively, compared with V/HepG2 cells (Table II). Furthermore, MRP4 rendered 1.78- to 9.70-fold resistance to CPT, CPT-11, and SN-38 in carboxylate form when the cells were exposed to the tested CPT analog for 4 and 48 h. Based on the resistance folds from the MTT assay with 48 h exposure time of the test drug, MRP4 conferred resistance to CPTs tested in the order 10-OH-CPT (14.21) > SN-38 carboxylate (9.70) > rubitecan (9.06) > SN-38 lactone (8.91) > CPT lactone (7.33) > CPT-11 lactone (5.64) > CPT carboxylate (4.30) > CPT-11 carboxylate (2.68). MRP4 showed the highest resistance to 10-OH-CPT, whereas the lowest resistance to CPT-11 carboxylate.

The cytotoxic effects of various CPTs tested in both V/HepG2 and MRP4/HepG2 cells were statistically analyzed by conducting two-way ANOVA using "CPT analog concentration" and "cell line" as within and between-sample factors, respectively. Generally, the CPT analog concentration effect was significant with variation ($P < 0.05$) for all CPTs tested in this study in V/HepG2 and MRP4/HepG2 cells, indicating that the cytotoxicity of these CPT analogs to V/HepG2 and MRP4/HepG2 cells was drug concentration dependent. In addition, the cell line effect was significant with variation ($P < 0.05$) for all CPTs tested with 4 and 48 h drug exposure time, indicating that there were significantly different cytotoxic profiles between MRP4/HepG2 and V/HepG2 cells when incubated with either of the test CPT analogs.

Table III. Cytotoxicity of Various Anticancer Drugs in HepG2 Cell Expressing MRP4 or with Insertion of Empty Vector

Drug	Drug exposure time (h)	IC_{50} (μM)*		Fold resistance	*N*
		V/HepG2	MRP4/HepG2		
Etoposide	48	0.809 ± 0.159	0.943 ± 0.183	1.17	6
	4	2.496 ± 0.483	2.829 ± 0.196	1.13	5
Cyclophosphamide	48	10.411 ± 1.051	41.678 ± 7.318[a]	4.00	4
	4	38.309 ± 6.916	121.382 ± 13.615[a]	3.17	5
5-Fluorouracil	48	1.027 ± 0.055	1.074 ± 0.157	1.04	6
	4	6.136 ± 0.012	6.700 ± 0.109	1.09	3
Norcantharidin	48	14.925 ± 0.024	15.508 ± 1.124	1.04	5
	4	24.433 ± 3.886	24.945 ± 1.554	1.02	4
Carboplatin	48	5.791 ± 0.492	6.009 ± 0.811	1.04	3
	4	59.481 ± 1.068	58.505 ± 5.770	0.98	3
Vincristine	48	0.772 ± 0.141	0.745 ± 0.177	0.97	6
	4	1.730 ± 0.393	1.804 ± 0.233	1.04	5
Vinblastine	48	0.232 ± 0.026	0.288 ± 0.034	1.24	4
	4	2.130 ± 0.279	2.071 ± 0.146	0.97	4
Paclitaxel	48	3.548 ± 0.044	3.753 ± 0.091	1.06	5
	4	21.765 ± 1.274	21.266 ± 0.563	0.98	4
Cyclosporine**	48	0.700 ± 0.178	0.754 ± 0.130	1.08	4
	4	6.988 ± 0.647	7.552 ± 0.904	1.08	4

Data are the means ± SD. 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 3–6 times.
[a] $P < 0.05$ by Student's *t* test, MRP4/HepG2 *vs.* V/HepG2.
*IC_{50} was in micromoles per liter for all drugs, except vincristine, vinblastine, and paclitaxel for which nanomoles per liter was used.
**It is not an anticancer agent.

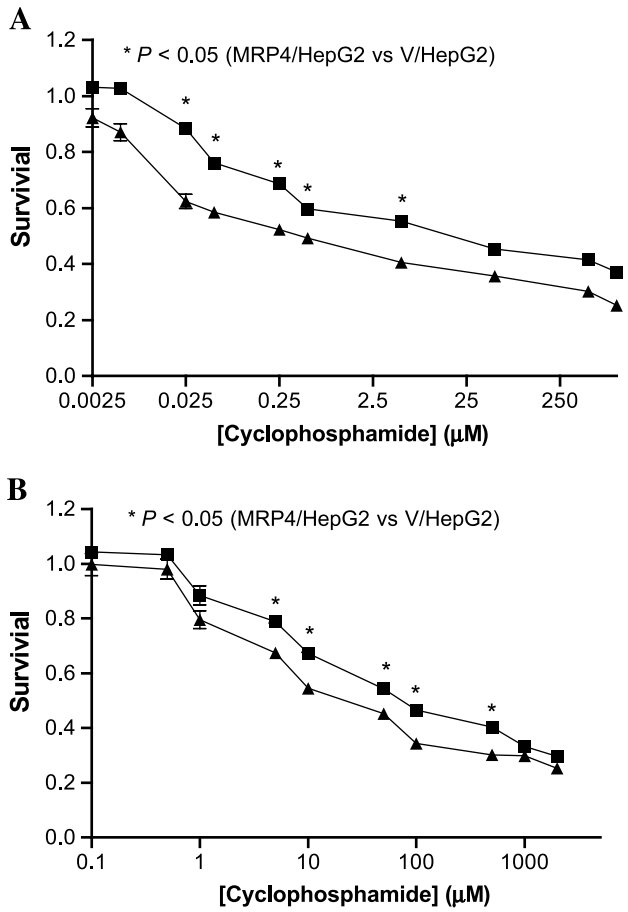


Fig. 5. Representative cytotoxicity profiles of cyclophosphamide when the cells were treated with the drug for 48 h (A) or 4 h (B) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Two-way ANOVA test was used. *$P < 0.05$.

Effect of BSO on MRP4-Mediated Resistance to CPT Analogs

The effects of the GSH synthesis inhibitor BSO on the cytotoxicity of CPT analogs in V/HepG2- and MRP4-expressing cells were investigated. The addition of BSO significantly decreased the IC_{50} values of CPT lactone (by 52.4 and 62.7% when the cells were exposed to CPT lactone for 48 and 4 h, respectively), CPT-11 lactone (by 44.0%, 4-h exposure assay), and SN-38 lactone (by 17.2 and 72.6% when the cells were exposed to SN-38 lactone for 48 and 4 h, respectively) in MRP4/HepG2 cells (Table II). BSO also significantly reduced the IC_{50} values of rubitecan (by 80.1 and 71.4% when the cells were exposed to rubitecan for 48 and 4 h, respectively) and 10-OH-CPT lactone (by 58.1 and 76.4% when the cells were exposed to 10-OH-CPT for 48 and 4 h, respectively) in HepG2 cell expressing MRP4. However, the presence of BSO reduced the cytotoxicity of CPT-11 lactone by 69.9%. The pretreatment of HepG2 cells expressing MRP4 with BSO for 24 h resulted in cytotoxicity profiles for CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT lactone, similar to those observed with V/HepG2 cells. These results indicate that BSO can partially reverse the MRP4-mediated resistance to CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT lactone.

In contrast, the presence of BSO significantly reduced the cytotoxicity of CPT and SN-38 lactone with respective increase of 25 to 259% in IC_{50} values in V/HepG2 cells when the cells were exposed to 10-OH-CPT for 4 and 48 h. This is consistent with the results with bis-POM-PMEA and MTX where reduced cytotoxicity was observed with the addition of BSO. BSO did not significantly affect the IC_{50} values of CPT-11, rubitecan, and 10-OH-CPT lactone when the cells were exposed to the CPT analog for 4 and 48 h. It seems that BSO had a confounding effect on the cytotoxicity of CPT and SN-38 lactone in V/HepG2 cells. The reduced cytotoxicity by BSO may be due to BSO-mediated functional modification of transporters other than MRP4 (e.g., MRP1-3) that play an important role in the efflux of CPTs.

Inhibition of MRP4-Mediated Resistance to CPT Analogs by Various Inhibitors

The effects of MK571, celecoxib, and diclofenac on the cytotoxicity of CPT-11, SN-38, and rubitecan lactone in V/HepG2 and MRP4/HepG2 cells were examined. Proincubation with celecoxib, diclofenac, or MK571 for 2 h significantly decreased the IC_{50} values of CPT-11, SN-38, and rubitecan lactone in MRP4/HepG2 cells (Table II). Celecoxib signifi-

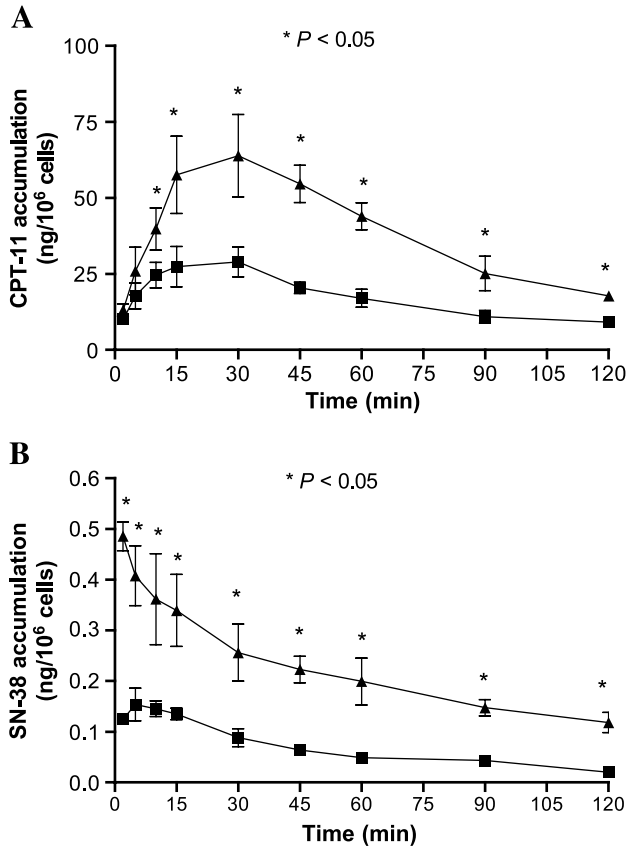


Fig. 6. The intracellular accumulation of CPT-11 (A) and SN-38 (B) lactone over 120 min in V/HepG2 (▲) and MRP4/HepG2 (■) cells. About 10^7 cells were exposed to 5 μM CPT-11 or 1 μM SN-38 lactone. At the indicated time, cells were rinsed with ice-cold PBS. Cells were then harvested, sonicated, and acidified. The concentrations of CPT-11 and SN-38 were determined by validated HPLC with fluorescence detection. The results are the mean of three independent experiments. Two-way ANOVA test was used. *$P < 0.05$.

cantly decreased the resistance fold by 76.9–78.0% for CPT-11 lactone, 52.9–57.0% for SN-38 lactone, and 75.9% for rubitecan lactone. Addition of diclofenac resulted in significantly decreased IC_{50} of CPT-11 lactone by 40.6–71.7%, SN-38 lactone by 50.6–56.2%, and rubitecan lactone by 59.9%. Furthermore, MK571 significantly reduced the IC_{50} of CPT-11 lactone by 53.2–73.5%, SN-38 lactone by 50.5–58.6%, and rubitecan lactone by 69.2%. By contrast, all inhibitors used had little effect on the cytotoxicity of CPT-11, SN-38, and rubitecan in V/HepG2 cells.

Resistance Profiles of Other Anticancer Drugs in Human MRP4

The cytotoxicity of a panel of other anticancer agents in MRP4/HepG2 and V/HepG2 cells was examined and the IC_{50} values are listed in Table III. MRP4 conferred 4.00- and 3.17-fold resistance to cyclophosphamide when the cells were exposed to cyclophosphamide for 48 and 4 h, respectively (Fig. 5). A two-way ANOVA indicated that the cytotoxicity of cyclophosphamide to V/HepG2 and MRP4/HepG2 cells was drug concentration dependent and there was a significantly different cytotoxic profile between MRP4/HepG2 and V/HepG2 cells when incubated with cyclophosphamide. Addition of BSO significantly decreased the IC_{50} of cyclophosphamide by 50.5 and 75.6% when the cells were exposed to cyclophosphamide for 48 and 4 h, respectively. However, MRP4 did not exhibit any significant resistance to other anticancer drugs including vinblastine, vincristine, etoposide, carboplatin, norcantharidin, 5-fluorouracil, and paclitaxel in cytotoxicity assays with 4 and 48 h drug exposure time, as indicated by similar IC_{50} values in both MRP4/HepG2 and V/HepG2 cells. A two-way ANOVA indicated that the cytotoxicity of these compounds to V/HepG2 and MRP4/HepG2 cells was drug concentration dependent, but there was an insignificantly different cytotoxic profile between MRP4/HepG2 and V/HepG2 cells. Moreover, there was no significant difference in the cytotoxicity of cyclosporine A, a known PgP substrate (51,52), in the two strains of cells (Table III).

Cellular Accumulation of CPT-11 and SN-38

The accumulation of CPT-11 and SN-38 lactone in MRP4/HepG2 and V/HepG2 cells was examined. As shown in Fig. 6A and B, the intracellular accumulation of CPT-11 and SN-38 lactone in MRP4/HepG2 cells over 120 min was significantly lower than in V/HepG2 for most time points ($P < 0.05$, by ANOVA). For CPT-11 lactone, the uptake by both V/HepG2 and MRP4/HepG2 cells achieved the maximum within 30 min, and then declined up to 120 min. MRP4/HepG2 cells accumulated 2- to 4-fold more CPT-11 than V/HepG2 cells. Notably, the uptake profile of SN-38 lactone was significantly different from that of CPT-11 lactone. Maximal SN-38 uptake was rapidly achieved within 2–5 min in MRP4/HepG2 and V/HepG2 cells; and then declined over the rest of the time. Overall, MRP4/HepG2 cells accumulated 2- to 4-fold lesser SN-38 than V/HepG2 cells.

The effects of preincubation with 200 μM BSO, 50 μM celecoxib, or 100 μM MK571 on the accumulation of CPT-11 and SN-38 lactone in both MRP4/HepG2 and V/HepG2 cells are shown in Fig. 7A and B. Pretreatment of the MRP4/HepG2 cells with 200 μM BSO for 24 h resulted in significantly ($P < 0.05$) increased accumulation of CPT-11 by 25.5% (control *vs.* treatment with BSO: 26.85 ± 1.13 *vs.* 39.30 ± 2.11 ng/10^6 cells) (Fig. 7A). Preincubation of MRP4/HepG2 cells with celecoxib (50 μM) or MK571 (100 μM) for 2 h also significantly increased the accumulation of CPT-11 lactone by 35.0% and SN-38 lactone by 38.1% ($P < 0.05$) (control *vs.* treatment with celecoxib or MK571: 23.44 ± 1.13 *vs.* 37.12 ± 3.24 or 38.33 ± 4.03 ng/10^6 cells). Similarly, preincubation of BSO, celecoxib, or MK571 significantly ($P < 0.05$) increased the accumulation of SN-38 lactone in MRP4/HepG2 cells by 29.5% (control *vs.* treatment: 0.150 ± 0.012 *vs.* 0.195 ± 0.033 ng/10^6 cells), 35.0% (control *vs.* treatment: 0.146 ± 0.024 *vs.* 0.197 ± 0.032 ng/10^6 cells), and 71.1% (control *vs.* treatment: 0.146 ± 0.024 *vs.* 0.250 ± 0.041 ng/10^6

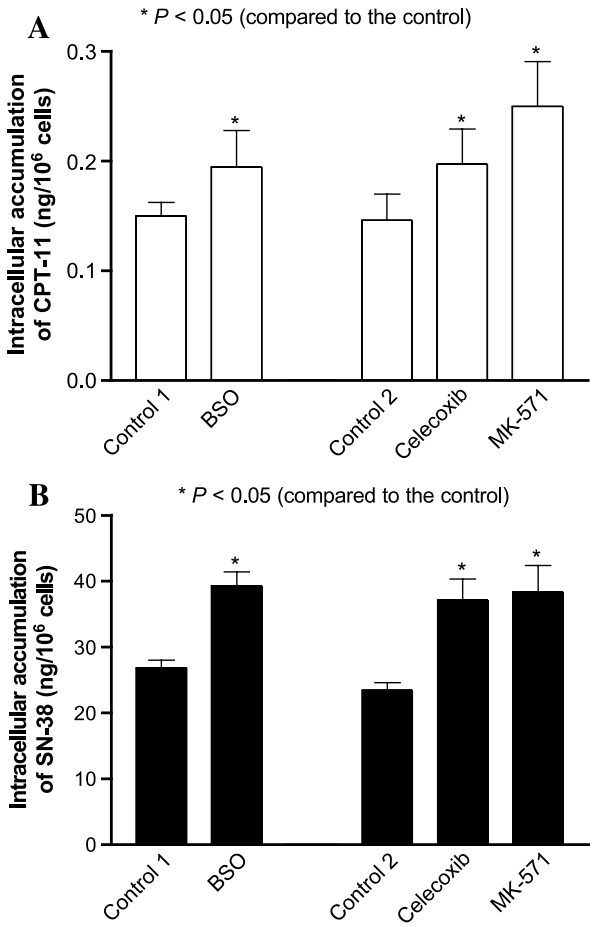


Fig. 7. Effects of preincubation of MRP4/HepG2 cells with DL-buthionine-(*S*,*R*)-sulfoximine (BSO) at 200 μM, celecoxib at 50 μM, or MK571 at 100 μM on the accumulation of CPT-11 (A) and SN-38 (B) lactone. Both celecoxib and MK571 were prepared by dissolving in DMSO and diluted by PBS, whereas BSO was dissolved in sterile Milli-Q water. Celecoxib or MK571 was preincubated with cells for 2 h, whereas BSO was preincubated for 24 h. Thereafter, cells were washed four times with warm PBS buffer. After continued incubation for 2 min for SN-38-treated cells and 30 min for CPT-11-treated cells, cells were washed five times with warm PBS. The cells were then harvested, lysed by sonication, and extracted using ice-cold acetonitrile/methanol mixture. The supernatant was injected into HPLC for the determination of CPT-11 and SN-38. The results are the mean of three independent experiments. Two-way ANOVA test was used. *$P < 0.05$.

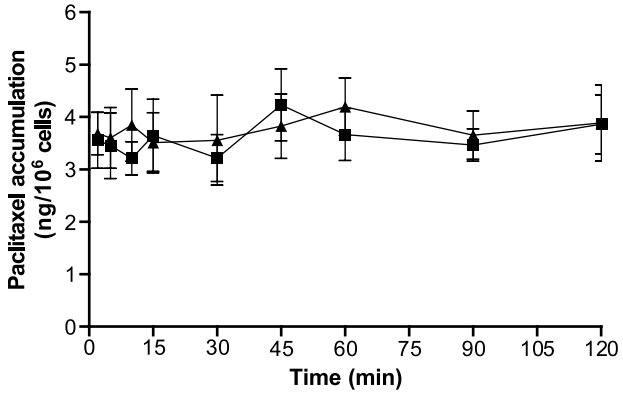


Fig. 8. The intracellular accumulation of paclitaxel over 120 min in V/HepG2 (▲) and MRP4/HepG2 (■) cells. About 10^7 cells were exposed to 0.05 μM paclitaxel. At the indicated time, cells were rinsed with ice-cold PBS. Cells were then harvested, sonicated, and acidified. The concentration of paclitaxel was determined by validated HPLC with UV detection. The results are the mean of three independent experiments. Two-way ANOVA test was used for comparison.

cells), respectively (Fig. 7B). Notably, although the resistance was significantly overcome in the presence of various MRP4 inhibitors tested, the cellular accumulation in MRP4/HepG2 cells in the presence of MRP4 inhibitors was still lesser than that by control V/HepG2 cells. Such discrepancy may be because the intracellular accumulation of CPT-11 and SN-38 was a more complicated process than the resistance observed. Resistance phenotype reflects reduced direct cell-killing effect of drugs that bind to the targets (Top1), whereas intracellular accumulation associated with cytotoxic effect is mainly determined by passive and active transport of the drug, subcellular distribution and compartmentation, metabolism (e.g., glucuronidation of SN-38 and hydrolysis of CPT-11), and binding to organelles.

However, preincubation of either celecoxib, BSO, or MK571 had little effect on CPT-11 and SN-38 accumulation in V/HepG2 cells (data not shown), which may provide an explanation for the negligible effect of celecoxib, BSO, and MK571 on the cytotoxicity of CPT-11 and SN-38 in these cells. These findings also indicated that MRP4 as well as MRP1-3 did not participate in the cellular uptake of CPT-11 and SN-38 in V/HepG2 cells because MK571 is a known inhibitor for MRP1, MRP2, MRP3, and MRP4 (32,33).

Cellular Accumulation of Paclitaxel

To examine whether low-level expression of PgP influences the transport of CPT-11 and SN-38 in MRP4/HepG2 and V/HepG2 cells, the accumulation of paclitaxel, a known PgP substrate (53,54), was investigated. There was no significant difference in intracellular accumulation of paclitaxel in V/HepG2 and MRP4/HepG2 cells over 120 min ($P > 0.05$, by two-way ANOVA) (Fig. 8). Paclitaxel seemed to achieve saturable accumulation in both V/HepG2 and MRP4/HepG2 cells within 2 to 5 min and the accumulated drug was 3.2–4.2 ng/10^6 cells over 120 min. In addition, similar IC_{50} values were observed with paclitaxel in the V/HepG2 and MRP4/HepG2 cells (IC_{50}: 3.548 ± 0.044 *vs.* 3.753 ± 0.091 nM for the MTT assay with 48 h drug exposure time; 21.765 ± 1.274 *vs.* 21.266 ± 0.563 nM for the MTT assay with 4 h drug exposure time).

DISCUSSION

In this study, we checked for the function of established cells using bis-POM-PMEA and MTX, which are known substrates for MPR4 (47,48). bis-POM-PMEA was chosen in this study as it first spontaneously hydrolyzed to mono-POM-PMEA (55), which is then rapidly converted to PMEA by cellular esterases (56). The established HepG2 cell expressing human MRP4 conferred significant resistance to bis-POM-PMEA when the cells were treated with bis-POM-PMEA for 4 and 48 h and the presence of the GSH synthesis inhibitor BSO partially reversed the resistance in the present study (Table I and Fig. 2). The intracellular conversion efficiency of bis-POM-PMEA *in vitro* to PMEA is on the order of 80−90% for initial substrate concentrations of 0.125−1.0 μM (57,58). Thus, the observed cytotoxicity for bis-POM-PMEA is due to the action of PMEA. Because PMEA does not seem to be a substrate for PgP or BCRP (59), nor does it interact with typical substrates for MRP1–3 (47,60), the observed resistance to bis-POM-PMEA by MRP4/HepG2 cells is thus mediated by MRP4. As to MTX, MRP4 displayed resistance that was reversed by BSO when the drug exposure time was 4 h only, but resistance was not observed when the drug exposure time was prolonged to 48 h (Table I and Fig. 2). This is consistent with the reported results for these two compounds obtained from other cells with overexpression of MRP4 (34,47,48,61). Although MRP4 shows resistance to MTX, the magnitude is lower than that of MRP1-3, whose resistance levels are reported to be 21- to 78-fold (34,62). MTX is transported by MRP1-4 with comparable affinities. Together with another study using the same cellular model where MRP4/HepG2 demonstrated significant resistance to PMEA and 6-thioguanine and transported GSH efficiently (38), our results indicate the validity of the HepG2 cells expressing human MRP4 as a model in the study of potential resistance to camptothecin analogs.

Camptothecins clearly represent one important group of anticancer drugs developed in the last few decades. A wealth of information has become available that has yielded valuable insight into their mechanism of action, pharmacokinetics, toxicities, and tumor resistance. Resistance to camptothecins is a major clinical problem often resulting in therapeutic failure. Detailed investigations aimed at identification of resistant proteins and circumventing approaches of intrinsic drug resistance are thus warranted. The present study provided solid evidence that MRP4 conferred significant resistance to various CPTs including CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT in both lactone and carboxylate forms using the validated cellular model, HepG2, with stable overexpression of MRP4 (Table II, Figs. 3 and 4). The addition of BSO, a GSH synthesis inhibitor, significantly reversed MRP4-mediated resistance to these CPTs. This indicated that GSH played an important role in MRP4-mediated efflux of CPTs. Other inhibitors, including diclofenac, celecoxib, and MK571, also significantly reduced the resistance of MRP4 to CPT-11 and SN-38.

Of the tested Top1 inhibitors, CPT-11 and SN-38 are clearly substrates for MRP4 transporter. This is supported by the following: (a) MRP4/HepG2 cells had lesser sensitivity to CPT-11 and SN-38 in lactone or carboxylate form than V/HepG2 cells; (b) MRP4-mediated resistance to CPT-11 and SN-38 was inhibited by BSO; (c) MRP4-mediated resistance to CPT-11 and SN-38 was inhibited by diclofenac, celecoxib, and MK571; (d) CPT-11 and SN-38 in MRP4/HepG2 cells had lesser accumulation than V/HepG2 cells (Fig. 6); and (e) the accumulation of CPT-11 and SN-38 in MRP4/HepG2 cells was significantly increased with preincubation of celecoxib, BSO, or MK571 (Fig. 7). Other members of CPTs including CPT, rubitecan, and 10-OH-CPT are also highly possible substrates for MRP4. The differential resistance folds for various CPTs tested in this study are mainly due to different lipophilicity, transport across cell membrane, intracellular accumulation, and binding affinity to MRP4. Estimated from the resistance folds for the MTT assay with 48 h drug exposure time of the test CPTs (Table II), the substrate affinity of MRP4 seems to decrease from 10-OH-CPT (14.21) > SN-38 carboxylate (9.70) > rubitecan (9.06) > SN-38 lactone (8.91) > CPT lactone (7.33) > CPT-11 lactone (5.64) > CPT carboxylate (4.30) > CPT-11 carboxylate (2.68). 10-OH-CPT seems to have the highest affinity to MRP4, whereas CPT-11 carboxylate might have the lowest affinity. These observations may have important implications in the further development of CPTs and optimization of chemotherapy for cancer patients when new CPT analogs without binding affinity to MRP4 are synthesized.

The resistance magnitude of MRP4 to CPT, CPT-11, and SN-38 in lactone and carboxylate form is different (Table II). Thus, MRP4 is considered to have different affinity and transport capacity to their lactone and carboxylate forms. This may have important implications in the transport and disposition of these camptothecins given that CPTs undergo rapid interconversion *in vivo*. The half-life of CPT, CPT-11, and SN-38 lactone varies from 29 to 32 min at pH 7.3, and the equilibrium lactone content is from 15% to 23% (63). For SN-38, both lactone and carboxylate forms exist considerably *in vivo*, with the lactone accounting for 54−64% of the total area under the plasma concentration *vs.* time curve (64,65). The lactone and carboxylate of CPTs possess different affinities for drug transporters. CPT-11 and SN-38 lactone were both passively transported, with significantly more rapidly taken up than their carboxylate forms in HT29 cells, whereas their respective carboxylate forms were primarily transported via an ATP-dependent mechanism (66). The intestinal uptake of CPT-11 and SN-38 lactone is about ten times greater than that of the carboxylate form (67). Active uptake of the CPT-11 carboxylate instead of lactone was also observed in KB-C2 membrane vesicles (68). Mrp2 is responsible for the biliary excretion of the carboxylate forms of CPT-11 and SN-38 as well as the lactone and carboxylate forms of SN-38 glucuronide in rats (24,69). These findings indicate that the uptake rate of the non-ionic form (lactone) of CPTs is higher than that of the respective anionic form (carboxylate) and thus the pH is one of the determining factors affecting the uptake rate of CPTs. Furthermore, the lactone and carboxylate of CPTs exhibit differences in pharmacokinetics in animals and humans. SN-38 lactone has a greater volume of distribution and binds with much higher affinity to albumin when compared with SN-38 carboxylate in rats and cancer patients (70–72). In Eisai hyperbilirubinemic rats deficient in Mrp2, the plasma clearance of SN-38 carboxylate biliary clearance was reduced, whereas it remained unchanged for the corresponding lactone form (69). CPT-11 lactone is metabolized to SN-38 at a greater rate than its carboxylate form (73), and SN-38 lactone is glucuronidated up to 6-fold greater than the carboxylate in human liver microsomes and recombinant UGT isoenzymes (74). Furthermore, the tumor inhibitory activity of the lactone form of CPTs is significantly greater than the carboxylate form (4,5,11). Thus, the lactone and carboxylate form of CPTs can be considered two pharmacokinetically and pharmacodynamically distinct compounds and further studies are warranted for the differences in their interaction with drug transporters including PgP, BCRP, and MRPs.

All members of the ABC transporter family use ATP to translocate substrates. Efficient transport of some substrates by several of the MRP protein family members, such as MRP1, MRP2, MRP4, and MRP5 also requires physiological concentrations of the antioxidant GSH, which is cotransported with another substrate (38,50,58). Similarly, in MRP4-overexpressing HepG2 cells used in the present study, GSH depletion by BSO (200 μM) significantly reversed the resistance to bis-POM-PMEA, MTX, and CPTs (Tables I and II). When a high concentration of BSO (500 μM) was used, MRP4-transfected HepG2 cells showed altered drug resistance in the presence of 200−400 μM PMEA (38). However, GSH does not seem to play a significant role in PMEA-mediated efflux in MRP4-overexpressing HEK293 (75) cells or rat microglial MLS-8 cell (48). The reasons for such discrepancies in effects of GSH are unclear, but the differences in cell lines used, intracellular GSH levels, and MRP4 and other transporter levels may be involved.

There is a possibility that other transporters instead of MRP4 play a role in the resistance to CPTs observed. However, PgP is excluded as a transporter for CPT resistance in this study by the fact that there was no difference in the IC_{50} and accumulation of paclitaxel (a known PgP substrate) in MRP4/HepG2 and V/HepG2 cells (Table III and Fig. 8). This is further supported by the similar IC_{50} values in the two strains of cells with cyclosporine, a known PgP substrate (51). Furthermore, our study did not find resistance of MRP4 to vincristine, vinblastine, and etoposide (Table III), which are typical substrates of PgP and MRP1–3 (32,76). It has been reported that MRP4 does not interact with typical substrates of MRP1–3, including vincristine, etoposide, daunorubicin, and cisplatin (47,60). Thus, the resistance to CPTs observed in this study is mediated by MRP4, which is functionally distinct from other transporter proteins.

Targeting Top1 to kill tumor cells requires high enough intracellular accumulation of the active lactone form of CPTs (77). The mechanism for the uptake of CPT-11 and SN-38 by HepG2 cells is unknown, but both active and passive transport are implicated. CPT-11 and SN-38 can be readily taken up by human intestinal Caco-2 cells through passive diffusion (78), whereas the influx of topotecan and SN-38 by ovarian tumor cells requires active transporters and disrupted influx results in drug resistance (79). To date, CPTs have been shown to be substrates for various including PgP, BCRP, OATP1B1, MRP1, and MRP2 (see Fig. 9). The differential accumulation

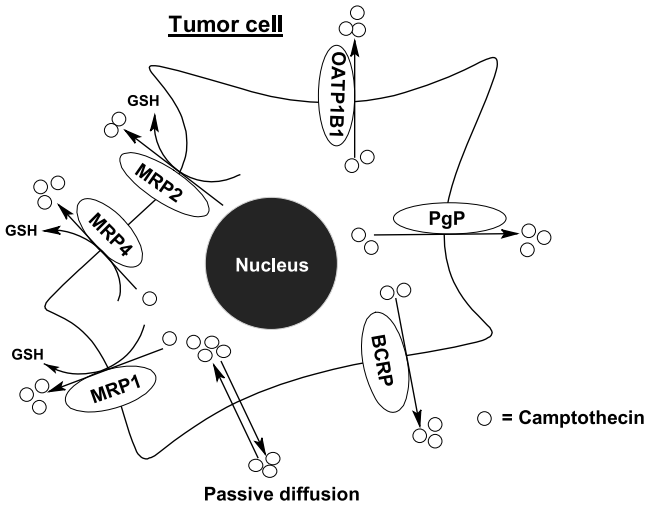


Fig. 9. A proposed schematic diagram showing multiple transporters involved in the efflux of camptothecins (CPTs) from tumor cells. In addition to passive diffusion, active efflux of CPT molecules from tumor cells occurs by glutathione (GSH)-independent transporters including P-glycoprotein (PgP) and the breast cancer resistance protein (BCRP/MXR) and GSH-dependent transporters such as MRP1, MRP2, and MRP4. Efflux of CPTs by MRP1, MRP2, and MRP4 requires GSH cotransport. The organic anion transporter OATP1B1 may efflux CPTs in a GSH-independent manner. However, little is known about the mechanisms for the uptake of CPTs by these transporters. It seems that adequate cellular accumulation of CPTs is crucial for killing tumor cells, whereas cellular resistance to CPTs occurs when efflux is greater than influx.

of CPT-11 and SN-38 in MRP4/Hepg2 and V/Hepg2 cells (Fig. 6) provides partial explanation for the different cytotoxicity observed. Nevertheless, the differential accumulation cannot be attributable to damage of the plasma membrane, which in turn could increase drug influx. The tumor cells remained viable during drug accumulation studies over 120 min as measured using trypan blue exclusion. Interestingly, the accumulation profiles of CPT-11 are different from those of SN-38. It seems that CPT-11 enters tumor cells at a moderate rate, and are then distributed within cells and bound by subcellular organelles, drug-metabolizing enzymes located in the endoplasm reticulum and drug target in the nucleus (Top1). The organelles may represent a store of active drug. The drug is finally cleared from the cells by metabolism and transporter-mediated efflux. However, SN-38 seems to enter cells more rapidly than CPT-11 and is then removed from the cells just as rapidly. This may be due mainly to the higher lipophilicity of SN-38 than CPT-11. Differential lipophilicity can result in different uptake, subcellular compartmentation, metabolism, and efflux of the two drugs. For example, the water-soluble topotecan is mainly localized in mitochondria when incubated with HT-29 cells, whereas gimatecan (ST1481, a water-insoluble CPT analog) is mainly distributed into lysosomes (80). Furthermore, different uptake rates and extent of CPT-11 and SN-38 have been observed in intestinal and lung cancer cells (68,81). Moreover, metabolism is considered an important determinant for the cellular accumulation of CPT-11 and SN-38. CPT-11 is hydrolyzed by cellular carboxylesterases, whereas SN-38 is readily conjugated by UGT1A1/1A9, which is associated with increased efflux of the drug from HT29 and HCT116 cells (82).

MRP4-mediated resistance to CPTs and identification of CPTs as MRP4 substrates have important clinical implications. First, MRP4 enhances the ability of tumor cells to efflux CPTs out of cells to reduce the cellular drug concentration leading to drug resistance. Thus, MRP4 expression level in tumor cells may serve as an important determinant for the antitumor efficacy. Although caution should be taken when extrapolating these *in vitro* results of CPT resistance to the tumor *in vivo*, MRP4 expression seems to be one important factor that impinges on the efficacy of CPTs in specific tumors. Recently, it was found that the expression level of MRP4, instead of MRP1-3 or PgP, was associated with poor prognosis in patients with neuroblastoma (28). Second, the inhibition studies can help to identify potential MRP4 inhibitors useful in clinical chemotherapy. These MRP4 modifiers may be combined with CPTs in tumors in which MRP4 is frequently overexpressed. Celecoxib enhanced the antitumor activity of CPT-11 in nude mice bearing HT-29 or colon-26 tumor (83) and modulation of MRP4-mediated efflux is one of the possible mechanisms. Third, modulation of MRP4 may have important pharmacokinetic implications for CPTs. MRP4 is expressed at low levels in most organs such as liver, gut, lung, and brain, but substantial MRP4 is detected in the kidneys and prostate (84). Its expression is subject to induction and inhibition of a number of compounds. A recent study in infected human macrophages indicates that azidiothymidine (AZT) treatment induces MRP4 mRNA (85). Thus, the pharmacokinetics of CPTs may be changed and drug interactions may occur due to altered MRP4 expression and activity. Finally, MRP4 seems to act as a protective barrier in the brain; MRP4 alteration may affect the distribution of their substrates including CPTs, thus altering therapeutics or toxicology. *Mrp4*-deficient mice had enhanced accumulation of topotecan in brain tissue and cerebrospinal fluid (39). On the other hand, modulation of MRP4 in the blood–brain barrier may facilitate the management of diseases of the central nervous system by enhancing penetration of drugs into the brain. Such MRP4-based barrier may be circumvented by targeted site-specific drug delivery systems using immunoliposomes and nanoparticles. MRP4 may also play a role in the gastrointestinal and hematologic toxicities of CPTs. With the accumulation of information on the drug-resistance profile and physiological function of MRP4, the relationship between drug selectivity and MRP4 level will be significant and helpful in clinical cancer management using CPTs and development of novel CPT analogs that overcome MRP4 overexpression.

Notably, MRP4 conferred resistance to cyclophosphamide (Table III and Fig. 5), an alkylating agent, and this was partially reserved by BSO. Cyclophosphamide is a non-multidrug-resistant cytotoxic drug and it is usually used as part of combination chemotherapy protocols. It is classified as nitrogen mustard and requires metabolic activation by cytochrome P450s. The toxic metabolite of cyclophosphamide, acrolein, was found to completely reverse the MRP1-mediated daunorubicin and vinblastine accumulation deficit *in vitro*, which was ascribed to GSH depletion (86). MRP1 can transport the conjugates of cyclophosphamide, melphalan and chlorambucil (32,33). Thus, cellular GSH level, MRP1, and MRP4 may serve as determinants of the antitumor activity of cyclophos-

phamide. MRP4-mediated resistance to cyclophosphamide may have important clinical implications in alkylating agent resistance. Further studies are required to examine the interaction of cyclophosphamide with MRP4 and other MRPs.

Overall, this *in vitro* study is significantly different from that by Norris *et al.* (28), wherein overexpression of MRP4 was found to increase the IC_{50} of CPT-11 and SN-38 by 5.9- to 6.0-fold and MRP4 was overexpressed in neuroblastoma. The study by Norris *et al.* (28) did not examine the relationship between increased resistance with decreased intracellular accumulation of CPT-11 and SN-38 and the effects of various MRP4 inhibitors including BSO, celecoxib, and MK571 on the cytotoxicity and accumulation of CPT-11 and SN-38. However, our study has made several important new findings: (a) the increased resistance to CPT-11 and SN-38 was associated with decreased intracellular accumulation; (b) a panel of camptothecins including CPT, 10-OH-CPT, and rubitecan in addition to CPT-11 and SN-38 were studied; (c) both lactone and carboxylate forms of all CPT analogs tested were checked for their cytotoxicity and significant differences were observed between the two forms; (d) the established cellular model with stable MRP4 overexpression (MRP4/HepG2) was well validated using known MRP4 substrates such as MTX and known PgP substrate (paclitaxel); (e) a number of other anticancer agents (e.g., cyclophosphamide, vinblastine, vincristine, etoposide, carboplatin, norcantharidin, 5-fluorouracil, and paclitaxel) were also included in this study; and (f) we found that MRP4 conferred resistance to cyclophosphamide, probably providing new insight into the mechanism for resistance to oxazaphosphorine anticancer drugs.

In summary, the findings from this study indicate that MRP4 overexpression conferred significant resistance to cyclophosphamide, CPT, CPT-11, SN-38, rubitecan, and 10-OH-CPT, and that CPT-11 and SN-38 are substrates for MRP4. Further studies are needed to explore the role of MRP4 expression in cancer chemotherapy involving camptothecin analogs and oxazaphosphorines in patients. Elucidation of the role of MRP4 together with other relevant transporters in the pharmacokinetic and pharmacodynamic behavior of CPTs and oxazaphosphorines such as cyclophosphamide will allow rational optimization of cancer chemotherapy and further development of novel CPTs and oxazaphosphorines.

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