INTERACTIONS OF HUMAN MULTIDRUG RESISTANCE PROTEIN 4 WITH CAMPTOTHECINS

TIAN QUAN

(*MSc, Shenyang Pharmaceutical University, P.R. China*)

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List of Papers and Conference Proceedings

Papers

1. **Tian Q**, Zhang J, Chan SY, Tan TM, Duan W, Huang M, Zhu YZ, Chan E, Yu Q, Nie YQ, Ho PC, Li Q, Ng LK, Yang HY, Hong W, Bian JS, and Zhou SF. Topotecan is a substrate for multidrug resistance associated protein 4. *Curr Drug Metab* 2006; **7(1)**: 105-118.
2. **Tian Q**, Zhang J, Tan TM, Chan E, Duan W, Chan SY, Boelsterli UA, Ho PC, Yang H, Bian JS, Huang M, Zhu YZ, Xiong WP, Li XT, and Zhou SF. Human multidrug resistance associated protein 4 confers resistance to camptothecins. *Pharm Res* 2005; **22(11)**: 1837-1853.
3. **Tian Q**, Zhang J, Chan E, Duan W, and Zhou SF. Multidrug resistance proteins (MRPs) and implication in drug development. *Drug Dev Res* 2005; **51(2)**:1-18.
4. Zhang J, **Tian Q**, Zhu YZ, Xu AL, and Zhou SF. Reversal of resistance to oxazaphosphorines. *Curr Cancer Drug Targets*. 2006; **6(5)**:385-407.
5. Zhang J, **Tian Q**, and Zhou SF. Clinical pharmacology of cyclophosphamide and ifosfamide. *Curr Drug Ther* 2006; **1(1)**:104-168.
6. Zhang J, **Tian Q**, Chan SY, Chuen Li S, Zhou SF, Duan W, and Zhu YZ. Metabolism and transport of oxazaphosphorines and the clinical implications. *Drug Metab Rev* 2005; **37 (4)**:611-703.
7. Zhang J, **Tian Q**, Chan SY, Duan W, and Zhou SF. Insights into oxazaphosphorine resistance and possible approaches to its circumvention. *Drug Resist Update* 2005; **8(5)**: 271-297.

Conference Abstracts

1. **Tian Q**, Zhang J, Tan MQ, Chan E, Chan SY, and Zhou SF. Resistance profiles of camptothecins in HepG2 cells with overexpression of MRP4. 17th Singapore Pharmacy Congress, 30 June-3 July 2005, Singapore.
2. **Tian Q**, Tan MQ, Chan E, Hung Y, Lim LY, and Zhou SF. Sensitivity of camptothecins in HepG2 cells overexpressing multidrug resistance associated protein 4. 3rd International Conference on Structural Biology and Functional Genomics, 2-4 December 2004, Singapore.
3. **Tian Q**, Tan MQ, Chan E, Hung Y, Lim LY, and Zhou SF. Resistance profile of CPTs in multidrug resistance associated protein 4. 5th HUGO Pacific Meeting & 6th Asia-Pacific Conference on Human Genetics, 17-20 November 2004, Singapore.
4. **Tian Q**, Tan MQ, and Zhou SF. Overexpression of multidrug resistance associated protein 4 (MRP4) confers resistance to camptothecins in HepG2 cells. The 3rd Pacific Anti-ageing Conference and Exhibition, 24-27 June 2004, Singapore.
5. **Tian Q**, Tan MQ, and Zhou SF. Overexpression of MRP4 confers resistance to camptothecins in HepG2. HSA-NUS Joint Scientific Symposium, 19 May 2004, Singapore.
6. Tan MC, Yang F, Bai J, Zhang J, **Tian Q**, and Zhou SF. The aromatic residues Trp230, Trp995 and Phe324 contribute to the MRP4-mediated transport and drug resistance. The FEBS special meeting-ATP binding cassette proteins: from multidrug resistance to genetic diseases, Innsbruck, 4-10 March 2006, Austria.

Table of Contents

Summary

The multidrug resistance protein 4 (MRP4) is a member of the MRP subfamily, which in turn belongs to the ATP-binding cassette (ABC) transporter superfamily. MRP4 has specific tissue distribution, substrate and inhibitor specificity. MRP4 is capable of transporting physiological and endogenous compounds, as well as anticancer agents. As a MRP4 substrate, topotecan belongs to a group of compounds known as camptothecins (CPTs) which have shown substantial anticancer activity by inhibiting DNA topoisomerase I, but the success of CPT-related treatment is often limited by tumor resistance. Some ABC transporters, including P-glycoprotein (PgP), breast cancer resistance protein (BCRP), MRP1 and MRP2, have been implicated in resistance to various CPT analogs. Emerging evidence has also shown that MRP4 may be involved with CPTs. Our interest is thus to elucidate more clearly the role of MRP4 in CPTs resistance. The overall hypothesis tested is that CPTs act as substrates of MRP4 and interact with MRP4 on cellular levels to mediate multidrug resistance in cancer. In this thesis, we aimed to investigate the interaction between MRP4 and CPTs by identifying whether CPTs were substrates for MRP4 and exploring their effects on MRP4 function. We examined the cytotoxicity profiles of a panel of CPTs including CPT, CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT in HepG2 cells overexpressing human MRP4 (MRP4/HepG2) and empty vector (V/HepG2). We found that the cytotoxicity of CPTs in V/HepG2 was much higher than that in MRP4/HepG2 cells, suggesting that MRP4 overexpression conferred significant resistance to CPTs. The resistance to CPTs by MRP4 was significantly reversed by the glutathione (GSH) synthesis inhibitor buthionine sulfoximine (BSO), and the MRP4 inhibitors including MK571, celecoxib, and diclofenac. However, MRP4 did not display resistance to selected anticancer drugs in cytotoxicity assays, except

cyclophosphamide. The intracellular accumulations of CPT analogs were further investigated. The intracellular concentrations of CPT analogs (except 10-OH-CPT) tested in MRP4/HepG2 were significantly lower than in V/HepG2 in both time-dependent and concentration-dependent accumulation assays, and these concentrations could be effectively increased by inhibitors that reversed drug resistance in our cytotoxicity assays. In addition, various single amino acid changes were introduced into MRP4 and examined for their influence in the intracellular accumulation of CPT-11 and SN-38. Our findings strongly suggested that MRP4 overexpression is involved in the resistance to CPTs by significantly decreasing the cellular accumulation. Since MRP4 transports a number of its substrates in a GSH-dependent manner, effects of CPT analogs on GSH efflux were investigated. Among CPT analogs tested, only CPT-11 at a high concentration of 10 μM significantly inhibited GSH efflux in MRP4/HepG2. Moreover, BSO showed a specific inhibitory effect on the MRP4-mediated GSH efflux. The possible involvement of some related ABC transporters (PgP, MRP1, MRP2, MRP3, MRP5 and BCRP) in GSH efflux and resistance to CPTs was excluded by their undetectable expression level in western blot assays. The resistance to CPTs in cytotoxicity assays, differences in drug uptake and GSH efflux can be mainly attributed to a high expression level of MRP4. Our findings are significant and may be useful in designing novel non-cross resistant CPT-related drugs.

List of Tables

List of Figures

List of Abbreviations

ABC transporters	ATP-binding cassette transporters
9-AC	9-aminocamptothecin, 9-NH_2-CPT,
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AIDS	acquired immune deficiency syndrome
APC	7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin
AUC	area under the plasma concentration-time curve
AZT	azidiothymidine
AZT-MP	azidiothymidine monophosphate
BCRP	breast cancer resistance protein
bimane-GS	bimane-glutathione
bis-POM-PMEA	bis(pivaloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine, adefovir dipivoxil
BSO	DL-buthionine-(*S*,*R*)-sulfoximine
cAMP	cyclic adenosine monophosphate
CCD	charged coupling device
CDNB	1-chloro-2,4-dinitrobenzene
CEs	carboxylestesterases
cGMP	cyclic guanosine monophosphate
CMMDC	7-chloromethyl-10,11-methylenedioxy-20(S)-camptothecin
cMOAT	canalicular multispecific organic anion transporter
cMVs	canalicular membrane vesicles
CPT(s)	camptothecin(s)
CPT-11	irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin
CYP3A	cytochrome P450 isoform 3A

DHEAS	dehydroepiandrosterone 3-sulfate
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
$E_2$17βG	estradiol 17-β-D-glucuronide
FA	folic acid
FDA	Food and Drug Administration
FPGS	folylpolyglutamate synthetase
GCV	ganciclovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanine
GSH	glutathione
GST	glutathione S-transferase
HBSS	Hanks balanced salt solution
HIV-1	human immunodeficiency virus type 1
HPLC	high-performance liquid chromatography
HSA	human serum albumin
HSV-TK	herpes simplex virus thymidine kinase
MCB	monochlorobimane
MCB-GS	monochlorobimane glutathione
MDR	multidrug resistance
MK571	(E)-3-((3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)(3-(dimethylamino)-3-oxopropylthio)methylthio)propanoic acid
6-MP	6-mercaptopurine
MRP(s)	multidrug resistance protein(s)
MSDs	membrane spanning domains
MTD	maximum tolerated dose
MTX	methotrexate
MX	mitoxantrone

MXR	mitoxantrone resistance
NBDs	nucleotide binding domains
NBMPR	nitrobenzylmercaptopurine ribonucleoside
NPC	7-ethyl-10-[4-(1-piperidino)-1-amino-carbonyloxycamptothecin
OATP	organic anion transporting polypeptide
10-OH-CPT	10-hydroxycamptothecin, HCPT
9-OH-CPT-G	glucuronide of 9-hydroxy-CPT
PAK-104P	2-[4-Diphenylmethyl)-1-piperazinyl]ethyl-5-(trans-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6- dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P-oxide
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PGEs	prostaglandins
PgP	P-glycoprotein
PK	protein kinase
PMEA	9-(2-phosphonylmethoxyethyl)adenine, adefovir
RBT	rubitecan, 9-NO_2-CPT, 9-nitrocamptothecin
RFC	reduced folate carrier
SDS	sodium dodecyl sulfate
SLDR	sublethal damage recovery
SN-38	7-ethyl-10-hydroxycampotothecin
SN-38G	the β-glucuronide conjugate of SN-38, 7-ethyl-10-3,4,5-trihydroxy-pyran-2-carboxylic acid]camptothecin
SNPs	single nucleotide polymorphisms
6-TG	6-thioguanine
TM	transmembrane
TMDs	transmembrane domains
Top 1	topoisomerase I

TPT	topotecan, hycamtin, 9-dimethylaminomethyl-10-hydroxycamptothecin
TPT-G	topotecan-*O*-glucuronide
TXB_2	thromboxane B2
UGT	uridine diphosphate glucuronosyltransferanse

CHAPTER 1 INTRODUCTION

Cancer remains the main cause of death for both men and women worldwide. To date, the major modalities for treating cancer include surgery, radiation, chemotherapy and immunotherapy (Gibbs, 2000). More often than not, these therapies provide a cure only when the cancer is detected at an early stage, or applied to certain types of cancer (e.g. leukemia). The target of chemotherapy is to kill or disable tumor cells by direct or indirect mechanisms, while preserving the normal cells (Fidler and Ellis, 2000). However, chemotherapeutic agents generally have a narrow margin of safety, and are usually given at a maximum tolerated dose (MTD) to achieve maximum cancer cell killing. Their mechanisms of actions vary and may include direct cytotoxicity, activation of host immune responses, inhibition of the proliferation processes of tumor cells, and/or induction of apoptosis (Gibbs, 2000).

Unfortunately, the success of chemotherapy is often impeded by multidrug resistance (MDR). Tumor cells can acquire MDR *via* a number of factors associated with the tumor, host and drug. Host factors include sanctuary sites for tumors, lack of bioactivation and/or dose-limiting normal tissue toxicity, leading to inadequate tumor cell exposure to chemotherapeutic agents. Specific conditions may produce sanctuary sites, which are areas where the tumor is inaccessible to anticancer drugs and/or the drug accumulation over time is insufficient to kill cells (Tan et al., 2000). Tumor-related (cellular) factors, on the other hand, include defective drug transport (reduced drug transport or increased drug efflux), altered drug activation or inactivation, and/or enhanced repair or tolerance to DNA damage (Gottesman et al., 2002). In this respect, it has been well-established that ATP-binding cassette (ABC) transporters play an important part in mediating MDR by decreasing intracellular drug concentrations

through transporting substrates across cell membranes using the energy of ATP-binding/hydrolysis (Haimeur et al., 2004). Their increased expression in tumor cells has been well associated with resistance to a wide number of important chemotherapeutic agents. Besides, these transporters play an important role in the absorption, disposition and elimination of chemotherapeutic agents in the body by affecting the penetration and transmembrane transport of endogenous compounds and their metabolites. The P-glycoprotein (PgP), which is a 170 kDa plasma glycoprotein encoded by the human *MDR1* gene (Ambudkar et al., 1999; Borst and Elferink, 2002; Schinkel and Jonker, 2003; Marzolini et al., 2004), was the first of such transporters to be discovered. PgP can cause considerable resistance to a number of anticancer drugs, including paclitaxel, anthracyclines (*e.g.* daunorubicin and doxorubicin), camptothecins, and vinca alkaloids (vincristine and vinblastine) (Zaman et al., 1994; Zhu et al., 1997; Loe et al., 1998). With an increased understanding of the function of efflux transporters, the multidrug resistance protein (MRP) family was next identified (Haimeur et al., 2004), with the breast cancer resistance protein (BCRP) being the most recently identified (Allikmets et al., 1998). The focus of this thesis is, however, on the fourth member of the MRP family, MRP4, which has specific tissue distribution and substrate selectivity, and we will describe this transporter in detail in the following section.

1.1. MULTIDRUG RESISTANCE PROTEIN 4

The multidrug resistance proteins (MRPs) are important members of the superfamily of ABC transporters (Borst and Elferink, 2002). The ABC transporters are able to transport drugs at the cost of ATP hydrolysis. The ABC superfamily of transporters consists of a large number of functionally diverse transmembrane proteins which have

been subdivided into seven families designated A through G (Borst and Elferink, 2002). They are capable of transporting a structurally diverse array of endo- and xenobiotics and their metabolites across cell membranes. They facilitate unidirectional translocation of chemically diverse substrates including amino acids, lipids, inorganic ions, peptides, saccharides, metals, drugs, and proteins. Energy derived from the hydrolysis of ATP is used to transport the substrate across the membrane against a concentration gradient (Jones and George, 2004). These transporters are present in almost all tissues and cell types in different amounts. A typical ABC transporter is characterized by the presence of three peptide motifs: Walker A and B sequences and the so-called ABC-signature sequence ("ALSGGQ", or 'C' motif) (Klein et al., 1999; Locher, 2004). Most ABC proteins from eukaryotes encode full transporters, consisting of two ATP-binding domains and 12 membrane-spanning regions or half transporters, which are presumed to dimerize (Borst et al., 2000).

As the C subfamily of ABC-transporters, MRP family contains at least nine members (MRP1-9) with sizes ranging from 1325 to 1545 amino acids. All MRP members have membrane spanning domains (MSDs; transmembrane domains, TMDs) and cytoplasmic ATP-binding domains (or nucleotide binding domains, NBDs) (Higgins, 1992). The NBDs are responsible for the ATP-binding/hydrolysis that drives drug transport, and their structure is conserved independently of the degree of primary-sequence homology (Altenberg, 2004). The MSDs contain the drug-binding sites that are likely located in a flexible internal chamber that is sufficiently large to accommodate different drugs. Each MSD typically includes six hydrophobic transmembrane (TM) helices, but may range from 5 to 10 (Higgins, 1992). The critical functional unit of these transporters consists of two MSDs and two NBDs

(**Figure 1-1**). In addition, the MRPs can be divided into two different subgroups based on their membrane topologies: MRP1-3 and MRP6-7 contain three MSDs and two NBDs (Bera et al., 2001; Hopper et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001); MRP4-5 and MRP8-9 contain only two MSDs and two NBDs. MRPs with the third NH_2-terminal MSD (MRP1-3 and MRP6-7) have the ability to transport conjugates, while MRPs without it are able to transport cyclic nucleotides (Belinsky et al., 1998; Borst et al., 1999; Dean et al., 2001; Kruh and Belinsky, 2003; Deeley et al., 2006). Not surprisingly, the various MRPs show considerable differences in their tissue distribution, substrate specificities, and proposed physiological and pharmacological functions. MRP4 (ABCC4) has particular tissue expression profile, drug resistance selectivity, and substrate and inhibitor specificity, in comparison with other MRPs.

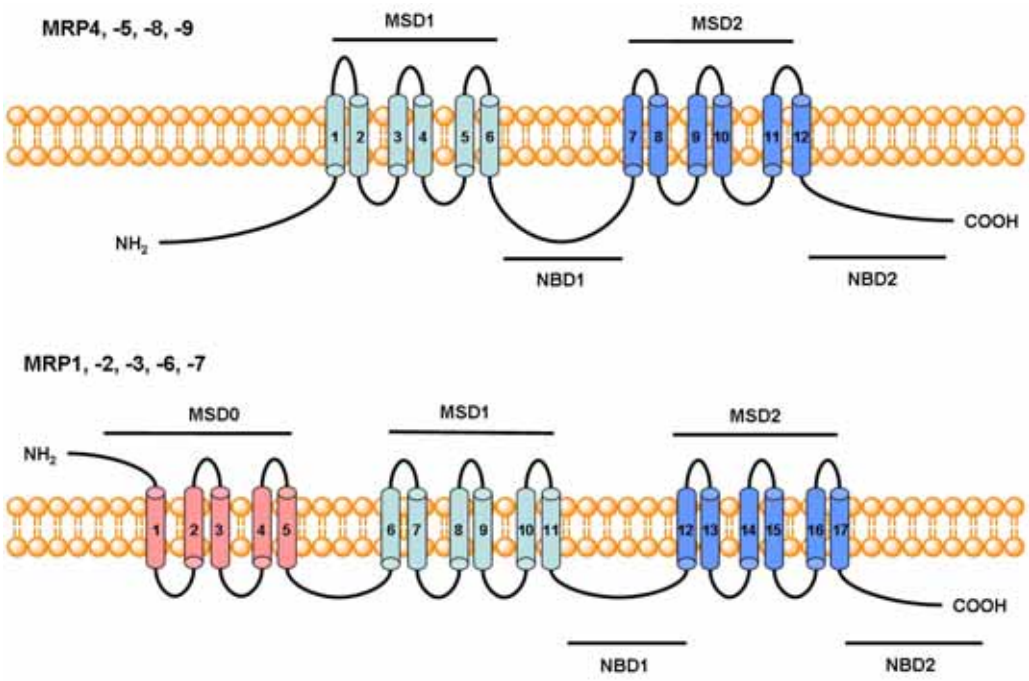


Figure 1-1. Topology of short and long MRP family members. "Short" MRPs, such as MRP4-5 and 8-9, have 12 TM helices and two ATP-binding sites. "Long" MRPs, MRP 1-3 and 6-7, are similar in that they contain two ATP-binding regions. They also contain an additional domain with a length of five TM segments at the N-terminal end, giving them a total of 17 TM helices (Gottesman et al., 2002; Deeley et al., 2006).

1.1.1. Structure and tissue distribution of MRP4

MRP4, encoded by the gene *ABCC4*, was first identified as a homolog of MRP1 (*ABCC1*) by the screening of databases of human expressed sequence tags (Kool et al., 1997). MRP4 was found overexpressed by gene duplication in the human T-lymphoid CEM cells which were resistant to nucleoside monophosphate analogs (Schuetz et al., 1999). Increased MRP4 expression in these cells was responsible for the increased efflux of 9-(2-phosphonylmethoxyethyl)adenine (PMEA, adefovir) and azidiothymidine (AZT), leading to the low cytotoxicity of these drugs and other nucleoside analogs, such as lamivudine (Schuetz et al., 1999). The MRP4 gene is located on the long arm of chromosome 13 at 13q32 (Kool et al., 1997; Lee et al., 1998; Schuetz et al., 1999), while the MRP4 protein has a length of 1,325 amino acids, and resembles the secondary structure of MRP5 (Borst et al., 2000). In comparison with MRP5, MRP8 and MRP9, the N-terminus of MRP4 is 87-225 amino acids shorter, but its C-terminus is 40 amino acids longer and has a PDZ domain (conserved 80-90 amino acid sequence elements found in PSD95, the Dlg-A, and the ZO-1 proteins) (Kocher et al., 1999; Adachi et al., 2002a; Nies et al., 2002). The gap of about 50 amino acids shortly after TM6 might be the most special, which is absent in the long MRPs (MRP1-3 and MRP6-7) (Deeley et al., 2006). The amino acids in TM6 of MRP4 are conserved among different species, but not among other MRPs. Two phenylalanines at position 368 and 369 in MRP4 are specific and absent in other MRPs. MRP4 also contains a positively charged residue in TM8 (Arg782) that is absent in long MRPs (Deeley et al., 2006).

MRP4 mRNA expression has been found in several tissues, including prostate (Lee et al., 1998; Lee et al., 2000), liver (Lee et al., 1998), testis (Lee et al., 1998), ovary (Lee

et al., 1998), kidney (Lee et al., 1998; van Aubel et al., 2002; Langmann et al., 2003; Nishimura and Naito, 2005), lung (Nishimura and Naito, 2005), pancreas (Konig et al., 2005), adrenal gland (Chen et al., 2001; Zelcer et al., 2003), and brain (Nies et al., 2004). Although *MRP4* mRNA is present in most normal tissues, the expression levels are highly variable (Nishimura and Naito, 2005). It is highly expressed in prostate (Lee et al., 2000), kidney (van Aubel et al., 2002), and lung (Nishimura and Naito, 2005); while being present in very low or having no expression in liver, and placenta (Lee et al., 1998; Nishimura and Naito, 2005). *MRP4* mRNA was found in the human brain by quantitative RT-PCR analysis (Nies et al., 2004), while it was not detected earlier in human brain by the RNase protection assay (Kool et al., 1997). Similar to normal organs, human *MRP4* expression in tumors is also highly variable (Szakacs et al., 2004). *MRP4* is highly expressed in normal lung tissue (Nishimura and Naito, 2005), whereas some lung cancer cell lines, such as HCI-H460, have very low levels of *MRP4* (Szakacs et al., 2004). In rats and mice, *Mrp4* mRNA was also present in many tissues with highest levels in kidney (Chen and Klaassen, 2004; Maher et al., 2005). *Mrp4* was found in rat glial cells, and in bovine brain microvessel endothelial cells by RT-PCR (Zhang et al., 2000c). It has been reported that the activation of the constitutive androsterone receptor was capable of increasing *Mrp4* expression in the mice kidney (Assem et al., 2004). Similarly, *Mrp4* expression was also found to be increased in the kidney when mice were fed with ursodeoxycholate (Zollner et al., 2006).

MRP4 protein, on the other hand, is mainly detected in the kidneys (Kool et al., 1997; Maher et al., 2005; Nishimura and Naito, 2005), and is highly expressed in the prostate (Jedlitschky et al., 2004), pancreas (Konig et al., 2005), hematopoietic stem cells and blood cells (Su et al., 2004). Unique to this member in the family, MRP4

protein displays dual membrane localization in polarized cell types. It is localized in the basolateral membrane of tubuloacinar cells in prostate tissues (Lee et al., 2000), and the basolateral hepatocyte membrane by immunofluorescence staining of rat liver sections (Rius et al., 2003), whereas it is expressed at the apical-membrane of the proximal tubules in human and rat kidney (van Aubel et al., 2002), and the luminal side of brain capillary endothelium (Leggas et al., 2004). The MRP transporters expression levels in cancer cells are basically unpredictable. It is important to identify whether the cells have the ability to respond to a xenobiotic stimulus *via* the overexpression of specific MRP transporters. Clinical studies suggest that cancer cells are able to rapidly adjust their MRP transporters expression in response to multiple stimuli (Zhou et al., 1995; Kruh, 2003).

Factors governing the substrate specificity of the MRP transporters are complex, as these are large membrane proteins that recognize a diverse variety of substrates. Studies have only recently begun to identify domains and individual amino acid residues in MRP transporters that are involved in determining substrate specificity. At least 25 non-synonymous single nucleotide polymorphisms (SNPs) have been identified for the *MRP4* gene (Gradhand et al., 2008). Trp residues in different region of MRP1 (position 1246, 459 and 553) have been shown to affect substrate specificity and transport efficiency (Ito et al., 2001; Koike et al., 2002; Deeley et al., 2006; Zhang et al., 2006). Despite differences in the topologies of MRP1-3 and MRP4, Trp residues are present at position 230 and 995 of MRP4 (analogous to Trp 459, and Trp 1246 in MRP1) and Phe is present at position 324 (analogous to Trp 553 in MRP1). It remains to be determined whether variances in polymorphisms of gene or amino acids of protein affect MRP4 expression and function and whether they influence drug metabolism.

1.1.2. Substrates of MRP4

MRP4 is a lipophilic anion pump capable of transporting a variety of compounds with diverse chemical structures (**Table 1-1** & **Figure 1-2**). For example, MRP4 has been reported to mediate the transport of endogenous molecules (Chen et al., 2002; van Aubel et al., 2002; Reid et al., 2003b; Zelcer et al., 2003; Denk et al., 2004), nucleoside/nucleotide analogs (Schuetz et al., 1999; Adachi et al., 2002b; Anderson et al., 2006; Imaoka et al., 2007), and anticancer drugs (Lee et al., 2000; Chen et al., 2002; Wielinga et al., 2002; Leggas et al., 2004).

The physiological and endogenous compounds that could be transported by MRP4 include prostaglandins, steroids, bile acids, cyclic nucleotides, folate and urate (Chen et al., 2001; Chen et al., 2002; van Aubel et al., 2002; Reid et al., 2003b; Zelcer et al., 2003; Denk et al., 2004; Van Aubel et al., 2005). The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) were among the first endogenous substrates identified for MRP4 (Chen et al., 2001; van Aubel et al., 2002; Wielinga et al., 2002; Van Aubel et al., 2005). cAMP and cGMP are intracellular second messengers that mediate a wide range of cellular responses to extracellular signals by activating the protein kinase (PK) A and PKG signaling pathways (Hofer and Lefkimmiatis, 2007). cGMP has a higher affinity to MRP4 than cAMP (Chen et al., 2001), but MRP4-mediated cGMP transport is inhibited by cAMP (van Aubel et al., 2002). Increased cGMP efflux was also observed in MRP4-transfected HEK293 cells, compared with the parental cells (Wielinga et al., 2003), providing evidence that MRP4 might be able to partially influence the intracellular concentrations of cGMP and cAMP by controlling their efflux. Besides, MRP4 has shown modulating effect on levels of cGMP and cAMP in

a microdomain underneath the surface membrane, but not the whole cell concentrations (Li et al., 2007).

The endogenous organic anions and steroid conjugates whose transport can be mediated by MRP4 include estradiol 17-β-glucuronide ($E_2$17βG) (Jedlitschky et al., 1996; Loe et al., 1996a; Zeng et al., 2000; Chen et al., 2001), and dehydroepiandrosterone-3-sulfate (DHEAS) which is the major circulating steroid made in the adrenal gland in humans (Zelcer et al., 2003). Although MRP5 is a high-affinity transporter of cGMP and cAMP as MRP4 (Jedlitschky et al., 2000; Chen et al., 2001), MRP5 is not able to transport $E_2$17βG. The affinity of MRP4 for $E_2$17βG is similar to that of MRP3, while lower than that of MRP1 and MRP2 (Jedlitschky et al., 1996; Loe et al., 1996a; Zeng et al., 2000). On the other hand, DHEAS shows a higher affinity to MRP4 than $E_2$17βG (Zelcer et al., 2003). MRP4-mediated transport of $E_2$17βG and DHEAS is inhibited by several steroid derivatives with a cholesterol backbone structure in a competitive manner, suggesting they are MRP4 substrates (Zelcer et al., 2003). Similar, bile acids have also been identified as MRP4 substrates (Zelcer et al., 2003; Rius et al., 2006). Increased MRP4 expression in the liver of human and rats with obstructive cholestasis could stimulate the elimination of excess bile salts (Denk et al., 2004).

Prostaglandins (PGEs) are critical mediators in the regulation of many physiological processes (Funk, 2001). Specifically, MRP4 is able to mediate the uptake of PGEs, PGE_1 and PGE_2, unlike MRP1-3 and MRP5 (Reid et al., 2003b). MRP4 has high affinity for PGE_1 and PGE_2, and is capable of reducing their steady state accumulation in MRP4-transfected HEK293 cells (Reid et al., 2003b). Some PGEs, including $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGA_1, and thromboxane B2 (TXB_2), are identified as high affinity

inhibitors of MRP4, and therefore presumably substrates of MRP4 (Reid et al., 2003b).

MRP4 is also an efflux pump for urate, the purine end metabolite (Van Aubel et al., 2005) and thioxanthosine monophosphate and thioinosine monophosphate (both thiopurine metabolites) (Wielinga et al., 2002). Urate is bidirectionally transported in the kidney proximal tubule (Enomoto et al., 2002) and overexpression of MRP4 is found to greatly increase urate efflux in HEK293 cells (Van Aubel et al., 2005). Although there is interaction between MRP4-mediated transport of cGMP and cAMP (van Aubel et al., 2002), MRP4 is able to simultaneously transport urate, cGMP and cAMP (Van Aubel et al., 2005). In addition, physiological folates, folic acid (FA) and N^5-formyltetrahydrofolic acid (leucovorin) are susceptible to be transported by MRP4, but not by MRP5 (Chen et al., 2001; Chen et al., 2002; van Aubel et al., 2002).

Apart from the above-mentioned physiological substrates, a variety of nucleoside (purine and pyrimidine) analogues has been found to be substrates for MRP4. These include 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir, GCV) (Adachi et al., 2002b), azidiothymidine monophosphate (AZT-MP) (Sampath et al., 2002), PMEA (Lee et al., 2000; Sampath et al., 2002), bis-POM-PMEA (adefovir dipivoxil, a lipophilic ester prodrug of adefovir) (Dallas et al., 2004), 6-mercaptopurine (6-MP), and 6-thioguanine (6-TG) (Chen et al., 2001; Wielinga et al., 2002). PMEA is an acyclic nucleotide derivative with a broad-spectrum antiviral activity and acts as a viral DNA polymerase-mediated chain terminator by inhibiting the replication of both hepadna- and herpetoviruses (De Clercq et al., 1986; De Clercq, 1993). Due to the low oral bioavailability in animals (Cundy et al., 1994b) and humans (Cundy et al.,

1995) of PMEA, prodrugs of PMEA have been designed to increase the lipophilicity of this compound. The use of bis-POM-PMEA is able to increase the transport of total PMEA across Caco-2 monolayers (Annaert et al., 1997). It has been demonstrated that bis-POM-PMEA has better permeability than PMEA across human intestinal cell membranes *in vitro* (Starrett et al., 1992; Annaert et al., 1997), and the oral bioavailability of PMEA from bis-POM-PMEA was 22-25% in monkeys, which was not limited by the dissolution rate of the prodrug (Cundy et al., 1994a). Bis-POM-PMEA is only metabolized to PMEA after oral administration to rats (Shaw et al., 1997). Amplification of both MRP4 gene and MRP4 protein is detected in CEM-r1, a selected human T-lymphoid cell line which confers high-level resistance to PMEA by 250-fold (Schuetz et al., 1999). In agreement with this report, NIH3T3 cells transfected with MRP4 displays an average of 2.3-fold resistance to PMEA in a 3-day continuous exposure growth assay (Lee et al., 2000). However, the great difference in the resistance capacity between the two reports is probably attributed to low expression of MRP4 in NIH3T3 transfectants or low sensitivity of NIH3T3 fibroblasts to PMEA (Lee et al., 2000). As a prodrug, bis-POM-PMEA has the advantage of faster uptake than the parent compound PMEA, and is metabolized to PMEA intracellularly before efflux (Gallant et al., 1994; Adachi et al., 2002b). It has been shown that the uptake of bis-POM-PMEA was decreased in MCF-7 cells with transfection of MRP4, and this decrease was inversely proportional to the quantity of MRP4 expression level (Adachi et al., 2002b). In mice, Mrp4 was able to protect the bone marrow, spleen, thymus and intestine from PMEA-induced injury, and inhibit the penetration of PMEA into the brain (Belinsky et al., 2007). CEM-r1 cells with overexpression of MRP4 showed lower sensitivity to GCV and have lower accumulation of the drug, compared with the parental CEM cells. GCV is first

converted to its monophosphate form by the herpes simplex virus thymidine kinase (HSV-TK) and then in turn metabolized by cellular kinases. Moreover, CEM-r1 cells still preserved a lower sensitivity to GCV than CEM cells, when drug activation was enhanced by increasing HSV-TK expression in both cell lines. This indicates MRP4 can transport both GCV and its phosphorylated metabolites, and MRP4 may reduce the antiviral efficacy of GCV therapy by changing its cellular retention and accumulation (Adachi et al., 2002b).

Last but not least, a limited number of anticancer agents, such as topotecan (Leggas et al., 2004) and methotrexate (MTX) (Kool et al., 1999; Lee et al., 2000; Chen et al., 2002), have also been reported as MRP4 substrates. Clearly, the importance of MRP4 as a xenobiotic pump cannot be overlooked as this has bearing on its role in drug efficacy. Notably, the substrate specificity of MRP4 is distinguished from that of MRP5 by the ability of MRP4 to transport $E_2$17βG (Chen et al., 2002). MRP4-transfected NIH3T3 cells were able to increase resistance to MTX by 5.5-fold in 4 h drug exposure assays, and decrease intracellular MTX concentration at both 4 h and 24 h. However, MRP4 did not confer significant resistance to MTX in long exposure assays (Lee et al., 2000). In membrane vesicles prepared from insect cells with transfection of MRP4, it has also been shown that MRP4 is a potent MTX transporter (Chen et al., 2002). However, MTX is not specific and has also been identified as the substrate of MRP 1-3 and MRP5 (Masuda et al., 1997; Hooijberg et al., 1999; Kool et al., 1999; Zeng et al., 1999; Wielinga et al., 2005). As for topotecan, increased accumulation has been observed in brain tissue and cerebrospinal fluid of Mrp4-knockout mice (Leggas et al., 2004). Overexpression of MRP4 in Saos-2 cells was able to reduce the sensitivity of cells to topotecan and decrease intracellular drug accumulation, suggesting Mrp4 confers resistance to topotecan (Leggas et al., 2004).

Given the diversity of substrate specificity, the role of MRP4 in affecting both endo- and xenobiotic efflux cannot be undermined.

Table 1-1. Reported substrates for MRP4.

Substrates	References
Cyclic nucleotides	
cAMP	(Chen et al., 2001; Chen et al., 2002; van Aubel et al., 2002)
cGMP	(Chen et al., 2001; Chen et al., 2002; van Aubel et al., 2002)
Folate	(Lai and Tan, 2002)
GSH	(Lai and Tan, 2002)
Bimane-GS conjugate	
MCB-GS	(Bai et al., 2004)
Steroid conjugates	
$E_2$17βG	(Jedlitschky et al., 1996; Lokiec et al., 1996; Zeng et al., 2000)
DHEAS	(Zelcer et al., 2003)
Bile salt and bile salt conjugates	
Cholyltaurine	(Rius et al., 2003)
Cholylglycine	(Rius et al., 2003)
Cholate	(Rius et al., 2003)
Prostaglandins	
PGE_1	(Reid et al., 2003b)
PGE_2	(Reid et al., 2003b)
Purine and pyrimidine analogues	
AZT	(Sampath et al., 2002)
bis-POM-PMEA	(Dallas et al., 2004)
GCV	(Adachi et al., 2002b)
6-Mercaptopurine	(Chen et al., 2001)
PMEA	(Lee et al., 2000; Sampath et al., 2002)
6-Thioguanine	(Chen et al., 2001)
Purine end and intermediate metabolite	
Thioxanthosine monophosphate	(Wielinga et al., 2002)
Thioinosine monophosphate	(Wielinga et al., 2002)
Urate	(Van Aubel et al., 2005)
Anticancer drugs	
Leucovorin	(Chen et al., 2002)
MTX	(Kool et al., 1999; Lee et al., 2000; Chen et al., 2002; Stewart et al., 2004)
Topotecan	(Leggas et al., 2004)

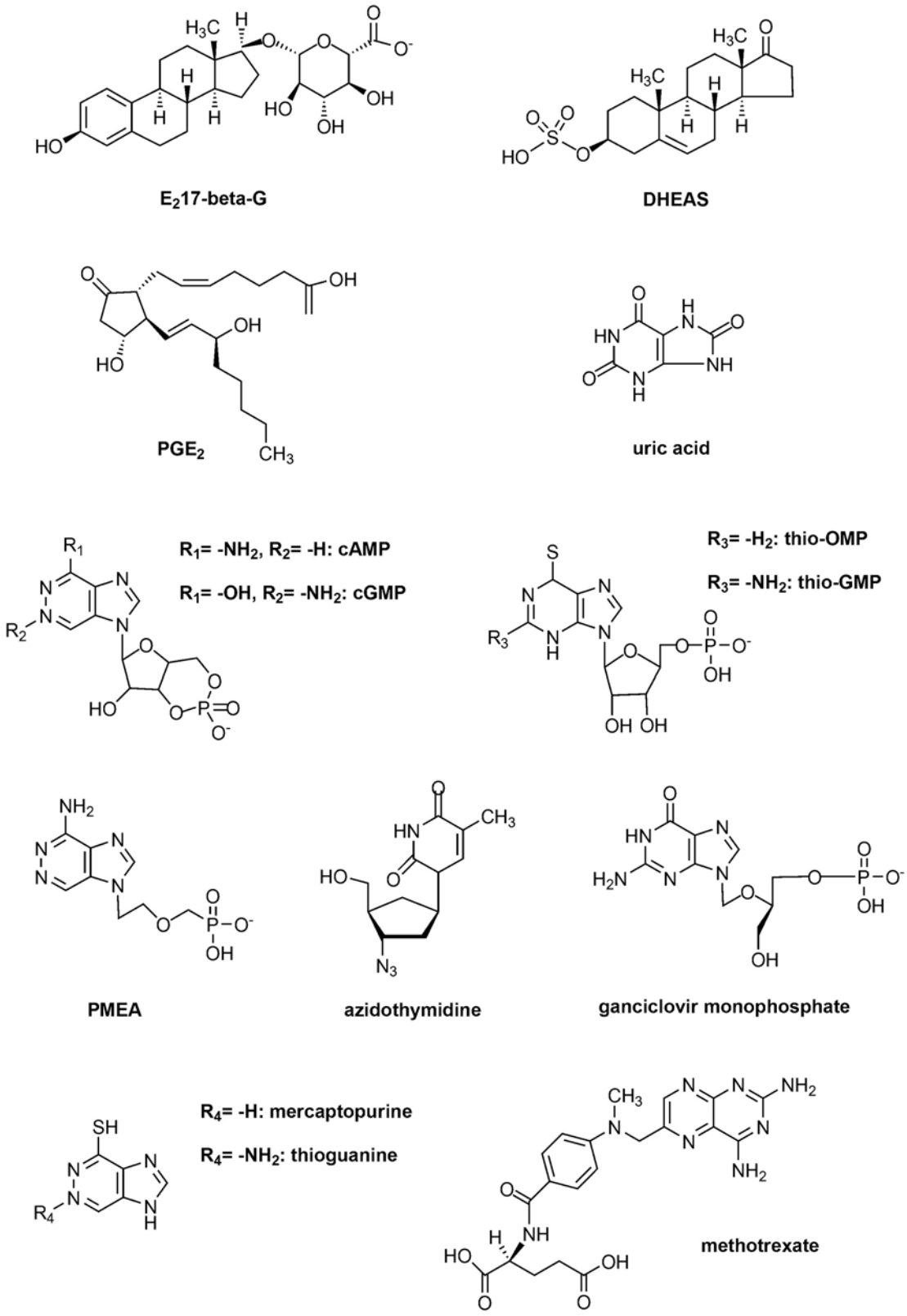


Figure 1-2. Chemical structures of selected MRP4 substrates, illustrating the endogenous and xenobiotic molecules with diverse structures that can interact with the transporter.

1.1.3. Inhibition of MRP4 activity

Since MRP4 can transport a number of important chemotherapeutic agents (Schuetz et al., 1999; Adachi et al., 2002b; Borst and Elferink, 2002; Sampath et al., 2002; van Aubel et al., 2002; Wielinga et al., 2002), the inhibition of MRP4-mediated transport might help to improve the efficacy of therapeutic substrate drugs in cancer treatment. A variety of inhibitors for MRP4 has been identified (**Figure 1-3**). Like MRP1 and MRP2, MRP4 is inhibited by the leukotriene antagonist (E)-3-((3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)(3-(dimethylamino)-3-oxopropylthio)methylthio) propanoic acid (MK571) (Chen et al., 2001; van Aubel et al., 2002). MRP4-mediated transport of PGE1 and PGE2 is inhibited by rofecoxib and celecoxib (both COX-2-specific inhibitors), and diclofenac (Reid et al., 2003b). Both probenecid and dipyridamol strongly inhibit MRP4-mediated cAMP and cGMP transport (van Aubel et al., 2002). MRP4-mediated PMEA transport is inhibited by benzbromarone, nitrobenzylmercaptopurine ribonucleoside (NBMPR), and dilazep (Reid et al., 2003b). The γ-glutamylcysteine synthetase inhibitor, DL-buthionine-(*S*,*R*)-sulfoximine (BSO), was able to reverse MRP4 resistance to PMEA and 6-TG by the regulation of GSH-related mechanism (Lai and Tan, 2002).

For the substrate bimane-GS, MRP4-mediated transport is totally inhibited in the presence of carbonylcyanide *m*-chlorophenylhydrasone (an uncoupler of oxidative phosphorylation) and 1-chloro-2,4-dinitrobenzene (CDNB) which is metabolized to the GSH conjugate after entry into cells. Significant inhibition is also observed with other known inhibitors of MRP transporters including benzbromarone, verapamil, indomethacin, MTX, and 6-TG (Bai et al., 2004). The intracellular accumulation of PMEA, on the other hand, could be increased over 600% in Saos-2 cells transfected

with MRP4 by the treatment of 100 μM indomethacin (Adachi et al., 2002b). Although 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) is not a strong inhibitor for MRP4, it seems to have relatively higher specificity for MRP4 than most of other inhibitors (de Wolf et al., 2007). Of interest, the transport of some MRP4 substrates can exert influence on each other. For instance, the cellular efflux of cGMP by MRP4 was inhibited by PGA_1 and PGE_1, the steroid progesterone and the anticancer drug estramustine (a combination of estrogen and mechlorethamine), all of which are MRP4 substrates (Wielinga et al., 2003). PGA_1 also inhibited the ATP-dependent efflux of MTX by MRP4 (Assaraf et al., 1999; Chen et al., 2002). PGEs, including PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGA_1, and TXB_2, strongly inhibited $E_2$17βG transport by MRP4, but showed no effect on transport by MRP1 and MRP3 (Reid et al., 2003b). Sulfinpyrazone is a potent inhibitor (IC_{50} = 420 μM) of PMEA efflux in MRP4-overexpressing HEK293 cells (Reid et al., 2003a), while MTX can inhibit the MRP4-mediated transport of $E_2$17βG (Chen et al., 2001). Glucuronide and GSH conjugates can also inhibit MRP4-mediated transport of MTX (Chen et al., 2002; van Aubel et al., 2002). Last but not least, the MRP4-mediated transport of $E_2$17βG is blocked in the presence of estradiol 3,17-disulphate, taurolithocholate 3-sulphate (Zelcer et al., 2003), or topotecan (Leggas et al., 2004).

Table 1-2. Reported inhibitors for MRP4.

Inhibitors	**References**
MK571	(Chen et al., 2001; van Aubel et al., 2002)
Rofecoxib	(Reid et al., 2003b)
Celecoxib	(Reid et al., 2003b)
Diclofenac	(Reid et al., 2003b)
Probenecid	(van Aubel et al., 2002)
Dipyridamol	(van Aubel et al., 2002; Reid et al., 2003b)
Benzbromarone	(Reid et al., 2003b)
Dilazep	(Reid et al., 2003b)
BSO	(Lai and Tan, 2002)
CDNB	(Bai et al., 2004)
Indomethacin	(Adachi et al., 2002b)
Verapamil	(Bai et al., 2004)
MTX	(Bai et al., 2004)
6-TG	(Bai et al., 2004)
AEBSF	(de Wolf et al., 2007)

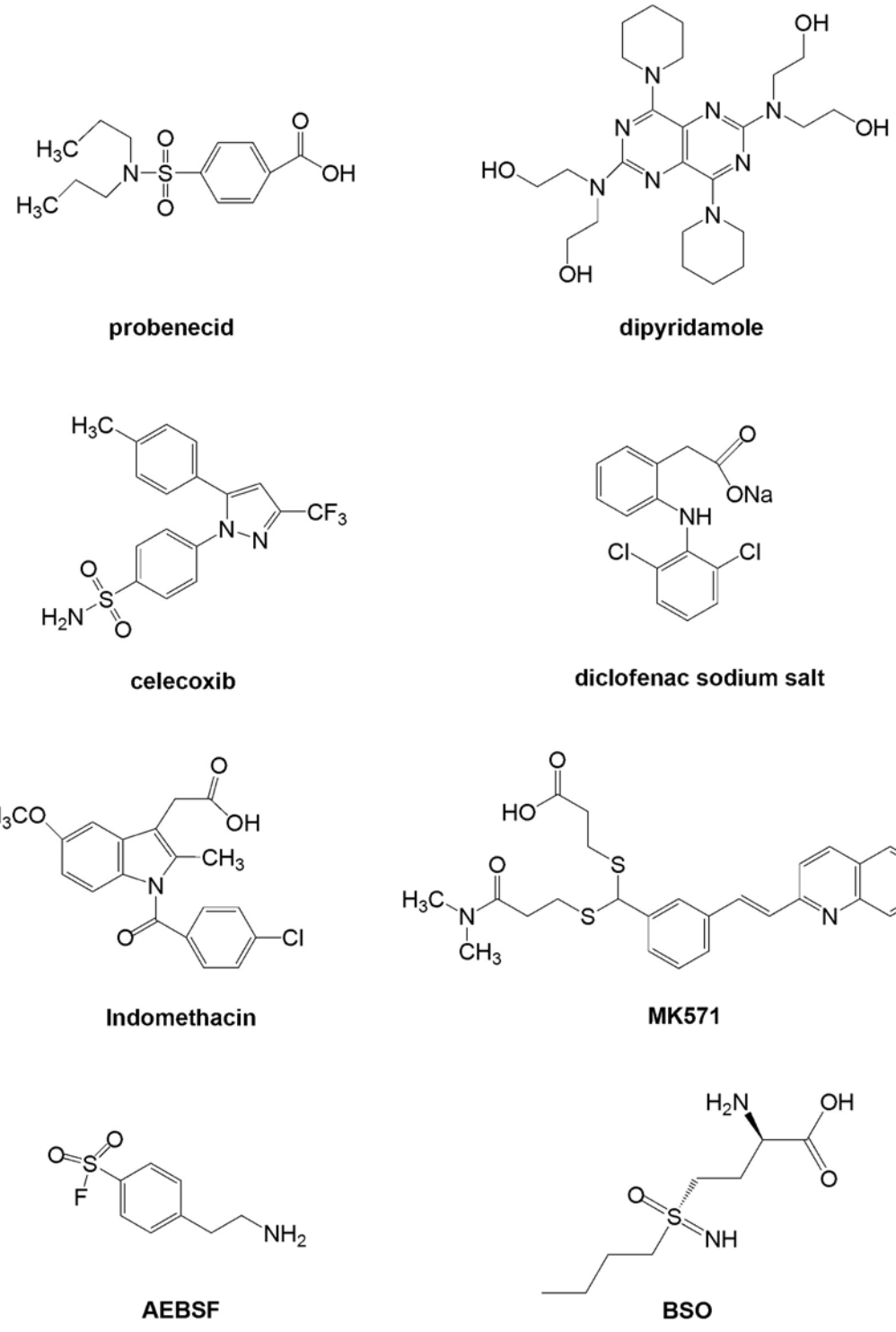


Figure 1-3. Chemical structures of selected compounds that can inhibit MRP4-mediated transport.

1.1.4. GSH and MRP4-mediated transport

The tripeptide GSH is required for various physiological functions such as proper DNA and protein synthesis, cell cycle regulation, storage and transport of cysteine, maintenance of the thiol redox status of the cell, protection against oxidative damage, and detoxification of endogenous and exogenous reactive metals and electrophiles (Meister and Anderson, 1983; Meister, 1984; DeLeve and Kaplowitz, 1990; Wang and Ballatori, 1998). GSH also plays a role in the regulation of gene expression, apoptosis, and membrane transport of both endogenous and exogenous compounds (Hammond et al., 2001). Of more relevance, the reducing capacity of intracellular thiols such as GSH has been shown to contribute to the mechanism of action or activity of many anticancer agents by interrupting pharmacological functions of corresponding transporters for drugs (Lai et al., 1989; Schneider et al., 1995; Sawyer and Bonner, 1996; Renes et al., 1999).

Hepatic GSH is predominantly transported across the basolateral membrane of hepatocytes into the blood circulation as the major source of GSH in plasma (DeLeve and Kaplowitz, 1990; Loe et al., 1996a). As a physiological antioxidant involved in many biochemical processes and as the major transport form of cysteine, GSH needs to be transported across the sinusoidal membrane of the hepatocytes to enable GSH delivery to other tissues (Ballatori et al., 2005). Several members of MRP family have been identified as GSH pumps, including MRP1 (Loe et al., 1996b; Loe et al., 2000), MRP2 (Paulusma et al., 1999), MRP4 (Rius et al., 2003) and MRP5 (Wijnholds et al., 2000). Moreover, efficient transport of some substrates by these transporters requires physiological concentrations of the antioxidant GSH (Paulusma et al., 1999; Renes et al., 1999; Evers et al., 2000; Mao et al., 2000; Wijnholds et al., 2000; Lai and Tan,

2002). In the co-transport by MRP1 and MRP4, GSH can be replaced by GSH analogs, including S-methyl-glutathione and ophthalmate (Loe et al., 2000; Rius et al., 2003). Specifically for MRP4, GSH plays a crucial role with MRP4 transporting many of its substrates in a GSH-dependent manner. MRP4 can mediate the efflux of GSH from hypatocytes into blood by co-transport with bile acids (glucocholate, taurocholate and cholate) (Rius et al., 2003). BSO, the GSH synthesis inhibitor, significantly decreased the intracellular GSH level in HepG2 cells at a high concentration (500 μM) (Zhang et al., 2001). Overexpression of MRP4 in HepG2 cells is associated with a greater increase in GSH export (Lai and Tan, 2002). BSO is able to decrease MRP4-mediated export of cAMP and increase cAMP intracellular accumulation. BSO could also abolish resistance to PMEA and 6-TG by MRP4 (Lai and Tan, 2002).

It has also been shown that MRP4-mediated ATP-dependent co-transport of GSH or S-methyl-GSH together with substrates such as cholyltaurine, cholylglycine, or cholate, and MRP4-mediated uptake of taurocholate could be stimulated by co-transport of reduced GSH or its S-methyl derivative (Rius et al., 2003). The locatization of MRP4 in human hepatocytes, together with the identification of co-substrates, cholytaurine and GSH, of MRP4, provide an alternative pathway to transport GSH and bile salts into the systemic circulation. Thus, MRP4 may play important role in the physiology and pathophysiology of the liver (Rius et al., 2003). In the presence of physiological concentrations of GSH, MRP4 has a high affinity for the taurine and glycine conjugates of the natural bile acids as well as the unconjugated bile acid cholate by using inside-out membrane vesicles (Rius et al., 2006). Chenodeoxycholyltaurine and chenodeoxycholylglycine were the GSH co-substrates with the highest affinities for MRP4. It is proposed that after GSH binds with MRP4

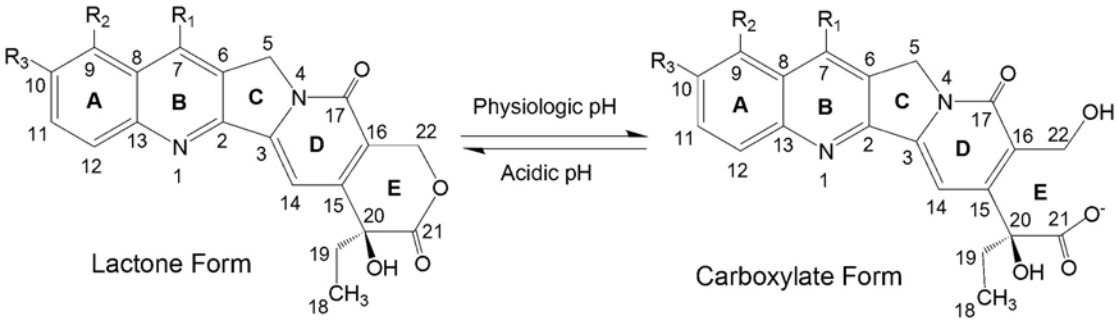
as an anionic moiety, the conformational change of MRP4 may allow the binding of bile acids to the other binding site, leading to the co-transport (Rius et al., 2006). However, GSH was considered to have no involvement in the transport of cAMP and cGMP in HEK293 cells (Wielinga et al., 2003), and did not appear to have a significant effect on PMEA efflux mediated by MRP4-5 in rat microglial MLS-8 cells (Dallas et al., 2004). The transport of DHEAS or $E_2$17βG mediated by MRP4 was found to be independent of GSH, while MRP1-mediated DHEAS transported required the presence of GSH (Zelcer et al., 2003). The reasons for such discrepancy in effects of GSH are unclear, although the differences in cell lines used, intracellular GSH levels, and levels of MRP4 and other transporters may have been causes (Zelcer et al., 2003).

To examine the influence of GSH, fluorescent probes have recently been widely used to detect intracellular GSH levels (Hedley and Chow, 1994; Coates and Tripp, 1995; Kosower and Kosower, 1995). Among them, monochlorobimane (MCB) is reported to be the most specific probe (Cook et al., 1991; Ublacker et al., 1991). MCB is cell-permeant and does not fluoresce. Within the cell, MCB is conjugated to GSH by glutathione S-transferase (GST) to form a cell impermeant and fluorescent bimane-glutathione (bimane-GS) conjugate. It has been shown that MRP4 was able to transport both GSH and bimane-GS (Lai and Tan, 2002; Bai et al., 2004). MRP4-mediated bimane-GS transport was inhibited by known inhibitors for MRP family members, including benzbromarone, verapamil and indomethacin. Known substrates for MRP4, MTX and 6-TG, reduced MRP4-mediated bimane-GS efflux by 40% at a high concentration of 100 μM (Bai et al., 2004). At present, there are no specific GSH transport regulators reported. The examination of newly discovered MRP4 substrates

on the function of GSH efflux may thus be useful for further clarifying the mechanism of substrate-substrate interactions in the corresponding transporters.

1.2. CAMPTOTHECINS

The camptothecins (CPTs), a relatively new group of compounds, have shown capability against a variety of tumors (Redinbo et al., 1998; Stewart et al., 1998; Jung and Zamboni, 2001; Pizzolato and Saltz, 2003). The parent compound, camptothecin (CPT), is a natural alkaloid isolated from the Chinese tree, *Camptotheca acuminata*, during a screen of plant extracts for anticancer agents (Wall et al., 1966). Initial studies on the mechanism of action of CPT suggested that its activity might be attributed to its inhibition of DNA and RNA synthesis (Horwitz et al., 1971; Kessel, 1971; Kessel et al., 1972), as well as cytotoxic selectivity to S-phase cells, G_2 phase cell cycle arrest and induction of chromosomal DNA fragmentation (Horwitz et al., 1971; Kessel et al., 1972). Over the past few years, intensive efforts in medicinal chemistry have led to the generation of a large number of derivatives of CPT, many of which showed improved water solubility and potent anticancer activity. The chemical structures of CPT and some important CPT analogs are given in **Figure 1-4**.



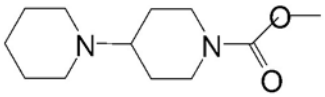
Name	R_1	R_2	R_3
CPT	H	H	H
CPT-11	C_2H_5	H	
SN-38	C_2H_5	H	OH
Topotecan	H	$CH_2N(CH_3)_2$	OH
9-Rubitecan	H	NO_2	H
9-NH_2-CPT	H	NH_2	H
10-OH-CPT	H	H	OH

Figure 1-4. Chemical structures of CPT and its analogs.

It is noted that CPTs have a dynamic equilibrium between the closed ring lactone form and open-ring carboxylate form, which is dependent on the pH of the environment, interaction with blood components and possible other factors. Under neutral or alkaline conditions, CPTs with a closed α-hydroxy-δ-lactone ring, undergo reversible, pH-dependent hydrolysis to yield the corresponding open-ring hydroxyl acid, namely, the more soluble carboxylate form (Fassberg and Stella, 1992). It has been shown that the lactone and carboxylate forms of CPT displayed obviously

different interaction with human serum albumin (HSA), which is abundant in human plasma (Mi and Burke, 1994a; Sriram et al., 2005). The lactone form binds HSA with moderate affinity, while the carboxylate form shows strong binding affinity to HSA, becomes stable and will not convert back to lactone form. After incubation in human plasma or HSA solution for 2 h, the active lactone form of CPT is almost totally converted to its inactive carboxylate form (Burke and Mi, 1994; Mi and Burke, 1994b). The percentage of various CPTs, including CPT, 9-aminocamptothecin (9-AC, IDEC-132) (Burke et al., 1995), topotecan, CPT-11 (irinotecan) and SN-38, present in their active lactone form was 13% -19% at 37°C in phosphate buffered saline (PBS) (Burke et al., 1995). In the presence of 40 mg/ml HSA, only about 1% of CPT and 9-AC are present in the lactone form at equilibrium (Burke et al., 1995; Loos et al., 1999). On the contrary, HSA is able to stabilize the lactone form of CPT-11 and SN-38, with 30% and 39% of the concentration, respectively, present as the lactone form, but shows little effect on the balance of topotecan (Burke et al., 1995; Mi et al., 1995; Loos et al., 1999). These findings suggest that various substitutions at the C7, C9 and C10 position of parental structure CPT are related to the prevention of their carboxylate form binding with HSA by stereo-hindrance. The differences in the lactone/carboxylate ratio of CPTs may greatly influence their pharmacological activity and therapeutic efficacy.

The cytotoxicity of CPTs depends on their effect on DNA synthesis. The lactone ring structure is essential for topoisomerase I (Top 1) binding, antitumor activity and the toxicity of CPTs (Chabner, 1992; Slichenmyer et al., 1993; Redinbo et al., 1998; Stewart et al., 1998), while the carboxylate form is biologically inactive for inhibiting Top 1 and only shows weak cytotoxicity (Hertzberg et al., 1990). This is evident in early clinical studies where the carboxylate form of CPT showed much lower

antitumor activity in comparison with the lactone form of CPT (Gottlieb et al., 1970; Moertel et al., 1972; Muggia et al., 1972). The 20(*S*) form of CPT and its derivatives has also been found to be more active than the corresponding 20(*R*) form in tumor cell models (Wani et al., 1987). Among the list of CPT derivatives, two have already been approved by the US Food and Drug Administration (FDA) for clinical treatment (CPT-11 and topotecan) (**Figure 1-4**). Topotecan was the first CPT derivative to receive US FDA approval for the treatment of advanced ovarian cancer in 1996 (Takimoto and Arbuck, 1997; ten Bokkel Huinink et al., 1997). In 2000, CPT-11 was approved by FDA as first-line treatment for advanced colorectal carcinoma (2000). With good aqueous solubility, CPT-11 and topotecan have shown broad-spectrum anticancer capability in preclinical tumor models, and are currently undergoing wide clinical trials. Additionally, other CPT derivatives are being tested in clinical trials, including 9-AC, rubitecan (9-nitrocamptothecin, 9-NC, RFS2000), 10-hydroxycamptothecin (10-OH-CPT, HCPT), silatecan (DB-67, 7-tert-butyldimethylsilyl-10-hydroxycamptothecin), and exatecan (DX-8951f, a hexacyclic analog of CPT) (Ulukan and Swaan, 2002; Pizzolato and Saltz, 2003). All these derivatives have been shown to induce tumor cell death by the stabilization of Top 1 complex and the generation of permanent DNA strand breaks, like CPT with similar or higher potency (Goldwasser et al., 1996; Ulukan and Swaan, 2002).

1.2.1. Mechanism of action

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 (Wang, 1996). There are two general types of topoisomerases, Type I and Type II. Type I cleaves and separates a single strand of DNA and alter the linkage quantity of DNA, whereas

Type II cleaves both strands of DNA and changes the linking number of DNA by two (Wang, 1996). Mammalian Top 1 is particularly important in supporting replication fork movement during DNA replication and to relax supercoils formed during transcription (Wang, 1996). There is an increased interest in topoisomerases since they were found to be targets for naturally occurring anticancer drugs. Human Top 2 isozymes are targets in tumor cells for anthracyclines (e.g. doxorubicin) and epipodophyllotoxins (e.g. etoposide) (Hande, 2003).

CPTs inhibit the relegation reaction of Top 1 (Hsiang et al., 1985; Hsiang and Liu, 1988; Svejstrup et al., 1991). The action mechanism of CPTs was explained by the replication collision model, whereby CPTs were considered to stabilize the transient cleavable DNA-Top 1 complex and produce an enzyme-drug-DNA cleavable complex, leading to the inhibition of cleavage reaction and relegation steps (**Figure 1-5**) (Svejstrup et al., 1991; Kjeldsen et al., 1992). The reversible stabilization can cause an irreversible arrest of replication fork and double-strand DNA breakage when a replication fork collides with a cleavable complex (Hsiang et al., 1989). The frequency of cleavable complexes and the number of DNA breakage were increased in correlation with the Top 1 level (Pommier, 1996). DNA breakage may eventually result in cell cycle arrest in the S/G_2-phase, activation of apoptosis pathways, and cell death (Hsiang et al., 1989). In comparison with G_1- or G_2/M cells, S-phased cells displayed higher sensitivity to Top 1 inhibitors, suggesting that CPTs have an S-phase specific cytotoxicity (Drewinko et al., 1974; D'Arpa et al., 1990; Takimoto et al., 2000). However, CPT has some other cellular effects independent from replication, including inhibition of RNA synthesis, multi-ubiquitination and degradation of Top 1, chromatin reorganization and activation of transcription factors (Kharbanda et al., 1991; Beidler and Cheng, 1995; Piret and Piette, 1996; Desai et al., 1997; Wu and

Liu, 1997). The cellular effects of CPT independent from S-phase were explained by the proposed transcription collision model, which suggested that the collision between the Top 1-cleavable complexes located on the template strand and the elongating RNA polymerase led to transcription arrest and conversion of Top 1-cleavable complexes into 'irreversible' strand breakage. As a result, the RNA transcript was terminated at the arrested site, resulting in double strand DNA damage and cell killing (Wu and Liu, 1997). Thus, Top 1 inhibitors may show cytotoxicity only in the presence of ongoing DNA replication or RNA transcription (Horwitz et al., 1971; Holm et al., 1989; Hsiang et al., 1989; D'Arpa et al., 1990).

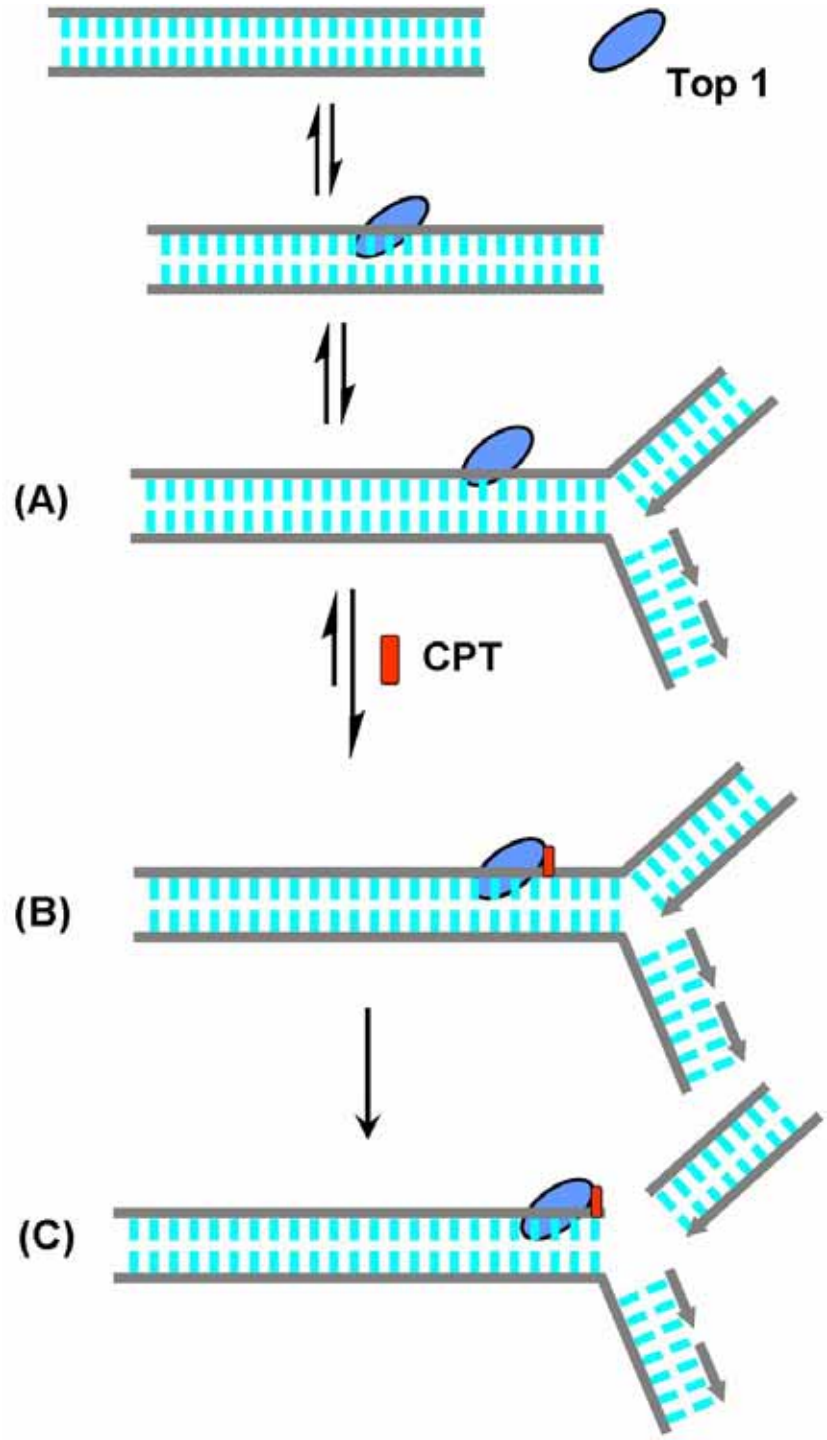


Figure 1-5. Mechanism of DNA damage mediated by Top 1. (A) Cleavage reaction of Top 1; (B) drug stabilized cleavable complex; (C) collision with the replication fork.

1.2.2. Pharmacokinetics and metabolism of CPT-11 and SN-38

CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) was the first water-soluble, semisynthetic CPT derivative to be used in clinical tests (Negoro et al., 1991; Ohe et al., 1992). Similar to CPT, the biological activity of CPT-11 results from its capacity to bind to the transient cleavable complex formed between DNA and Top 1, preventing dissociation of the DNA-Top 1 complex and thereby inhibiting enzyme activity (Jaxel et al., 1989; Vassal et al., 1996). CPT-11 has shown activity against a broad spectrum of cancer, including non-small cell lung cancer (Negoro et al., 1991), ovarian cancer (Takeuchi et al., 1991), non-Hodgkin's lymphoma (Tsuda et al., 1994), refractory leukemia and lymphoma (Ohno et al., 1990).

CPT-11 undergoes several metabolic pathways (**Figure 1-6**) to generate conjugated and unconjugated derivatives that could be excreted from the body. As a prodrug, CPT-11 is transformed to its active metabolite, SN-38 (7-ethyl-10-hydroxycampotothecin), by carboxylesterases (CEs). SN-38 exhibits at least a 1000-fold more potent anticancer effect compared to the parent drug (Kawato et al., 1991). SN-38 is further conjugated by uridine diphosphate glucuronosyltransferanse (UGT) to generate inactive SN-38G (the β-glucuronide conjugate of SN-38), which is considered as a detoxifying route for SN-38 (Iyer et al., 1998). CPT-11 is also oxidized by cytochrome P450 isoform 3A (CYP3A), yielding several pharmacologically inactive products such as APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin) and NPC (7-ethyl-10-[4-amino-1-piperidino]carbonyloxycamptothecin) (Santos et al., 2000). Both CPT-11 and SN-38 have a labile α-hydroxy-δ-lactone ring that undergoes pH-dependent reversible

hydrolysis. Both compounds exist in equilibrium depending on the pH and the presence of binding proteins (Burke and Mi, 1994; Rivory et al., 1994). At physiological pH and higher, equilibrium favors the less toxic carboxylate form, whereas at acidic pH, the more potent lactone form is favored. SN-38G may undergo β-glucuronidase-mediated hydrolysis in the intestine, releasing SN-38 (Takasuna et al., 1998). Evidence has indicated that the biliary excretion of both CPT-11 and SN-38 depends on the presence of drug-transporting proteins, notably PgP and MRP2, present on the hepatocyte canalicular membrane (Chu et al., 1997a). The major toxicities of CPT-11 are myelosuppression and gastrointestinal toxicity, in particular unpredictable severe diarrhea (Gupta et al., 1994).

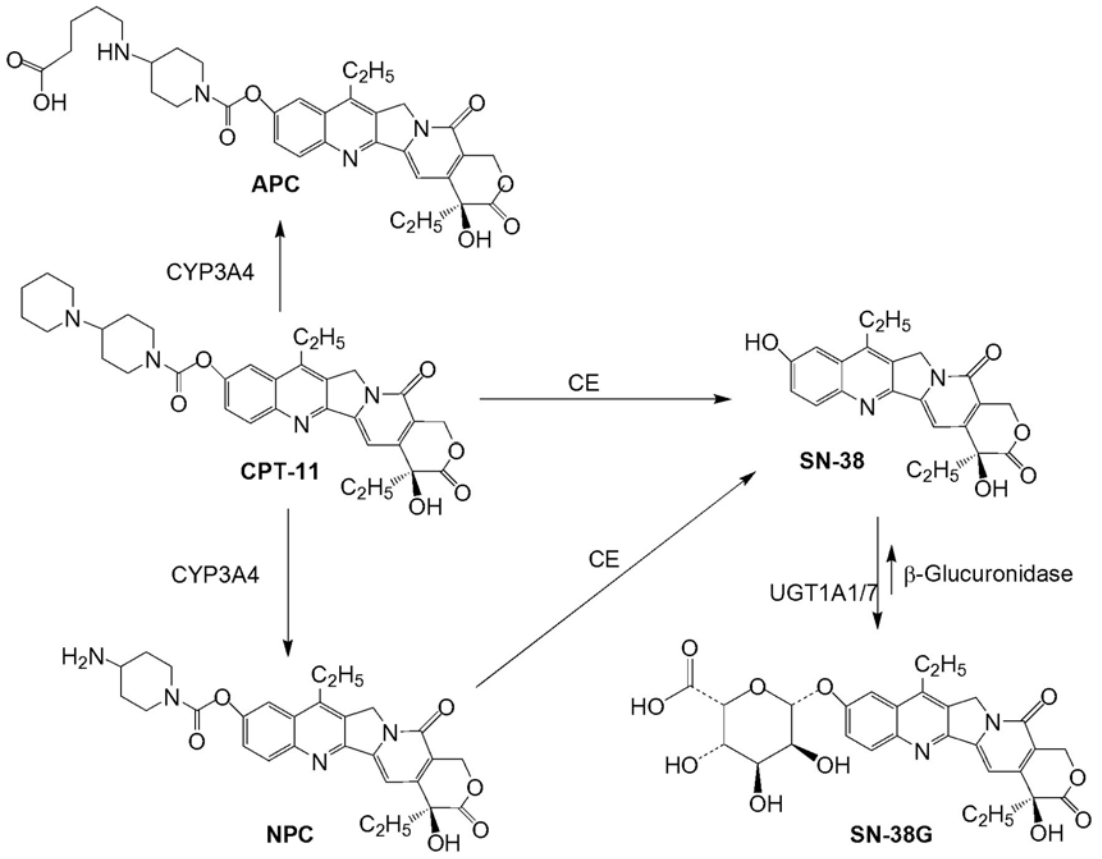


Figure 1-6. Metabolic pathways of CPT-11, showing known involved important metabolites and enzymes.

CE-Mediated Activation

CE enzymes, members of a group of serine esterases, play a critical role in the metabolism of numerous drugs. CPT-11 must be transformed *in vivo* to SN-38 by hydrolysis of the bulk di-piperidino side chain at C-10 of CPT-11 to exert its antitumor activity. The conversion of CPT-11 to SN-38 mediated by CEs has been studied extensively recently. Two similar human isozymes of liver CE, hCE-1 and hCE-2, have been characterized to be responsible for the activation of CPT-11 (Slatter et al., 1997; Humerickhouse et al., 2000). hCE-2 has a 12.5-fold higher affinity for CPT-11 and 5-fold higher maximal rate of CPT-11 hydrolysis than hCE-1. The catalytic efficiency of hCE-2 is 60-fold higher than that of hCE-1. This is evident in cytotoxicity assay using human head and neck squamous carcinoma cells SQ20b. When SQ20b cells were incubated with 1 μM CPT-11, the cell survival was only 38% in the presence of hCE-2, but increased to 88% in the presence of hCE-1 (Humerickhouse et al., 2000). Therefore, hCE-2 is likely to be a key enzyme in CPT-11 activation in human liver. Apart from hCE-1 and hCE-2, it has been suggested that two other human enzymes, hiCE (Khanna et al., 2000) and the butyrylcholinesterase (Morton et al., 1999), have the ability to activate CPT-11. The conversion from CPT-11 to SN-38 may result from any combination of these CEs, and possibly others yet to be identified. Looking at pharmacokinetic parameters, phase I studies of CPT-11 has shown that there was a linear increase of SN-38 with CPT-11 dose over the dose range studied (100-750 mg/m^2) (Chabot et al., 1995). The terminal disposition half lives of SN-38 lactone and total drug are significantly longer than those of CPT-11 (Kehrer et al., 2000). In a study of kinetics of transformation from CPT-11 to SN-38, twice as much SN-38 was generated when CPT-11 was added to human liver microsomes in its lactone form, compared with CPT-11 in its carboxylate form.

Because CPT-11 lactone is easier to change to SN-38 lactone, this phenomenon might in part contribute to the predominance of SN-38 in its lactone form in plasma (Haaz et al., 1997).

CYP3A-Mediated Oxidation

The involvement of CYP3A has been identified in the formation of diverse oxidative metabolites of CPT-11. The most abundant derivative is APC, resulting from a double oxidation of the terminal piperidine ring and generated by CYP3A4 (Haaz et al., 1998b). NPC, produced by cleavage of the distal piperidine ring of CPT-11 by CYP3A4, is another major metabolite which has been identified in the plasma of patients receiving CPT-11 (Dodds et al., 1998). In the study of the kinetic formation from CPT-11 to NPC with pooled human microsomes, the lactone form of CPT-11 was converted more rapidly than the corresponding carboxylate form because the former has a higher enzyme affinity than the latter (Haaz et al., 1998a). Both APC and NPC showed little cytotoxicity. Like CPT-11, APC and NPC were found to be a poor inhibitor of cell growth and a weak inducer of Top 1 DNA-cleavable complexes (100-fold less potent than SN-38) (Rivory et al., 1996b; Dodds et al., 1998). In comparison with CPT-11, APC was not hydrolyzed to SN-38 by human liver microsomes and purified human liver carboxylesterase. Moreover, APC did not inhibit the transformation from CPT-11 to SN-38. Therefore, APC probably does not play a direct role on the activity and toxicity of CPT-11 *in vivo* (Rivory et al., 1996b). However, unlike APC, NPC appeared to be functionally important because it can be a substrate of hCE and be transformed into SN-38, though to a lesser extent than CPT-11. It seems that NPC might contribute to the activity and toxicity profile of CPT-11 *in vivo* (Dodds et al., 1998). It has been reported recently that the subtype CYP3A5

was also capable of metabolizing CPT-11. However, the metabolism of CPT-11 by CYP3A5 was markedly different because neither APC nor NPC was produced, whereas a new (unidentified) metabolite was generated by de-ethylation of the CPT moiety (Santos et al., 2000). Kinetic studies, however, indicated that CPT-11 is preferentially metabolized by CYP3A4 and suggested the affinity of CPT-11 for CYP3A5 is lower than for CYP3A4. The catalytic activity of CYP3A5 was generally weaker than that of CYP3A4 (Santos et al., 2000). Furthermore, CYP3A5 has been demonstrated to be polymorphically expressed, because only 25-30% of human livers have been found to contain detectable levels of CYP3A5 (Shou et al., 1998). The recognition that CPT-11 is a substrate of CYP3A is an important finding, with the consequence of CPT-11 being subjected to a host of enzyme-mediated drug interactions. The conversion of CPT-11 to APC and NPC could be mostly abolished by the prototypical CYP3A inhibitors, ketoconazole and troleandomycine (Haaz et al., 1998a; Santos et al., 2000).

UGT-Mediated Detoxification

The main detoxifying pathway for CPT-11 is the formation of inactive SN-38G. It has been suggested that the accumulation of SN-38 in the intestine is responsible for the severe diarrhea, one of the major side effects of CPT-11 (Wiseman and Markham, 1996). Recently, the metabolism of CPT-11 *via* glucuronidation has been indicated to protect against CPT-11 induced gastrointestinal toxicity. An inverse relationship was observed between SN-38 glucuronidation rates and severity of diarrhea incidences in patients treated with CPT-11(Gupta et al., 1994). Therefore, the conversion of SN-38 to SN-38G by hepatic UGTs is a critical step in the sequential metabolic pathway of CPT-11. Hepatic glucuronidation results from the activities of a multigene family of

UGT enzymes, the members of which exhibit specificity for a variety of endogenous substrates and xenobiotics (Tephly et al., 1988; Burchell and Coughtrie, 1989). The UGT enzymes are broadly classified into two distinct families of protein termed UGT1 and UGT2 on the basis of amino acid sequences (Burchell et al., 1995; Burchell et al., 1998). UGT1A1 isoenzyme has been suggested to be the predominant human UGT involved in the formation of SN-38G. The lactone functionality of SN-38 could also be hydrolyzed after a pH-dependent equilibrium (Iyer et al., 1998; Gagne et al., 2002). In the reaction with SN-38, a UDP-activated glucuronic acid moiety is transferred at the C-10 O-aryl-hydroxy of SN-38. This not only inactivates the drug but also serves as an integral step in transforming the lipophilic SN-38 substrate into hydrophilic glucuronide, facilitating its subsequent biliary or renal elimination. Other UGT1A isoforms, UGT1A3 and UGT1A6 have also been shown to contribute to SN-38 glucuronidation in human liver (Hanioka et al., 2001). Recently, another major hepatic UGT, UGT1A9, and the extrahepatic UGT1A7, have been reported to be involved in SN-38 glucuronide formation (Gagne et al., 2002). Genetic polymorphism has been reported in the TATA box sequences of UGT1A1 (Ando et al., 1998). Normally, the box contains $(TA)_6TAA$ in its promoter region, and sometimes a box contains an extra TA repeat (Ando et al., 1998; Iyer et al., 1999). SN-38 glucuronidation rates were significantly lower in homozygotes $(TA)_7/(TA)_7$ and heterozygotes $(TA)_6/(TA)_7$ when compared with the wild type genotype $(TA)_6/(TA)_6$ (Iyer et al., 2002). UGT1A1 activity is inversely related to the number of TA repeats, since the transcriptional activity of the promoter decreases with the progressive increase in the number of TA repeats (Beutler et al., 1998).

β-Glucuronidase-Mediated Biotransformation

Several bacteria including *Escherichia coli*, *Bacteroides* species, and *Clostridium perfringens* can convert SN-38G in bile and intestine back to the active compound SN-38 by producing the enzyme β-glucuronidase (Skar et al., 1988; Sperker et al., 1997). Several studies have described (microflora-derived) β-glucuronidase activity in non-human (Takasuna et al., 1996; Takasuna et al., 1998) and human intestines (Fujisawa and Mori, 1997; Van Huyen et al., 1998). In rats, it has been suggested that SN-38, which results from the hydrolysis of SN-38 glucuronide by β-glucuronidase in the intestinal microflora, contributes considerably to the distribution of SN-38 in the large intestine tissue, and that inhibition of the β-glucuronidase activity by antibiotics results in decreased accumulation of SN-38 in the large intestine (Takasuna et al., 1998). Histopathological changes of rats in the descending colon, cecum, and ileum were observed after the daily administration of CPT-11 (Takasuna et al., 1996). Recently, these mucosal abnormalities were confirmed in human colon (Kehrer et al., 2001). It has been noticed that this histological damage showed a good correlation with β-glucuronidase activity in the intestinal lumen, but not with the intestinal tissue CE activity (Takasuna et al., 1996). The lesser damage found in small intestines compared with damage in the colon could be explained by a lower exposure of this tissue to SN-38. This may be attributable to a smaller amount of β-glucuronidase in small intestinal lumen (Takasuna et al., 1998).

Because many drugs could affect the functioning of the intestinal bacteria, co-medication may influence the chemotherapeutic treatment of patients with CPT-11 (Mallett et al., 1989). Because bacteria producing β-glucuronidase activity will be killed by antibiotics, it is anticipated that this will lead to a reduction in acute and

delayed diarrhea and cecal damage (Takasuna et al., 1996). It has been demonstrated that antibiotics had no effects on the plasma concentrations of CPT-11, SN-38, or SN-38G in mice (penicillin/streptomycin) (Takasuna et al., 1998) and in humans (neomycin) (Kehrer et al., 2001). This combined treatment also resulted in reduced SN-38 concentrations in the intestinal contents with concomitantly increased SN-38G levels (Takasuna et al., 1998; Kehrer et al., 2001). The decrease of bacterial β-glucuronidase-mediated deconjugation of SN-38 may be a potential approach for modulating CPT-11-induced intestinal toxicity.

1.2.3. Pharmacokinetics and metabolism of topotecan

Topotecan (TPT, Hycamtin, 9-dimethylaminomethyl-10-hydroxycamptothecin) is synthesized by the combination of 10-OH-CPT with a positively charged dimethyl-aminomethyl group at position 9, which can increase the aqueous solubility of the compound (**Figure 1-7**) (Hsiang et al., 1989; Kingsbury et al., 1991). As a potent Top 1 inhibitor, topotecan induces tumor cell death by stabilization of Top 1 complex and generation of permanent DNA strand breaks (Grochow et al., 1992; Wall et al., 1992; Tanizawa et al., 1994; Kollmannsberger et al., 1999). However, the molecular mechanisms by which topotecan treatment inhibits cancer cell proliferation and induces cancer cell apoptosis are not fully elucidated. In addition to the approval for the treatment of metastatic ovarian cancer (Pizzolato and Saltz, 2003), topotecan is used in the treatment of many other tumors including small cell lung cancer (Perez-Soler et al., 1996; von Pawel et al., 1999; Takeda et al., 2003; Ardizzoni, 2004), non-small cell lung cancer (Weitz et al., 2000; White et al., 2000), breast cancer (Levine et al., 1999), neuroblastoma (Santana et al., 2005), uterine carcinoma (Miller et al., 2002), refractory glioblastoma multiforme (Pipas et al., 2005), carcinoma of the

uterine cervix (Long et al., 2005; Monk et al., 2005), and chronic myelomonocytic leukemia (Beran et al., 2003) in clinical practice.

Topotecan is irreversibly converted by hepatic CYP3A to form a less active metabolite, *N*-desmethyl topotecan (**Figure 1-7**) (Rosing et al., 1997), which is detected at low level in plasma and excreta (Herben et al., 2002), indicating that topotecan is only metabolized to a minor extent. A total of 71.3% topotecan and 5.1% *N*-desmethyl topotecan was traced in excretion products of patients within 9 days after topotecan infusion, and both compounds were present in active lactone form and inactive carboxylate form (Rosing et al., 1999). Both topotecan and *N*-desmethyl topotecan can undergo UGT-mediated glucuronidation to generate corresponding *O*-glucuronides (TPT-G) which can be reconverted to the respectively parent compounds by β-glucuronidase (**Figure 1-7**) (Rosing et al., 1998). In rat liver and human prostate carcinoma cell lines, topotecan might be transformed to a new metabolite by CYP450, whose structure and clinical importance remains unclear presently (Platzer et al., 1998a; Platzer et al., 1998b).

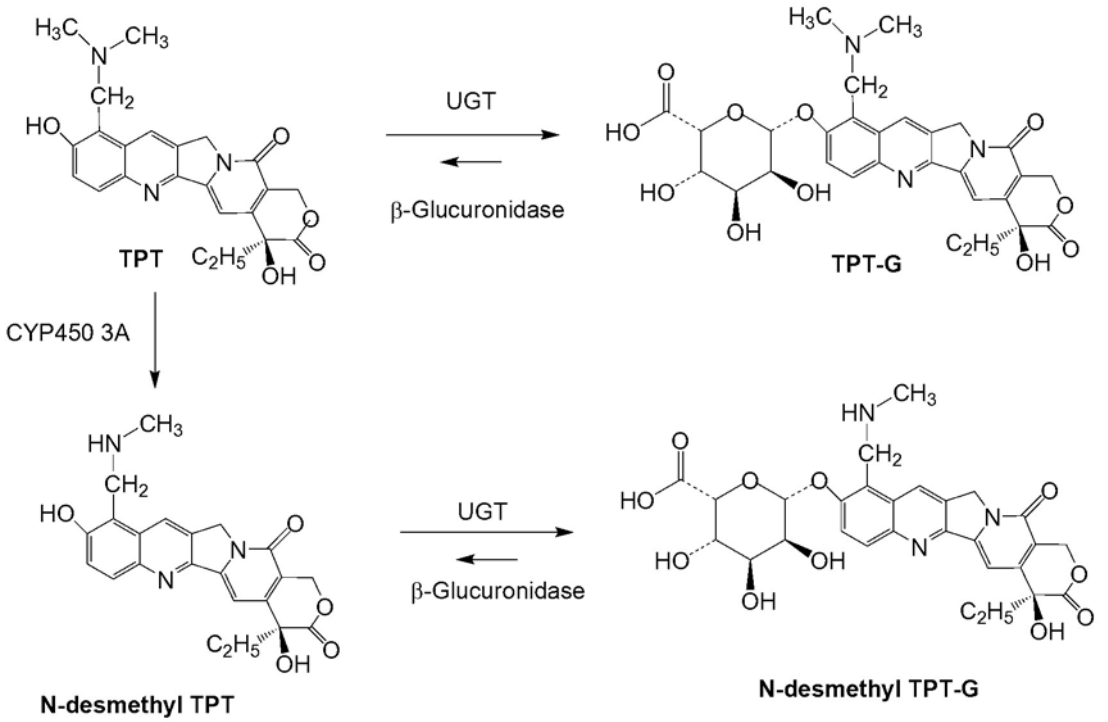


Figure 1-7. Metabolic pathways of topotecan (TPT), showing known involved important metabolites and enzymes.

Topotecan is widely distributed into the peripheral space including cerebrospinal fluid, with a mean volume of distribution at steady-state of 75 L/m^2 (Herben et al., 1996). There is a large interindividual variability in the exposure and clearance of topotecan in cancer patients, with a mean total body clearance of the lactone of 30 L/h/m^2, and a mean elimination half-life of 3 h when administered intravenously (Grochow et al., 1992; Wall et al., 1992; Loos et al., 2000a; Daw et al., 2004). Topotecan is mainly excreted into the urine accounting for 40-70% of the administered dose and to a lesser extent into the feces (18-33% of the total dose) in cancer patients (Loos et al., 2000a; Loos et al., 2000b; Herben et al., 2002). There is an increased interest in the oral administration of topotecan in cancer patients in recent years as it can provide a more convenient way of achieving prolonged exposure. However, the oral bioavailability of topotecan is moderate (30-44%) with a

substantially increased interpatient variability in its systemic exposure (Schellens et al., 1996; Zamboni et al., 1999).

1.2.4. Pharmacokinetics and metabolism of rubitecan

Rubitecan (RBT), an aqueous-insoluble derivative of CPT, has a wide anticancer potency *in vitro* and *in vivo* (**Figure 1-8**) (Verschraegen et al., 1998; Amorino et al., 2000; Sands et al., 2002). In pharmacological studies, rubitecan conferred higher anti-tumor activity than CPT in human tumors xenografted in nude mice (Giovanella et al., 1991). Rubitecan conferred obvious antitumor effect against various cancers in preclinical evaluation, such as lung, colorectal, breast, pancreatic, ovarian, prostate, stomach, melanoma and leukemia (Giovanella et al., 2002). In addition, rubitecan showed inhibitory effect on human immunodeficiency virus type 1 (HIV-1) replication in primary human lymphocytes, suggesting potential clinical utility for treatment of HIV-infection AIDS (acquired immune deficiency syndrome) (Hung et al., 2001). Rubitecan is safe and efficacious as first-line therapy for the treatment of advanced pancreatic cancer. It has also displayed some modest success as second-line therapy in treating gemcitabine failures (Stehlin et al., 1999). It has also been shown with H460 human lung carcinoma *in vitro* cell culture and *in vivo* xenografts that rubitecan could enhance the effects of radiation in human lung cancer. The sublethal damage recovery (SLDR) was found to be inhibited by rubitecan *in vitro*, suggesting that radiation therapy efficacy increased by rubitecan may possibly be related with the block of SLDR (Amorino et al., 2000).

Rubitecan is partially metabolized to 9-AC *in vitro* and *in vivo* (Pantazis et al., 1994; Schoemaker et al., 2002). It has been verified that rubitecan was converted to 9-AC in humans, dogs, and mice by oral administration (Hinz et al., 1994). Incubation of

mouse liver, spleen, kidney, brain, and muscle tissue with rubitecan all showed conversion to 9-AC, but no conversion was detected in cell-free plasma from human or mouse blood (Hinz et al., 1994). Seven metabolites (M1-M7) were identified in rats administered with rubitecan (**Figure 1-8**) (Li et al., 2003). The metabolites in the bile were parent drug, 9-AC (M3), 9-acetamino-CPT (M4), and the glucuronide of 9-OH-CPT (M1, 9-OH-CPT-G). Two other metabolites (M2 and M5) were found in the bile. M5 possibly was the GSH conjugate of 10-OH-CPT, and M2 may be produced by the loss of glutamic acid from M5. Metabolites M1 and M3 were also present in the urine and M4 in the feces. Fecal metabolite M7 was 9-OH-CPT. The mercapturic acid conjugate of 10-OH-CPT (M6) was found in the urine and feces (Li et al., 2003). However, 9-OH-CPT was found to be the main biliary metabolite in the feces, which might result from the hydrolysis of the glucuronide conjugate of 9-OH-CPT (Li et al., 2003).

In Caco-2 cells, the absorptive transport of rubitecan was found to be pH dependent and the transport was increased at weakly acidic pH on the apical side. No concentration dependence and saturation were observed for the absorptive transport of rubitecan at concentrations up to 250 μM (Sha and Fang, 2004). In the everted gut sacs, the absorption of rubitecan occurred *via* passive diffusion and had little difference in different gut regions. pH and efflux transporters were able to mediate the absorption and efflux of rubitecan, and they may contribute to the low oral absorption of rubitecan (Sha and Fang, 2004). The uptake of rubitecan is positively correlated to uptake time, and negatively correlated to pH and temperature. PgP had significant efflux effects on the uptake and transepithelial transport of rubitecan. The PgP inhibitors, cyclosporin A and verapamil, significantly increased the uptake amount of rubitecan (Sha et al., 2004).

Rubitecan may be tolerated for continuous periods of time, but has the potential for significant hematologic, gastrointestinal and urinary bladder toxicity (Verschraegen et al., 1998). The MTD of rubitecan given orally has been estimated at 1.5 mg/m^2/day for five consecutive days weekly (Verschraegen et al., 1998). Primary dose-limiting toxicities of rubitecan were myelosuppression and interstitial cystitis (Stehlin et al., 1999). In a phase I clinical and pharmacological studies of patients with refractory cancers, rubitecan showed substantial dose limiting toxicities, including myelosuppression and hemorrhagic cystitis (Natelson et al., 1996). For a phase II clinical study, no complete or partial responses were observed for patients with metastatic cutaneous and uveal melanoma, who orally administered rubitecan. Hematologic toxicity was moderate. Gastrointestinal side effects were diarrhea and vomiting. In this trial, no patients developed chemical cystitis with gross hematuria. Although preclinical studies have shown potent activity of rubitecan for melanoma, rubitecan, used at a starting dose of 1.5 mg/m^2/day for 5 consecutive days of each week in this trial, is significantly toxic and is not active for metastatic melanoma of cutaneous or uveal origin (Ellerhorst et al., 2002). Another phase II study was performed in patients with heavily refractory ovarian, tubal or peritoneal cancer. Side effects were hematologic toxicity (including anemia neutropenia and thrombocytopenia) and non-hematologic toxic effects (including nausea and vomiting, diarrhea, weight loss, chemical cystitis and neutropenic sepsis) (Verschraegen et al., 1999). Rubitecan administered orally to patients with advanced pancreatic cancer gave promising results, while the toxicity of the therapy was mild and readily overcome (Konstadoulakis et al., 2001).

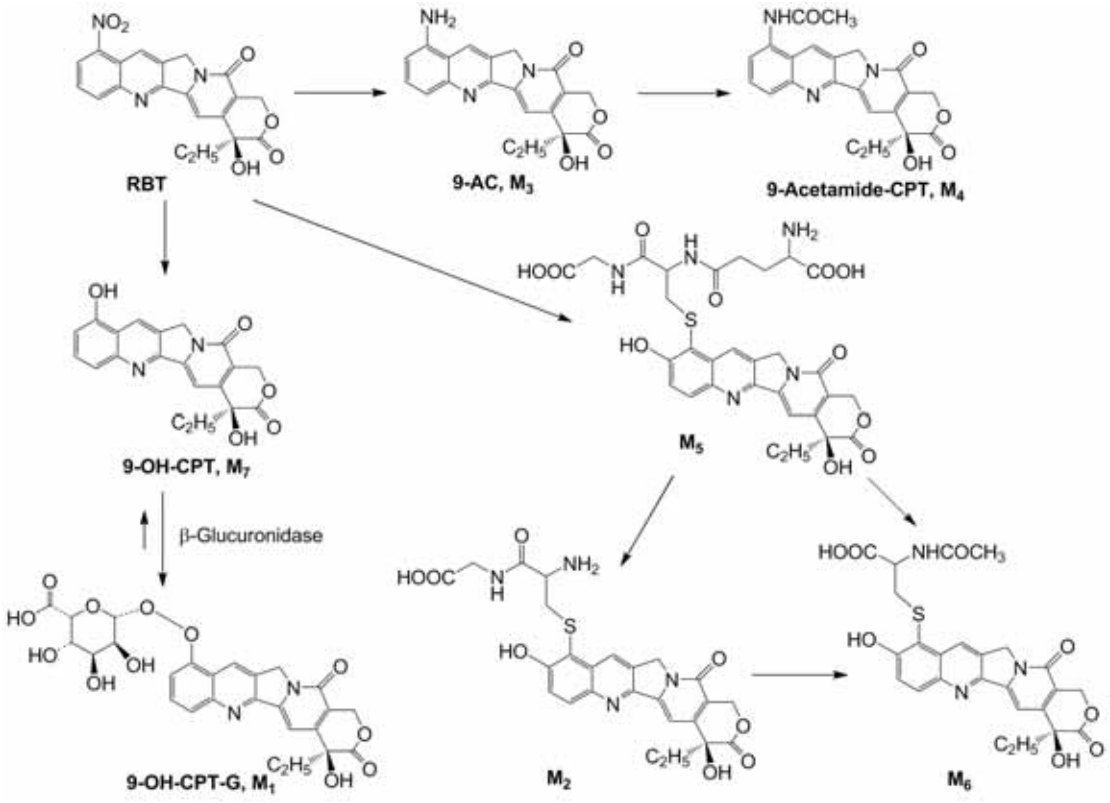


Figure 1-8. Metabolic pathways of rubitecan (RBT), showing known involved important metabolites and enzymes.

1.2.5. Pharmacokinetics and metabolism of 10-OH-CPT

10-OH-CPT, a natural derivative of CPT, is a potent anticancer agent in preclinical stage. In addition to be a Top 1 inhibitor, 10-OH-CPT can induce human hepatoma HepG2 cell differentiation and apoptosis (Zhang et al., 1999; Zhang et al., 2000a; Zhang et al., 2000b; Platzer et al., 2001). The differentiation of HepG2 cells induced by 10-OH-CPT is related to specific G_2/M cell cycle arrest, down-regulation of the proliferating cell nuclear antigen (PCNA) and up-regulation of wild-type protein p53 (Zhang et al., 2000b). 10-OH-CPT could arrest cells in G_2/M phase at low concentration or in S phase at high concentration in HepG2 cells (Zhang et al., 1999). It was found that in HepG2 cells, 0.1 μg/ml 10-OH-CPT could increase the level of p53, c-Myc and Bax proteins, and decrease the level of Bcl-2 protein and α-

fetoprotein (AFP). The activation of caspase-1 and caspase-3 induced by 10-OH-CPT may contribute most to the apoptosis of cells (Zhang and Xu, 2000). In the human breast cancer cell lines MCF-7 and MDA-MB-468, the apoptosis induced by 10-OH-CPT and CPT was shown to be dose- and time-dependent. 10-OH-CPT and CPT may increase the expression of p21WAF1/CIP1 protein and mRNA, and induce apoptosis in human breast cancer cells through both p53-dependent and -independent manners (Liu and Zhang, 1998).

10-OH-CPT is more active and less toxic than CPT (Wani et al., 1986; Ling et al., 1993; Ling and Xu, 1993; Han, 1994). 10-OH-CPT showed definite anticancer activity on murine leukemia L1210 cells (Wani et al., 1986), murine bladder carcinoma MBT-2 cells (Ling et al., 1993), and rodent tumors (Han, 1994). In murine bladder carcinoma MBT-2 cells, 50 μM 10-OH-CPT decreased protein formation in the matrix and other nuclear subfractions. 10-OH-CPT-induced ultrastructural changes in nuclei and nuclear matrix were specifically related with DNA replication or RNA transcription (Ling et al., 1993). In murine ascites hepatoma cells, 10-OH-CPT decreased DNA syntheses and inhibited phosphorylation in histone time-dependently. Moreover, 10-OH-CPT preferably inhibited phosphorylation of histone H1 and H3. *In vivo*, 10-OH-CPT also had inhibitory effect on histone H1 and H3 phosphorylation. Cell death induced by 10-OH-CPT may be related with the inhibition of histone H1 and H3 phosphorylation (Ling and Xu, 1993).

In liver model of male Wistar rats, 10-OH-CPT mainly has three metabolites, which are 10-OH-CPT glucuronide, hydroxyl-10-OH-CPT glucuronide and hydroxyl-10-OH-CPT (**Figure 1-9**). 10-OH-CPT preferred a non-biliary secretion, and conjugated 10-OH-CPT metabolites preferred biliary elimination (Platzer et al., 2001). After *i.v.*

administration to rats, lactone form of 10-OH-CPT was changed to its carboxylate form in the blood, and at the same time distributed to other organs widely, including liver, kidney and bone marrow. Urinary excretion was the main elimination pathway of 10-OH-CPT in the first 3 h, and major metabolites in the urine were the carboxylate form and glucuronides. Thereafter, fecal excretion was the major elimination pathway. Less active carboxylate form of 10-OH-CPT had a longer elimination half-time than its active lactone form. 10-OH-CPT is mainly biotransformed to its carboxylate form and glucuronide conjugates *in vivo*. 10-OH-CPT glucuronides were found in plasma, urine, feces and various organs. Thus the elimination time of 10-OH-CPT is increased by its carboxylate form. With *i.v.* administration, the toxicity of 10-OH-CPT is increased in accordance with dose (Zhang et al., 1998).

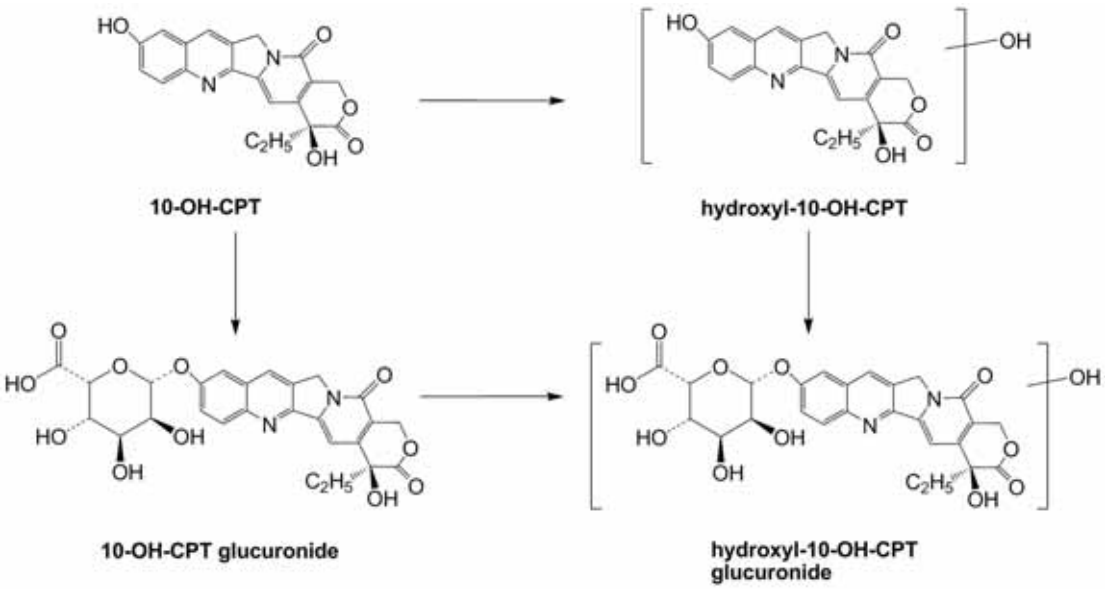


Figure 1-9. Metabolic pathways of 10-OH-CPT, showing known involved important metabolites.

1.3. RESISTANCE TO CPTS

Both severe and unpredictable dose-limiting toxicity and tumor resistance are major reasons for the failure of CPT analogs-based chemotherapy (Rasheed and Rubin, 2003). For example, CPT-11 could only give an objective response in about 20% treated patients with advanced colorectal or lung cancer (Gupta et al., 1997; Kudoh et al., 1998). Topotecan could give 35–38% response rates in recurrent ovarian carcinoma (ten Bokkel Huinink et al., 1997) and ~10% in breast cancer (Levine et al., 1999). The mechanisms for tumor resistance to CPTs are complicated and a number of tumor-, drug- and host-related factors have been implicated (Rasheed and Rubin, 2003; Beretta et al., 2006). These include: a) inadequate accumulation of drug in the tumor; b) alterations in the target (Top 1); c) alterations in the cellular response to the Top 1-CPT interaction. Intracellular accumulation of the active form of CPTs is the final outcome of processes such as cellular influx, metabolism and efflux. Both passive diffusion and transporter-mediated transport are involved in intestinal uptake of CPT (Gupta et al., 2000). In addition, cellular metabolism may be a significant factor to determine the intracellular concentrations of drugs. For instance, lack or low activity of bioactivating enzymes will result in insufficient conversion of drugs to its active forms and lower activity than expected. The toxicity of CPTs will also become lower when the formation of the cleavable complexes is decreased by alteration in Top 1 protein levels or gene mutations (Larsen and Skladanowski, 1998; Staker et al., 2002; Tsurutani et al., 2002). Finally, the increased efflux of CPTs by several drug transporters greatly contributes to tumor resistance. The action of several ABC drug transporters in this respect will be described below.

1.3.1. PgP-mediated resistance

Overexpression of PgP has been linked to the clinical multidrug resistance phenotype and poor prognosis for a number of cancers (Ambudkar et al., 1999; Borst and Elferink, 2002; Gottesman et al., 2002; Schinkel and Jonker, 2003; Marzolini et al., 2004). PgP conferred minor to moderate resistance to topotecan and diminished topotecan accumulation (Chen et al., 1991), but the effects were substantially smaller than the effects of PgP overexpression on the accumulation and cytotoxicity of daunorubicin and etoposide (Hendricks et al., 1992). Cells with overexpression of PgP conferred little resistant ability to CPT, whose cytotoxic levels were similar to the drug-sensitive parental cell line, KB 3-1, and its multidrug-resistant derivative, KB V1. Other uncharged CPT derivatives, including 9-AC and 10,11-methylenedioxy-camptothecin, had similar results compared with CPT. However, topotecan, the charged CPT derivative, showed reduced cytotoxicity against PgP-overexpressing KB V1 cells. For KB V1 cells, the resistance to topotecan was due to the overexpression of PgP. These results suggest that the charge on CPT may influence the drug resistance of PgP (Chen et al., 1991). CHRC5 (colchicine-resistant) and AuxB1 (parental) Chinese hamster ovary cells were also used to examine the effect of PgP overexpression on topotecan cytotoxicity. CHRC5 cells conferred 15-fold resistance to topotecan after a 1 h exposure and 3.2-fold resistance in continuous exposure in the examination of IC_{50} values by colony forming assays (Hendricks et al., 1992). Similar effects were detected in PgP-expressing human cells, MCF-7/Adriar^{r} (doxorubicin-resistant derivative of MCF-7 cell line) breast cancer cells and KG1a human acute myelogenous leukemia cells (Hendricks et al., 1992). Quinidine or verapamil increased intracellular topotecan levels in both of these cell lines but had no effect in parental MCF-7 cells or a variety of human leukemia cell lines without

overexpression of PgP. Quinidine also increased cytotoxicity for topotecan in the PgP-overexpressing cell lines, but not in the parental lines. These results suggest that PgP overexpression lowered topotecan accumulation and topotecan cytotoxicity in both hamster and human cells (Hendricks et al., 1992). In another study using the two kinds of Chinese hamster ovary cell lines, CHRC5 cells with overexpression of PgP conferred significant resistance only to topotecan, SN-38 and 9-AC, in comparison with the control cell line AuxB1. There was no significant cytotoxicity difference between the two cell lines when they were exposed to CPT, 10-OH-CPT, and 10,11-methylenedioxycamptothecin (Mattern et al., 1993). Hoki *et al* examined the relationship between PgP and resistance to CPT analogs by using two cell models, which were MDR KB-V1 cells selected by vinblastine resistance, and NIH3T3 cells with transfection of PgP (NIH-MDR-G185). Both KB-V1 and NIH-MDR-G185 cells were resistant to topotecan, and topotecan-induced cleavable complexes were reduced in KB-V1 cells, consistent with PgP-mediated resistance to topotecan. However, NIH-MDR-G185 cells did not show obvious resistance to CPT, 9-AC, 10, 11-methylenedioxycamptothecin, or SN-38. Although KB-V1 cells were cross-resistant to these compounds, the cleavable complexes level was similar to that of parental KB-3-1 cells. These data indicated that PgP only conferred resistance to topotecan in this assay. The resistance to other CPT derivatives observed in KB-V1 cells can probably be attributable to additional resistance factors besides PgP (Hoki et al., 1997). Human malignant cell lines (BRO/mdr1 and 2780AD) with overexpression of PgP showed a low cross-resistance to CPT-11 and SN-38, which was obvious in human tumor xenografts only with high-expression PgP *in vivo* (Jansen et al., 1998; Van Hattum et al., 2000). However, PgP showed no resistance to karenitecin ((7-[(2-

trimethylsilyl)ethyl]-20(S)camptothecin), BNP1350), a CPT derivative (Van Hattum et al., 2000).

As a known substrate and inhibitor of PgP, cyclosporin A increases the area under the plasma concentration-time curve (AUC) of CPT-11 and its metabolites in rats. Both renal and nonrenal clearances of CPT-11 are decreased in rats with pretreatment of cyclosporin A (Gupta et al., 1996). CPT-11 and its metabolites were also found to interact with PgP with the use of a photoaffinity analogue of verapamil (Gupta et al., 1996). *In vivo*, it was found that PgP may be involved in the biliary excretion of CPT-11 (Sugiyama et al., 1998). The ATP-dependent uptake of the carboxylate form of CPT-11 (5 μM) was found in membrane vesicles isolated from KB-C2 cells, a human epidermoid carcinoma KB-derived cell line with overexpression of PgP (Chu et al., 1999b). Moreover, PgP is considered as the high-affinity component in the biliary excretion of the carboxylate form of CPT-11 (Chu et al., 1999a). In isolated rat canalicular membrane vesicles (cMVs), both cyclosporin A and verapamil could inhibit the ATP-dependent uptake of the carboxylate form of CPT-11 at 5 μM, at which the high-affinity component is responsible for CPT-11 transport (Chu et al., 1997b; Chu et al., 1999a). On the contrary, when the concentration of CPT-11 carboxylate form was increased to 250 μM, at which the low-affinity component was the main factor for drug transport, the inhibitory effect of cyclosporin A and verapamil was greatly reduced (Chu et al., 1999a). It was found that the efflux of CPT-11 was decreased by PgP inhibitors, GF120918 (elacridar) and verapamil, in MDCKII cells with overexpression of PgP, indicating that PgP was involved in the efflux of CPT-11 *in vitro* (Luo et al., 2002).

1.3.2. MRPs-mediated resistance

The first member of MRP family, MRP1 (ABCC1), was found in 1992 in a lung cancer cell line conferring resistance to doxorubicin which was not related to PgP (Cole et al., 1992). MRP1 is nearly present in all major tissues and in all peripheral blood cell types (Zaman et al., 1993; Burger et al., 1994). In human epidermoid KB-3-1-derived cell lines with overexpression of MRP1, cytotoxicity of CPT-11 and SN-38 was significantly decreased, which indicated that MRP1 may contribute the active efflux of CPT-11 and SN-38 (Chu et al., 1999a). C-A120 cells and KB/MRP cells with overexpression of MRP1 were efficiently more resistant to CPT-11 and SN-38 than parental KB-3-1 cells (Chen et al., 1999). 2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(trans-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6- dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P-oxide (PAK-104P) and MK571 could increase the accumulation of CPT-11 and SN-38 in C-A120 and KB/MRP cells, and inhibit the drug resistance. However PAK-104P and MK571 had little effect on the drug sensitivity and accumulation in parental KB-3-1 cells (Chen et al., 1999). Furthermore, the metabolism of CPT-11 to SN-38 in C-A120 and parental KB-3-1 cell lines was similar. These data suggested that MRP1 may be related to the transport of CPT-11 and SN-38 and contribute to drug resistance (Chen et al., 1999). However, MRP1 expressed in human malignant cells (BRO/mdr1 and 2780AD) only showed resistance to SN-38, while no cross-resistance was observed for CPT-11 (Jansen et al., 1998). Resistance to topotecan in the tumor cell lines was also associated with the expression of the MRP1 (Jonsson et al., 1997).

MRP2 (ABCC2) was previously known as the canalicular multispecific organic anion transporter (cMOAT). The amino acids of MRP2 have 49% identity with MRP1

(Evers et al., 1998). The location of MRP2 is unique, as it is present on the apical plasma membranes of polarized cells such as hepatocytes, pneumocytes, kidney proximal tubules, and specialized cells in the intestine and brain (Keppler et al., 1997; Masuda et al., 1997), while other MRPs are in general located on the basolateral membrane of polarized cells. Based on its localization and substrate specificity, it is proposed that the primary physiological function of MRP2 is to export amphiphilic organic anions and xenobiotics into bile and into the lumen of excretory organs (Keppler and Kartenbeck, 1996). MRP2 has been shown to affect the biliary excretion of CPT-11 and SN-38 (Chu et al., 1997b). In Mrp2-deficientrats, MTX, CPT-11 and SN-38 showed decreased biliary clearance (Masuda et al., 1997; Chu et al., 1998; Sugiyama et al., 1998). In another report, MRP2 was also identified to mediate the biliary excretion of CPT-11 and its metabolites, by using isolated liver bile cMVs (Sugiyama et al., 1998). The carboxylate forms of SN-38G and SN-38 significantly inhibit the uptake of leukotriene C4 mediated by MRP2, while inhibition by the carboxylate form of CPT-11 is moderate (Chu et al., 1998). The efflux of CPT-11 was much greater in MDCKII cells overexpressing MRP2 than in wild-type MDCKII cells, and this phenomenon could be efficiently reduced by MRP2 inhibitors. It is suggested that MRP2 is partly responsible for the efflux of CPT-11 *in vitro* (Luo et al., 2002). More recently, it was found that the brain concentration of topotecan in Mrp4 knockout mice was significantly increased compared to the wild type, indicating that topotecan is a potential substrate for mouse Mrp4 (Leggas et al., 2004). Topotecan was able to inhibit MRP4-mediated transport of $E_2$17βG. Overexpression of MRP4 in Saos-2 cells decreased the intracellular accumulation of topotecan, suggesting that topotecan is a new substrate for MRP4 (Leggas et al., 2004).

However, it is not clear whether MRP4 is invovled in resistance to other CPT analogs which have been identified as the substrates for PgP, MRP1-2.

1.3.3. BCRP-mediated resistance

BCRP is a 655-amino acid protein of about 72 kDa. It is the second member of the G family of ABC transporters (ABCG2) (Allikmets et al., 1998) and also named as ABCP or mitoxantrone (MX) resistance gene, MXR (Mao and Unadkat, 2005). BCRP was first identified in a highly doxorubicin-resistant MCF-7 breast cancer cell subline (MCF-1/AdrVp) (Doyle et al., 1998). Overexpression of BCRP was also detected in multidrug-resistant cell lines selected with mitoxantrone (Ross et al., 1999). The transfection of BCRP cDNA in MCF-7 breast cancer cells caused the resistance to mitoxantrone, doxorubicin and daunorubicin, the decreased daunorubicin accumulation and retention in cells, and the increased efflux of rhodamine 123 (Doyle et al., 1998). In multidrug resistant cell lines, the expression of BCRP was correlated with great reduction in the intracellular content of mitoxantrone, daunorubicin, bisantrene, topotecan, prazosin, and rhodamine 123 (Litman et al., 2000).

BCRP confers resistance to SN-38 and topotecan (Lage and Dietel, 2000). Moreover, two sublines of multidrug-resistant human colon (S1) and breast (MCF-7) cancer cell lines with high BCRP expression, S1-M1-80 and MCF-7 AdVp3000, showed more higher resistance ability to topotecan than cells with PgP-mediated drug resistance (Litman et al., 2000). Topotecan-selected T8 and mitoxantrone-selected MX3 cell lines, derived from the human IGROV1 ovarian cancer cells, were resistant to topotecan and SN-38. The expression levels of BCRP protein in both cell lines were in proportion to the resistance ability to topotecan and SN-38, which suggested that BCRP may increase the transport of drugs out of cells. In vitro, 70% of topotecan in

T8 or MX3 cells was effluxed out in 30 sec. Thus, BCRP mRNA levels in the human IGROV1 ovarian cancer cells may be modulated by topotecan (Maliepaard et al., 1999). Mouse Bcrp1 was found to be overexpressed in topotecan-resistant mouse fibroblast cell lines, which lacked functional Mdr1a, Mdr1b, and Mrp1 genes. In resistant cells, the intracellular drug levels were significantly reduced and GF120918 inhibited resistance function of cells. Thus, mouse Bcrp1 may act as a multidrug transporter like the human BCRP (Allen et al., 1999). Similarly, GF120918 increased the intracellular concentration of topotecan in the human ovarian tumor cell lines T8 and MX3 to the drug level in the parental IGROV1 cells. GF120918 could almost totally inhibit BCRP-mediated drug-resistance to CPT analogs (Maliepaard et al., 2001). In vivo, mouse Bcrp1 is found to be responsible for the transport of topotecan, to reduce drug bioavailability and avoid drug harm to fetuses. The established BCRP inhibitor GF120918 could greatly reduce the resistance of topotecan in mice (Jonker et al., 2000). It is also reported that GF120918 efficiently increased oral bioavailability of topotecan in patients, possibly by increasing absorption and decreasing clearance of topotecan. (Kruijtzer et al., 2002).

A mitoxantrone-resistant human MCF-7 breast cancer subline (MCF/MX) conferred cross-resistance to topotecan, CPT-11 and SN-38, which was not clearly related to PgP or MRP1-mediated drug resistance (Nakagawa et al., 1992; Yang et al., 1995). A topotecan-resistant subline MCF-7/TPT300, derived from MCF-7 wild-type cells, showed an increase of topotecan efflux compared with the parental cells. The expression levels of PgP and MRP1 were similar in topotecan-resistant and parental cells, while the BCRP was overexpressed in MCF-7/TPT300 cells. Therefore, the topotecan-resistant activity of MCF-7/TPT300 cells may be caused by the increased topotecan efflux which possibly was mediated by BCRP (Yang et al., 2000). A

mitoxantrone-resistant HT29 colon carcinoma cell line (HT29/MIT) showed great resistance to topotecan and SN-38, which was not related with PgP or other MRPs. Overexpression of BCRP in HT29/MIT contributed to the reduction of intracellular levels of topotecan (Perego et al., 2001). CPT seems to be a poor substrate for BCRP, while its derivatives with potential for glucuronidation, such as SN-38, topotecan, 9-AC, are better BCRP substrates than CPT itself (Brangi et al., 1999; Mao and Unadkat, 2005). PgP, MRP1 and MRP2 showed little resistance to rubitecan and 9-AC, expression of both wild-type BCRP and R482T BCRP mutant displayed resistance to 9-AC, while not to rubitecan (Rajendra et al., 2003). Moreover, other CPT analogs, such as lurtotecan (NX211), exatecan mesylate (DX8951f), karenitecin (BNP1350) and gimatecan (ST1481), seem to be poor BCRP substrates (Mathijssen et al., 2001; Perego et al., 2001). The relationship between substrate selectivity of these transporters and compound structure still remain unclear. Studies aimed at identifying the specific regions responsible for the recognition of the different CPTs by transporters are expected to contribute to the development of new derivatives of overcoming specific mechanisms of drug resistance (Nakagawa et al., 2006).

1.3.4. GSH-mediated resistance

Intracellular GSH is a known determinant of sensitivity to alkyating agents and is involved in a general mechanism for drug detoxification (Gamcsik et al., 1999). GSH plays an important role in the resistance to alkylating CPT analogs by a series of processes including inhibition by formation of drug-GSH conjugates in spontaneous or enzyme-dependent reactions (Colvin et al., 1993). As such, GSH is proposed to be a determinant of cellular response to CPTs, by affecting the redox state of the tumor cells (Adams et al., 2000). GSH also showed influence on the sensitivity to other

nonalkylating CPT analogs. The relationship of CPTs sensitivity to GSH metabolism across cell lines is complicated, indicating that parameters such as GSH turnover rate or GSH utilization by MRPs, glutaredoxin or thioredoxin play a key role in the cellular sensitivity to CPTs (Adams et al., 2000). Several reports have found that reduction of intracellular GSH synthesis by treatment with BSO increases the cell sensitivity to CPT analogs, suggesting that GSH and its metabolism play a crucial role in the cytotoxicity of CPTs (Niimi et al., 1992; Matsumoto et al., 1995; Sawyer and Bonner, 1996; Gamcsik et al., 2001). Consistently, the intracellular GSH level was significantly increased in glioma cell lines (T98G/CPT-11, C6/CPT-11) with acquired resistance to CPT-11. In contrast, it was found that neither the difference of intracellular accumulation of CPT-11 nor the change in the total activity of Top 1 was involved in the CPT-11 resistance, implicating GSH might be partly responsible for the resistance to CPT-11 (Matsumoto et al., 1995). Furtherover, GSH is one crucial component in MRP4-mediated transport, being co-transported with MRP4 substrates (Rius et al., 2003; Rius et al., 2006). It seems that GSH may be involved in drug resistance in several ways and the study of the relationship between GSH and newly identified MRP4 substrates will be helpful in providing a clearer understanding of drug resistance mechanism and the refining of drug therapy in clinical studies.

CHAPTER 2 HYPOTHESIS & AIMS

CPTs are among the most promising antitumor agents endowed with a unique mechanism of action, because they act through inhibition of DNA Top 1, an enzyme involved in regulating critical cellular functions including DNA replication, transcription and recombination. On the premise of its pharmacological potential in cancer chemotherapy, medicinal chemistry has played a crucial role in the development of novel analogs, and recently some compounds have emerged as promising agents for clinical evaluation. A major limitation, however, to the clinical efficacy of CPT-containing therapies is represented by drug resistance. Although drug resistance is a multifactorial phenomenon involving both pharmacological and tumor-related factors, recent reports have pointed towards ABC transporters as important mediators of drug resistance in the context of CPTs. Specifically, transporters such as PgP, MRP1, MRP2 and BCRP have been implicated (Beretta et al., 2006; Ozben, 2006; Beretta and Zunino, 2007). Emerging evidence has also shown that MRP4 may be involved with limited members of this class of compounds (Stewart et al., 2004; Norris et al., 2005). Our interest is thus to elucidate more clearly the role of MRP4 in CPT resistance using an extensive panel of its analogs. The overall hypothesis tested is that CPT and its analogs act as substrates of MRP4 and interact with MRP4 on cellular levels to mediate multidrug resistance in cancer. The following aims were proposed to test this hypothesis:

(1) Determine the resistance profiles of CPT and its analogs in cancer cells overexpressing human MRP4 *in vitro*;

(2) Characterize MRP4-mediated uptake, accumulation, and transport of CPT and its analogs; and

(3) Examine the effect of CPT and its analogs on MRP4-mediated efflux of GSH.

Achieving the aims above will greatly enable us to better understand CPT resistance in cancer and hopefully devise new strategies to overcome drug resistance either through the molecular design of novel non-cross-resistant analogs or utilization of chemical features required for efficacy.

CHAPTER 3 MATERIALS & METHODS

3.1. GENERAL

3.1.1. Chemicals

CPT analogs (all in lactone form), including CPT, CPT-11, SN-38, topotecan, rubitecan, and 10-OH-CPT were purchased from SinoChem Ningbo Co. (Ningbo, China). All CPT analogs have a purity of > 99%. Vincristine, vinblastine, MTX, bis-POM-PMEA, celecoxib, and diclofenac sodium salt (all compounds with a purity > 99%) were also purchased from SinoChem Ningbo Co. (Ningbo, China). The GSH biosynthesis inhibitor, BSO, was from Sigma Chemical Co (St. Louis, MO, USA). The leukotriene antagonist, MK571, was a gift from Dr Ford-Hutchinson (Merck Frosst Canada, Inc., Kirkland, Quebec, Canada) (Laskey et al., 1989). Norcantharidin (with a purity of 99.5%), a protein phosphatase I inhibitor for cancer therapy, was a gift from Professor Andy Lee (Putuo District People's Hospital, Shanghai University of Traditional Chinese Medicine, Department of Oncology, China). Other anticancer drugs used such as etoposide (VP-16), 5-fluorouracil, cyclosporin A, carboplatin, paclitaxel, and mitoxantrone were obtained from Sigma Chemical Co (St. Louis, MO, USA).

3.1.2. Cell culture

The human hepatocellular carcinoma cells, V/HepG2 and MRP4/HepG2, were established by the stable transfection of empty vector and full length cDNA of the human MRP4, respectively, and were kindly provided by Dr Theresa Tan (National University of Singapore, Department of Biochemistry, Singapore) (Lai and Tan, 2002; Bai et al., 2004). 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 Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co, St. Louis, MO, USA) in the presence of 0.25 mg/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 (Lai and Tan, 2002). The established HepG2 cells with stable expression of MRP4 demonstrated significant resistance to PMEA and 6-TG (Lai and Tan, 2002). These cells are capable of transporting GSH (Lai and Tan, 2002) and bimane-GS conjugate (Bai et al., 2004). Seven point mutations (F324A, F324W, W230A, W230F, W216A, W995C, and W995F) were also made to the human MRP4 and the respective plasmids were stably transfected into HepG2 cells. All cells were cultured in DMEM containing 100 units/ml penicillin (Sigma Chemical Co, St. Louis, MO, USA), 100 μg/ml streptomycin (Sigma Chemical Co, St. Louis, MO, USA) and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), in the presence of 0.25 μg/ml blasticidin (Invitrogen, Carlsbad, CA, USA).

A series of Madin-Darby canine kidney (MDCKII) cell lines with the overexpression of PgP (MDCKII-PgP) , MRP1 (MDCKII-MRP1), MRP2 (MDCKII-MRP2), MRP3 (MDCKII-MRP3) and MRP5 (MDCKII-MRP5) were provided by Professor Piet Borst (Netherlands Cancer Institute, Amsterdam, Netherlands) (Bakos et al., 1998; Evers et al., 1998; Kool et al., 1999; Scheffer et al., 2000; Wijnholds et al., 2000). These MDCKII cell lines were cultured in a similar manner as HepG2 cells as stated above.

The breast carcinoma MCF/MX cells were obtained from A/P Go Mei Lin (National University of Singapore, Department of Pharmacy, Singapore) and cultured in RPMI medium (Invitrogen, Carlsbad, CA, USA) containing 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum, in the presence of 250 nM mitoxantrone.

All cell lines were grown at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Experiments were performed on cells within 10 passages. Viable cells were counted using the trypan blue exclusion method.

3.1.3. 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). The 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 with 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.

3.2. MRP4-MEDIATED RESISTANCE TO CPTS

3.2.1. Cytotoxicity assays

Drug effects on exponentially growing tumor cells were determined using MTT assay as described previously (Carmichael et al., 1987). V/HepG2 and MRP4/HepG2 cells were seeded at a density of 5,000 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 h or 48 h. Bis-POM-PMEA and MTX were freshly prepared by dissolving in dimethylsulfoxide (DMSO) and diluted with DMSO to a series of different concentrations. All CPTs (CPT, CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT) in lactone forms were freshly prepared by dissolving in DMSO. CPT analogs were protected from light exposure to avoid degradation. The carboxylate forms of CPT, CPT-11 and SN-38 were freshly obtained from their lactone forms respectively by incubation in PBS buffer at pH 9.0 overnight and at room temperature. These CPTs were 99% in lactone form at pH 3.0 and 98% in carboxylate form at pH 9.0 (Chu et al., 1997a; Chu et al., 1997b). Other anticancer drugs used in this study were prepared by dissolving in DMSO with the exception of cyclophosphamide which was dissolved in water. The final concentration of DMSO in cell culture medium was 1% (v/v) and such concentration showed little cytotoxicity to both strains of cells when incubated for 4 h 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, and fresh drug-free medium was added and the cells were incubated for a further 44 h. At 48 h after drug addition for both long and short drug exposures, 100 µl MTT reagent (0.5 mg/ml) (Duchefa Biochemie E.V., Haarlem, Netherlands) 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 was dissolved in 100 µl 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). Experiments were performed in eight replicate wells for each drug concentration and carried out independently at least three times. 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 replicate experiments.

3.2.2. *In vitro* cytotoxicity inhibition assays

BSO, MK571, celecoxib and diclofenac are all known inhibitors of MRP4 (Borst and Elferink, 2002; Tian et al., 2005). 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, with the exception of BSO which was dissolved in water. The final concentration of DMSO or water was 1% (v/v). All inhibitors at the concentrations used did not show any significant cytotoxicity when incubated with BSO for 24 h or with other inhibitors for 2 h. Before the cells were exposed to the model substrates for MRP4 or the CPT analogs, the medium with these inhibitors was removed, and the cells were washed twice with PBS. Then bis-POM-PMEA, MTX and CPT analogs were added and MTT assay performed as described previously in section 3.2.1.

3.2.3. Western blot analysis

For the western blot analysis of related ABC proteins in V/HepG2 and MRP4/HepG2 cell lines, the following procedures were followed. Logarithmic growth phase adherent cells were scraped and subsequently lysed in PBS buffer with 1% Triton, supplemented with protease inhibitors (Roche Diagnostics Asia Pacific Pte Ltd, Singapore). The cell mixture was centrifuged at 13,000 rpm at 4°C for 20 min. After that, the supernatant was taken out as the samples. The protein samples were stored at

-80°C. To ensure that the same amount of protein from different samples was applied, protein levels were determined by the Bradford method (Sigma Chemical Co, St. Louis, MO, USA) with bovine serum albumin (Sigma Chemical Co, St. Louis, MO, USA) as the standard. Briefly, protein samples were thawed, and then diluted appropriately with Milli-Q water to ensure that the concentrations are within measurable ranges of the calibration curve. Bovine serum albumin was dissolved in PBS buffer to prepare the standards in the concentration range of 0-1.4 mg/ml. The standards and protein samples were added in duplicates to a 96-well plate. The Bradford reagent (250 µl) was then added to each well and mixed on a shaker for 30 sec. The absorbance was read at 595 nm using a microplate reader (Tecan Instruments Inc., Research Triangle Park, NC, USA). Standard curves were obtained by plotting the net absorbance versus the protein concentration of standards. The protein concentration of the unknown samples was calculated using the standard curves.

Proteins were separated on a 7.5% polyacrylamide gel, and subsequently transferred electrophoretically to PVDF membrane. After blocking overnight, the membrane was incubated with primary antibody for 2 h, and then with the appropriate secondary antibody for 1 h in appropriate dilution (**Table 3-1**). The related transporters including PgP, MRP1, MRP2, MRP3, MRP5 and BCRP, were incubated with primary monoclonal antibodies of C219, MRPr1, M_2III-6, M_3II9, M_5II-54 and BXP-21, respectively (Signet Laboratories, Dedham, MA). MRP4 was incubated with primary monoclonal antibody of M_4I (Alexis Biochemicals, Lausen, Switzerland). β-actin was detected by primary monoclonal antibody of AC-74 (Sigma Chemical Co, St. Louis, MO, USA). Either HRP-conjugated anti-mouse (Bio-Rad Laboratories, Hercules, CA; Amersham Biosciences, Piscataway, USA) or anti-rat IgG (Sigma Chemical Co, St. Louis, MO, USA) was used as the secondary antibody (**Table 3-1**). Proteins were

detected using an enhanced chemiluminescence system (SuperSignal West Femto, Pierce, Rockford, IL). The images were acquired by Charged Coupling Device (CCD)-based detector of Alpha Innotech's FluorChem™ (Alpha Innotech Corporation**,** San Leandro). The expression of β-actin was used as internal standard.

Table 3-1. List of antibodies for transporter proteins.

Transporter proteins	Primary antibody (dilution)	Secondary antibody (dilution)
β-actin	Anti-β-actin (1:2000, Sigma)	Anti-mouse (1:3000, Biorad)
PgP	C219 (1:1000, Signet)	Anti-mouse (1:3000, Biorad)
MRP1	MRPr1 (1:1000, Signet)	Anti-rat (1:20,000, Sigma)
MRP2	M_2III-6 (1:1000, Signet)	Anti-mouse (1:10,000, Amersham)
MRP3	M_3II9 (1:1000, Signet)	Anti-mouse (1:10,000, Amersham)
MRP4	M_4I (1:2000, Alexis)	Anti-rat (1:20,000, Sigma)
MRP5	M_5II-54 (1:1000, Signet)	Anti-rat (1:20,000, Sigma)
BCRP	BXP-21 (1:1000, Signet)	Anti-mouse (1:10,000, Amersham)

3.3. UPTAKE & TRANSPORT ASSAYS

3.3.1. Intracellular accumulation of CPT analogs

The accumulation of CPT analogs (CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT) in V/HepG2 and MRP4/HepG2 cells was examined in confluent cell cultures grown in BD Falcon^{TM} cell culture dishes (BD Biosciences, San Jose, CA USA). Briefly, exponentially growing cells were exposed to CPT analogs lactone form (5 μM CPT-11 or rubitecan lactone, 1 μM SN-38, topotecan, or 10-OH-CPT lactone) over 120 min at 37^{o}C. The medium was aspirated off at the indicated times, and the dishes were rapidly rinsed 6 times with 5 ml of ice-cold PBS. The reverse-phase high-performance liquid chromatography (HPLC) analysis of final washes ensured that

they contained no residual CPT analogs. After washing with ice-cold PBS, the cells were harvested and each cell pellet was suspended in 100-200 µl extraction solution (acetonitrile:methanol = 1:1, v/v, with 0.01 N hydrochloric acid) with the addition of 5-10 µl internal standard correspondingly. The extraction solution volume for CPT-11, SN-38 topotecan, and 10-OH-CPT was 200 µl, and for rubitecan was 100 µl. CPT (2.0 µg/ml) was the internal standard for CPT-11, SN-38, and rubitecan. SN-38 (0.5 µg/ml) was internal standard for topotecan and 10-OH-CPT. The acidity of the extraction solution ensures the conversion of the compounds to their lactone form. Subsequently, the mixture was sonicated, vortexed and centrifuged. The supernatant was then injected into HPLC for drug concentration determination. Viable cells were monitored using the trypan blue exclusion method and the accumulation of CPT analogs in time-dependent assays was expressed as ng/10^6 cells. The concentration effect of CPT analogs (0.1-10 µM CPT-11 for 30 min; 0.05-5 µM SN-38 or 10-OH-CPT for 5 min; 0.05-5 µM topotecan for 10 min; 1-25 µM rubitecan for 5 min) on the cellular accumulation in the two cell lines was also investigated using the above described method. The intracellular accumulation rate of CPT analogs in concentration-dependent assays was expressed as ng/min/10^6 cells. Similar procedures were followed for the determination of intracellular accumulation of CPT-11 and SN-38 in the MRP4 mutant cell lines, with MRP4/HepG2 cells being used as the control group. CPT-11 and SN-38 lactone form were used in these concentrations: 1 µM or 5 µM CPT-11; 0.2 µM or 1 µM SN-38 lactone, with fixed time points of 5 min and 30 min. The intracellular accumulation of CPT-11 and SN-38 in mutant cell lines was expressed as percentage relative to that in MRP4/HepG2.

3.3.2. Inhibition of intracellular uptake of CPT analogs

In addition, the effect of celecoxib (50 μM), BSO (200 μM) and MK-571 (100 μM) on CPT analogs accumulation was investigated. Both celecoxib and MK-571 were prepared by dissolving in DMSO, while 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 MK-571 or 200 μM BSO was preincubated for 24 h. Thereafter, cells were washed with warm PBS buffer for 4 times. Exponentially growing cells were then exposed to CPT analogs lactone form (5 μM CPT-11 or rubitecan lactone, 1 μM SN-38, topotecan, or 10-OH-CPT lactone) at 37^{o}C. After continued incubation for the fixed time (30 min for CPT-11-treated cells, and 5 min for SN-38, rubitecan and 10-OH-CPT-treated cells, 10 min for topotecan-treated cells), cells were washed 6 times with ice-cold PBS. The cells were then harvested, lysed by sonication and extracted using ice-cold acetonitrile:methanol mixture (1:1, v/v, with 0.01 N hydrochloric acid). The supernatant was injected into HPLC for the determination of CPT analogs.

3.3.3. Determination of CPT analogs 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, a SPD-M10A UV 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 (Phenomenex, 4×3.0 mm) (Phenomenex Co., Torrance, CA). The mobile phase was a mixture of acetonitrile with 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, SN-38, topotecan, and 10-OH-CPT were detected by a fluorescence detector (Shimadzu Scientific instrument) with an excitation wavelength at 380 nm and an emission wavelength at 540 nm, respectively. Rubitecan was determined by UV detector (Shimadzu Scientific Instrument) at the wavelength of 365-366 nm.

CPT-11 and SN-38

The mobile phase for CPT-11 and SN-38 was a mixture of 27% (v/v) acetonitrile with 73% (v/v) aqueous buffer containing 50 mM sodium hydrogen phosphate and 10 mM sodium heptane sulfonate (pH=3.0). The limit of quantification for CPT-11 and SN-38 were 0.20 ng and 0.01 ng respectively, when the injection volume was 100 µl.

Topotecan

The mobile phase for topotecan was a mixture of 22.5% (v/v) acetonitrile with 77.5% (v/v) aqueous buffer containing 50 mM sodium hydrogen phosphate and 10 mM sodium heptane sulfonate (pH=3.0). The limit of quantification for topotecan was 0.25 ng, when the injection volume was 100 µl.

Rubitecan

The mobile phase for rubitecan was a mixture of 27% (v/v) acetonitrile with 73% (v/v) aqueous buffer containing 50 mM sodium hydrogen phosphate and 10 mM

sodium heptane sulfonate (pH=3.0). The limit of quantification for rubitecan was 10 ng, when the injection volume was 100 µl.

10-OH-CPT

The mobile phase for 10-OH-CPT was a mixture of 24% (v/v) acetonitrile with 76% (v/v) aqueous buffer containing 50 mM sodium hydrogen phosphate and 10 mM sodium heptane sulfonate (pH=3.0). The limit of quantification for 10-OH-CPT was 0.1 ng, when the injection volume was 100 µl.

3.3.4. Determination of efflux kinetic parameters

The MRP4-mediated efflux of CPT analogs (CPT-11, SN-38, topotecan, and rubitecan, all in lactone form) in MRP4/HepG2 cells was estimated by subtracting the accumulation of CPT analogs in MRP4/HepG2 cells from that in V/HepG2 cells. One-binding site model was found to give the best fit for the resultant data. Kinetic parameters for the MRP4-mediated efflux of CPT analogs were obtained by fitting the uptake data to the following equation by a nonlinear method:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Where v is the initial efflux rate of substrate (pmol/min/10^6 cells), [*S*] is the substrate concentration in the medium (µM), V_{max} is the maximum efflux rate (pmol/min/10^6 cells), and K_m is the Michaelis-Menten constant.

3.4. GSH EFFLUX ASSAYS

3.4.1. Export assay of bimane-GS using MCB

The formation and efflux of bimane-GS was assessed by a previously published method (Bai et al., 2004). V/HepG2 and MRP4/HepG2 cells were seeded at a density of 6×10^6 cells/2 ml per well in 6-well plates and allowed to attach for 24 h at 37°C under 5% CO_2. The next day, the medium was removed and the cells were incubated with 1 ml of cell culture medium DMEM containing 100 μM MCB at 10°C for 1 h. After pretreatment with MCB (Invitrogen, Carlsbad, CA, USA), the plates were placed on ice and the medium was removed. Then the cells were washed twice with cold HBSS (Hanks balanced salt solution, containing 5.8 mM K^+, 143 mM Na^+, 1.3 mM Ca^{2+}, 0.8 mM Mg^{2+}, 146 mM Cl^-, 0.8 mM phosphate, 4.2 mM HCO_3^-, and 10 mM HEPES, pH=7.4) without glucose (1-2 ml). Cells were further incubated with 0.6 ml HBSS/with glucose (5.6 mM) at 37°C. At the designated times (0, 5, 10, 15 min), the plate was placed on ice and 200 μl aliquots of incubation buffer were collected and placed into a black 96-well plate. The cells were then lysed with 0.6 ml of 0.2% sodium dodecyl sulfate (SDS), and the cells were detached with a rubber policeman. 200 μl aliquots of cell lysate were collected and placed into a black 96-well plate. The bimane-GS content in the sample was measured by determining the fluorescence intensity at an excitation wavelength of 385 nm and an emission wavelength of 478 nm in a Tecan Infinite® 200 series microplate reader (Tecan Instruments Inc., Research Triangle Park, NC, USA).

To quantify the amount of bimane-GS, a series of bimane-GS standards were generated based on previously described procedure (Bai et al., 2004). 0-48 μM of GSH, 1 unit/ml equine liver GST in HBSS buffer (or in HBSS buffer containing 0.2%

SDS for cell lysate) were mixed and incubated until no increase in fluorescence was observed. After 30 min of incubation at 37°C, the fluorescence of the standards was read using an excitation wavelength of 385 nm and an emission wavelength of 478 nm in a Tecan Infinite® 200 series microplate reader (Tecan Instruments Inc., Research Triangle Park, NC, USA). A calibration curve was obtained and the fluorescence of the samples was then correlated with the calibration curve.

To examine the effect of CPT analogs (CPT-11, SN-38, topotecan, rubitecan, and 10-OH-CPT) on the transport of bimane-GS, cells were incubated in 1 ml DMEM containing 100 μM MCB at 10°C for 1 h together with CPT analogs. The medium was removed and cells were washed with cold HBSS. For time-dependent effects, the efflux was carried out with 0.6 ml HBSS/with glucose (5.6 mM) in the presence of CPT analogs at 37°C for designated times (0. 5, 10, 15 min). For concentration-dependent effects, the efflux was carried out in HBSS/5.6 mM glucose containing CPT analogs at 37°C for 10 min at which the difference of GSH efflux between two cell lines reached the peak. The CPT analogs used were replaced by 1% DMSO in the control group.

3.4.2. Effect of BSO on bimane-GS export assay

To determine the effect of BSO on the bimane-GS export, cells were seeded at a density of 6×10^6 cells/2 ml per well in 6-well plates with the presence of 200 μM BSO and allowed to attach for 24 h at 37°C under 5% CO_2. For control group, cells were seeded with the presence of 1% water. After the attachment period, the cells were incubated in 1 ml of DMEM containing 100 μM MCB and 200 μM BSO at 10°C for 1 h. The medium was then removed and efflux was carried out in HBSS/5.6

mM glucose containing 200 μM BSO at 37°C. The BSO was replaced by 1% water in the control group.

CHAPTER 4 RESULTS

4.1. MRP4-MEDIATED RESISTANCE TO CPTS

The aim of the first part of the study was to determine the resistance profiles of CPT and its analogs in cancer cells overexpressing human MRP4 *in vitro*. The strategy chosen was to first validate the functionality of MRP4 in our cell model system using established MRP4 substrates such as bis-POM-PMEA and MTX and then screen time- and concentration-dependent cytotoxicity effects of CPTs *in vitro*. The effect of known inhibitors on MRP4-mediated resistance of CPTs was also examined.

4.1.1. Functionality of MRP4 in cell model system

HepG2 cells were stably transfected with empty vector and full-length cDNA of human MRP4 and were kindly provided by Dr Theresa Tan (National University of Singapore, Department of Biochemistry). This cell model was chosen based on the following reasons: (1) *MRP4* mRNA is highly expressed in kidney, lung and prostate, but absent or present in low levels in liver, placenta, and adrenal gland (Nishimura and Naito, 2005). This low MRP4 expression background in liver cells enabled us to associate cell response to our manipulations to the involvement of exogenous MRP4; and (2) liver is the main tissue responsible for the metabolism of CPTs and the liver system and its enzymes are ideal used for the study of CPTs and metabolisms (Haaz et al., 1998b; Platzer et al., 2000). To validate the functionality of this cell model system, we assessed the cytotoxicity of both bis-POM-PMEA and MTX in these cells. As shown in **Table 4-1** and **Figure 4-1**, the forced expression of MRP4 in HepG2 cells conferred 3.9- and 10.4-fold resistance to bis-POM-PMEA when the cells were exposed to the drug for 4 h and 48 h, respectively. MRP4-transfected HepG2 cells

exposed to MTX for 4 h were 4.7-fold more resistant compared with V/HepG2 cells, whereas there was no significantly increased resistance when cells were exposed to MTX for 48 h. These results for MTX were consistent with that in MRP4-transfected NIH3T3 cells as reported by Lee *et al* (Lee et al., 2000). Comparing MTX and bis-POM-PMEA, MTX was 3-fold more potent than bis-POM-PMEA in V/HepG2 cells when the cells were exposed to the drugs for 48 h (IC_{50}: 0.372 ± 0.027 *vs.* 0.110 ± 0.017 μM). However, at 4 h of drug exposure, the toxicities of bis-POM-PMEA and MTX in V/HepG2 cells were close to each other (IC_{50}: 2.40 ± 0.20 *vs.* 2.59 ± 0.51 μM). We then analyzed the statistical significance of the cytotoxic effects of bis-POM-PMEA and MTX in both V/HepG2 and MRP4/HepG2 cells using a two-way ANOVA analysis with "Drug concentration" and "Cell line" as within- and between-sample factors, respectively. Results showed that 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. The "Cell line" effect was also significant with variation ($P < 0.05$) for both bis-POM-PMEA and MTX except for MTX at drug exposure time of 48 h, indicating that MRP4/HepG2 cells had different cytotoxic profiles when incubated with bis-POM-PMEA or MTX compared to V/HepG2 cells.

Statistical significance was only observed for MTX in MRP4/HepG2 when MTX exposure was limited to the first 4 h and not 48 h of the cytotoxicity assay (**Table 4-1**). This may be attributed to increased polyglutamylation of MTX in the continuous drug exposure. In long-term exposure to MTX, cells can accumulate long-chain polyglutamylated metabolites of MTX to high enough levels causing cell death (Hooijberg et al., 1999; Kool et al., 1999). For short-term drug exposure, however,

there may not have been enough time for the conversion to the long-chain polyglutamylated forms to take place, resulting in MTX being present in free drug form. It has also been demonstrated that MRP4 is not able to transport MTX polyglutamates out of cells (Chen et al., 2002). Thus, MRP4 could not display resistance to MTX in long-term exposure due to its inability to efflux MTX in long-chain polyglutamylated form, but show resistance in short-term exposure by transporting MTX present mainly in free drug form.

The effects of BSO on the cytotoxicity of bis-POM-PMEA and MTX in V/HepG2 and MRP4/HepG2 cells were also examined. This was performed because BSO was found to be able to modulate MRP4 function by decreasing the intracellular GSH level (Lai and Tan, 2002). Cells were preincubated with 200 μM BSO for 24 h before they were exposed to bis-POM-PMEA and MTX. In MRP4/HepG2 cells, the addition of BSO significantly increased the cytotoxicity of bis-POM-PMEA by 4.0- and 3.6-fold when the cells were exposed to bis-POM-PMEA for 48 h and 4 h, respectively (**Table 4-1** & **Figure 4-2**). BSO also significantly reversed the MRP4-mediated resistance to MTX with drug exposure time of 4 h, with the IC_{50} reduced from 12.1 ± 1.3 to 5.06 ± 0.96 μM ($P < 0.05$). Interestingly, the presence of BSO significantly decreased the cytotoxicity of bis-POM-PMEA in V/HepG2 cells by 2.6- and 1.3-fold when the cells were exposed to bis-POM-PMEA for 48 h and 4 h, respectively (**Table 4-1** & **Figure 4-2**). BSO also decreased the cytotoxicity of MTX by 1.5-fold in V/HepG2 cells for the 4 h exposure assay. In contrast, BSO had little effect on the cytotoxicity of MTX for both V/HepG2 and MRP4/HepG2 cells in 48 h drug exposure assay (**Table 4-1** & **Figure 4-2**). Since MRP4 did not display any effect with long-term drug exposure, it is expected that BSO will not have any inhibitory effect on MRP4-mediated transport. Overall, we have shown that the MRP4-

overexpressing HepG2 cells conferred significant resistance to established MRP4 substrates such as bis-POM-PMEA and MTX and that such resistance was effectively reversed by the addition of the GSH biosynthesis inhibitor, BSO. These results indicate that there is proper functionality of MRP4 in cell models used in our study.

Table 4-1. Drug sensitivity of MRP4-overexpressing HepG2 cells to bis-POM-PMEA and MTX.

Drugs	Drug exposure time (h)	IC_{50} (μM)		Fold resistance
		V/HepG2	MRP4/HepG2	
Bis-POM-PMEA	48	0.372 ± 0.027	3.86 ± 0.35[a]	10.4
+ BSO	48	0.98 ± 0.13[b]	0.96 ± 0.16[b]	0.98
Bis-POM-PMEA	4	2.40 ± 0.20	9.40 ± 0.37[a]	3.9
+ BSO	4	3.05 ± 0.77[b]	2.60 ± 0.10[a,b]	0.85
MTX	48	0.110 ± 0.017	0.130 ± 0.024	1.2
+ BSO	48	0.105 ± 0.029	0.128 ± 0.017	1.2
MTX	4	2.59 ± 0.51	12.1 ± 1.3[a]	4.7
+ BSO	4	3.82 ± 0.74[b]	5.06 ± 0.96[b]	1.3

Data are reported as means ± SD. Fold resistance is calculated as IC_{50} in MRP4/HepG2 cells divided by that in V/HepG2 cells. Each experiment was performed independently at least 3 times. Two-way ANOVA test was used.

[a]$P < 0.05$, MRP4/HepG2 *vs.* V/HepG2; [b]$P < 0.05$, test drug *vs.* test drug + BSO.

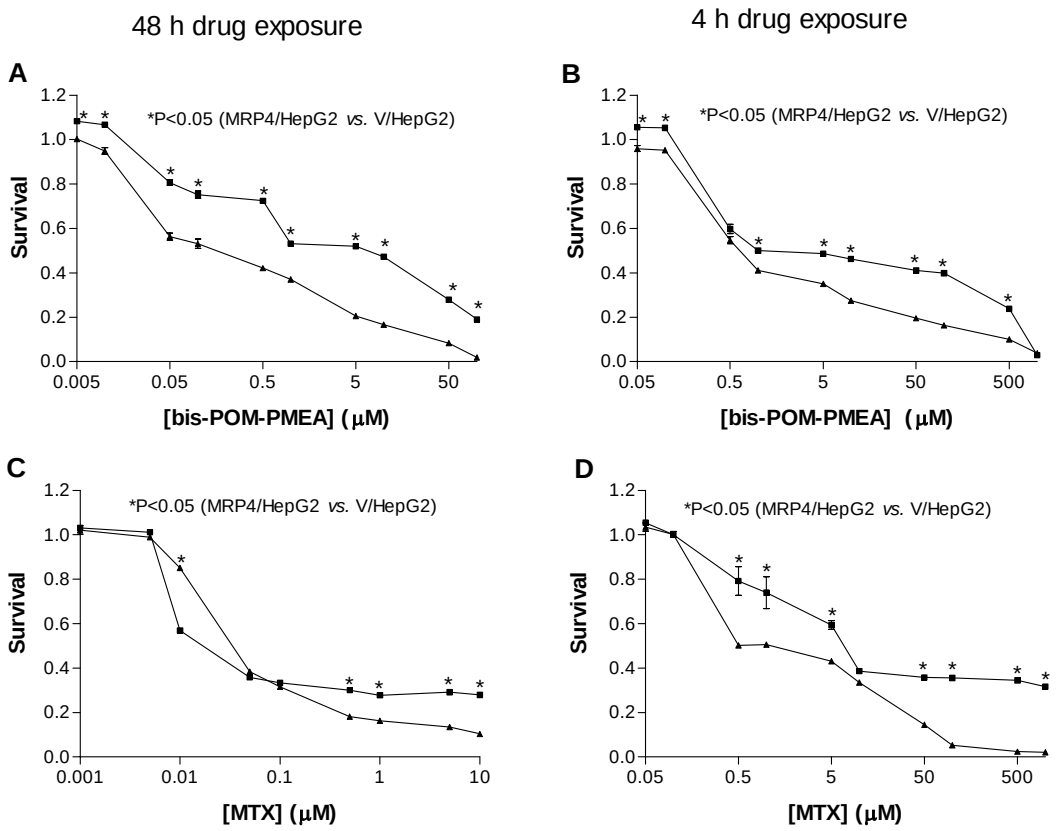


Figure 4-1. Representative cytotoxicity profiles of bis-POM-PMEA and MTX when the cells were treated with the drug for 48 h (A & C) or 4 h (B & D) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. **P* < 0.05.

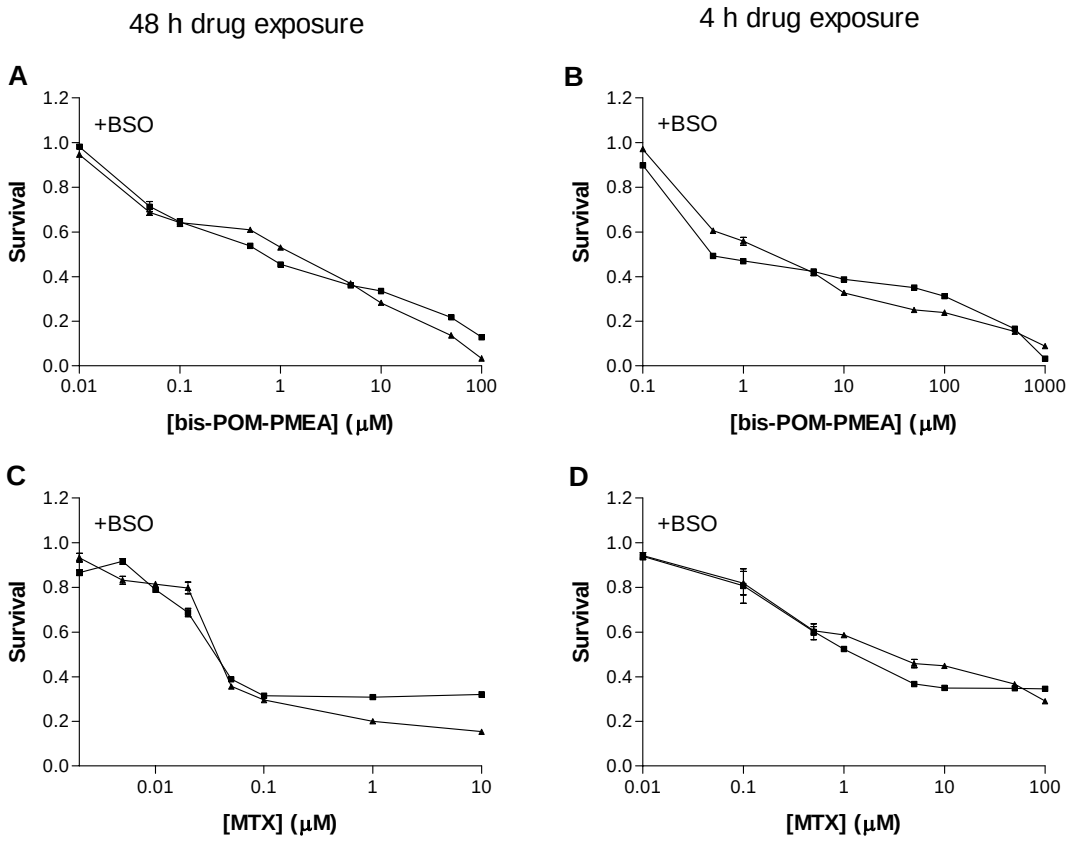


Figure 4-2. Representative cytotoxicity profiles of bis-POM-PMEA and MTX when the cells were preincubated with 200 μM BSO for 24 h and then treated with the drug for 48 h (A & C) or 4 h (B & D) in V/HepG2 (▲) and MRP4/HepG2 (■) cells.

4.1.2. Human MRP4 conferred resistance CPTs

The cytotoxicities of CPT, CPT-11 and SN-38 in lactone and carboxylate forms in both MRP4/HepG2 and V/HepG2 are shown in **Table 4-2**. We first examined their effects in V/HepG2 cells. Compared with CPT-11 lactone, CPT lactone showed 16.0- and 75.6-fold higher cytotoxicity while SN-38 lactone exhibited 13.7- and 29.2- fold higher cytotoxicity at drug exposure times of 4 h and 48 h, respectively. The lactone of CPT and SN-38 displayed similar cytotoxicity to V/HepG2 cells in 4 h drug exporsure assay, whereas CPT lactone had 2.6-fold higher cytotocixity to V/HepG2 than SN-38 latone in 48 h drug exposure assay (**Table 4-2** & **Figure 4-3**). Prolonged

drug exposure time from 4 h to 48 h significantly decreased the IC_{50} of CPT, CPT-11, and SN-38 in lactone form in V/HepG2 cells by 25.3-, 5.4-, and 11.4-fold, respectively.

CPT-11, CPT, and SN-38 in carboxylate form exhibited lesser but comparable cytotoxicity in both MRP4/HepG2 and V/HepG2 cells compared to their respective lactone (**Table 4-2** & **Figure 4-4**). This indicated that part of CPTs in carboxylate form at pH 7.4 medium was rapidly converted to active lactone form, whereas conversion to less active carboxylate occurred only when CPTs in lactone were loaded, resulting in a stable lactone percentages. The pH-dependent interconversion of lactone and carboxylate forms of CPT analogs has been widely reported; CPT in its lactone form was 11.7% at pH 7.6 (Fassberg and Stella, 1992), and 17% at pH 7.4 (Burke and Mi, 1994), while both CPT-11 and SN-38 existed in lactone form for about 13% at pH 7.4 (Burke and Mi, 1994). In V/HepG2 cells, CPT and SN-38 carboxylate displayed similar cytotoxicity. Compared with CPT-11 carboxylate, CPT carboxylate showed 24.2- and 54.3-fold higher cytotoxicity, whereas SN-38 carboxylate exhibited 30.1- and 49.5- fold greater toxicity in V/HepG2 cells at drug exposure times of 4 h and 48 h respectively (**Table 4-2** & **Figure 4-4**). It is noted that as the active metabolite of CPT-11, SN-38 in either lactone or carboxylate form showed much higher toxicity to cells than its parent drug. When the drug exposure time was changed from 4 h to 48 h, the IC_{50} of CPT, CPT-11, and SN-38 in carboxylate form in V/HepG2 cells decreased 11.7-, 5.2- and 8.5-fold respectively.

The cytotoxicities of topotecan, rubitecan, and 10-OH-CPT in lactone form in both MRP4/HepG2 and V/HepG2 cells are shown in **Table 4-3** and **Figure 4-5**. Topotecan lactone showed 24.1- and 14.3-fold higher cytotoxicity in V/HepG2 cells at drug

exposure times of 4 h and 48 h, respectively, compared with CPT-11 lactone. Rubitecan and SN-38 had similar cytotoxicity in V/HepG2 and MRP4/HepG2 cells (**Table 4-2** & **Table 4-3**). Rubitecan lactone exhibited 14.3- and 28.5-fold greater toxicity in V/HepG2 cells when the cells were exposed to rubitecan lactone for 4 h and 48 h respectively, compared with CPT-11 lactone. With a similar cytotoxicity of topotecan lactone, 10-OH-CPT displayed 19.9- and 17.8-fold higher cytotoxicity in V/HepG2 when the cells were exposed to 10-OH-CPT lactone for 4 h and 48 h respectively, compared with CPT-11 lactone (**Table 4-2** & **Table 4-3**). Prolonged drug exposure time from 4 h to 48 h significantly decreased the IC_{50} of topotecan, rubitecan, and 10-OH-CPT 3.2-, 10.6- and 4.8-fold in V/HepG2 cells respectively. For MRP4/HepG2 cells, the IC_{50} of topotecan, rubitecan, and 10-OH-CPT decreased 5.6-, 8.7- and 4.0-fold, respectively, when the drug exposure time was changed from 4 h to 48 h (**Table 4-3**).

MRP4 overexpression, on the other hand, conferred significant resistance to CPT, CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT in lactone form (7.2-, 5.6-, 8.9-, 6.9-, 9.1- , and 14.2-fold, respectively) when the exposure time of the cells for the test drugs was 48 h (**Figure 4-3** & **Figure 4-5**). At a shorter exposure time of 4 h, MRP4/HepG2 cells showed lower levels of resistance to CPT, CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT in lactone form (5.0-, 3.5-, 8.1-, 12.0-, 7.4-, and 11.9-fold, respectively) (**Figure 4-3** & **Figure 4-5**). Furthermore, MRP4 rendered 1.8- to 9.7-fold resistance to CPT, CPT-11 and SN-38 in carboxylate form when the cells were exposed to the tested CPT analog for 4 h and 48 h (**Figure 4-4**). Based on the resistance folds from the MTT assays with 48 h exposure time of the test drugs, MRP4 conferred resistance to CPTs tested in an order of from 10-OH-CPT lactone (14.2) > SN-38 carboxylate (9.7) > rubitecan lactone (9.1) > SN-38 lactone (8.9) >

CPT lactone (7.2) > topotecan lactone (6.9) > CPT-11 lactone (5.6) > CPT carboxylate (4.3) > CPT-11 carboxylate (2.7). MRP4 showed the highest resistance to 10-OH-CPT lactone, whereas CPT-11 carboxylate had the lowest resistance by MRP4 (**Table 4-2** & **Table 4-3**).

The cytotoxicity effects of various CPTs tested in both V/HepG2 and MRP4/HepG2 cells were statistically analyzed by conducting 2-way ANOVA analysis 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 h and 48 h drug exposure time, indicating that there were significantly different cytotoxicity profiles between MRP4/HepG2 and V/HepG2 cells when incubated with either of the test CPTs.

Table 4-2. Resistance profiles of CPT, CPT-11 and SN-38 in HepG2 cells overexpressing MRP4 or empty vector.

Drugs	Drug exposure time (h)	IC_{50} (μM)		Fold resistance
		V/HepG2	MRP4/HepG2	
CPT-11 (lactone)	48	2.420 ± 0.053	13.6 ± 1.3[a]	5.6
+ Celecoxib	48	2.834 ± 0.053	3.15 ± 0.50[b]	1.1
+ Diclofenac	48	4.4 ± 1.4[b]	8.10 ± 0.82[a,b]	1.8
+ MK-571	48	3.09 ± 0.13[b]	6.39 ± 0.65[a,b]	2.1
+ BSO	48	5.39 ± 0.55	4.908 ± 0.045[b]	0.91
CPT-11 (lactone)	4	12.9 ± 1.3	44.9 ± 8.0[a]	3.5
+ Celecoxib	4	10.6 ± 1.1	9.9 ± 1.7[b]	0.93
+ Diclofenac	4	10.81 ± 0.85	12.66 ± 0.23[b]	1.2
+ MK-571	4	12.9 ± 1.7	11.9 ± 1.9[b]	0.92
+ BSO	4	11.8 ± 2.0	14.4 ± 3.8[b]	1.2
CPT-11 (carboxylate)	48	3.91 ± 0.23	10.49 ± 0.48[b]	2.7
	4	20.3 ± 1.2[b]	36.1 ± 5.5[a,b]	1.8
SN-38 (lactone)	48	0.083 ± 0.005	0.741 ± 0.004[a]	8.9
+ Celecoxib	48	0.086 ± 0.001	0.349 ± 0.016[a,b]	4.1
+ Diclofenac	48	0.091 ± 0.014	0.366 ± 0.003[a,b]	4.0
+ MK-571	48	0.091 ± 0.002	0.367 ± 0.003[a,b]	4.0
+ BSO	48	0.194 ± 0.007[b]	0.207 ± 0.010[a,b]	1.1
SN-38 (lactone)	4	0.948 ± 0.009	7.70 ± 0.65	8.1
+ Celecoxib	4	0.970 ± 0.029	3.17 ± 0.13	3.3
+ Diclofenac	4	0.97 ± 0.21	3.37 ± 0.48	3.5
+ MK-571	4	0.938 ± 0.032	3.19 ± 0.20	3.4
+ BSO	4	0.95 ± 0.13	1.882 ± 0.089	2.0
SN-38 (carboxylate)	48	0.079 ± 0.007	0.766 ± 0.091[a]	9.7
	4	0.674 ± 0.061[b]	5.53 ± 0.63[a,b]	8.2
CPT (lactone)	48	0.032 ± 0.001	0.231 ± 0.050[a]	7.2
+ BSO	48	0.083 ± 0.005[b]	0.110 ± 0.013[a,b]	1.3
CPT (lactone)	4	0.808 ± 0.130	4.054 ± 0.221[a]	5.0
+ BSO	4	1.879 ± 0.017[b]	1.515 ± 0.030[a,b]	0.81
CPT (carboxylate)	48	0.072 ± 0.005	0.309 ± 0.021[a]	4.3
	4	0.840 ± 0.051[b]	2.449 ± 0.294[a,b]	2.9

Data are the means ± SD. Fold resistance is calculated as IC_{50} in MRP4/HepG2 cells over that in V/HepG2 cells. Each experiment was carried out independently at least 3 times. Two-way ANOVA test was used.

[a]$P < 0.05$, MRP4/HepG2 *vs*. V/HepG2; [b]$P < 0.05$, test drug *vs*. test drug + inhibitor.

Table 4-3. Resistance profiles of topotecan, rubitecan and 10-OH-CPT in HepG2 cells overexpressing MRP4 or empty vector.

Drug	Drug exposure time (h)	IC_{50} (μM)		Fold resistance
		V/HepG2	MRP4/HepG2	
Topotecan (lactone)	48	0.169 ± 0.011	1.16 ± 0.16[a]	6.9
+ BSO	48	0.192 ± 0.016	0.403 ± 0.047[a,b]	2.1
Topotecan (lactone)	4	0.537 ± 0.022	6.46 ± 0.21[a]	12.0
+ BSO	4	0.522 ± 0.067	1.143 ± 0.040[a,b]	2.2
Rubitecan (lactone)	48	0.085 ± 0.012	0.77 ± 0.14[a]	9.1
+ BSO	48	0.072 ± 0.002	0.153 ± 0.008[a,b]	2.1
Rubitecan (lactone)	4	0.904 ± 0.006	6.69 ± 0.36[a]	7.4
+ BSO	4	1.036 ± 0.096	1.91 ± 0.14[a,b]	1.8
10-OH-CPT (lactone)	48	0.136 ± 0.005	1.929 ± 0.021[a]	14.2
+ BSO	48	0.154 ± 0.002	0.809 ± 0.019[a,b]	5.3
10-OH-CPT (lactone)	4	0.651 ± 0.002	7.74 ± 0.78[a]	11.9
+ BSO	4	0.715 ± 0.016	1.822 ± 0.037[a,b]	2.6

Data are the means ± SD. Fold resistance is calculated as IC_{50} in MRP4/HepG2 cells over that in V/HepG2 cells. Each experiment was performed carried out independently at least 3 times. Two-way ANOVA test was used.

[a]$P < 0.05$, MRP4/HepG2 *vs*. V/HepG2; [b]$P < 0.05$, test drug *vs*. test drug + inhibitor.

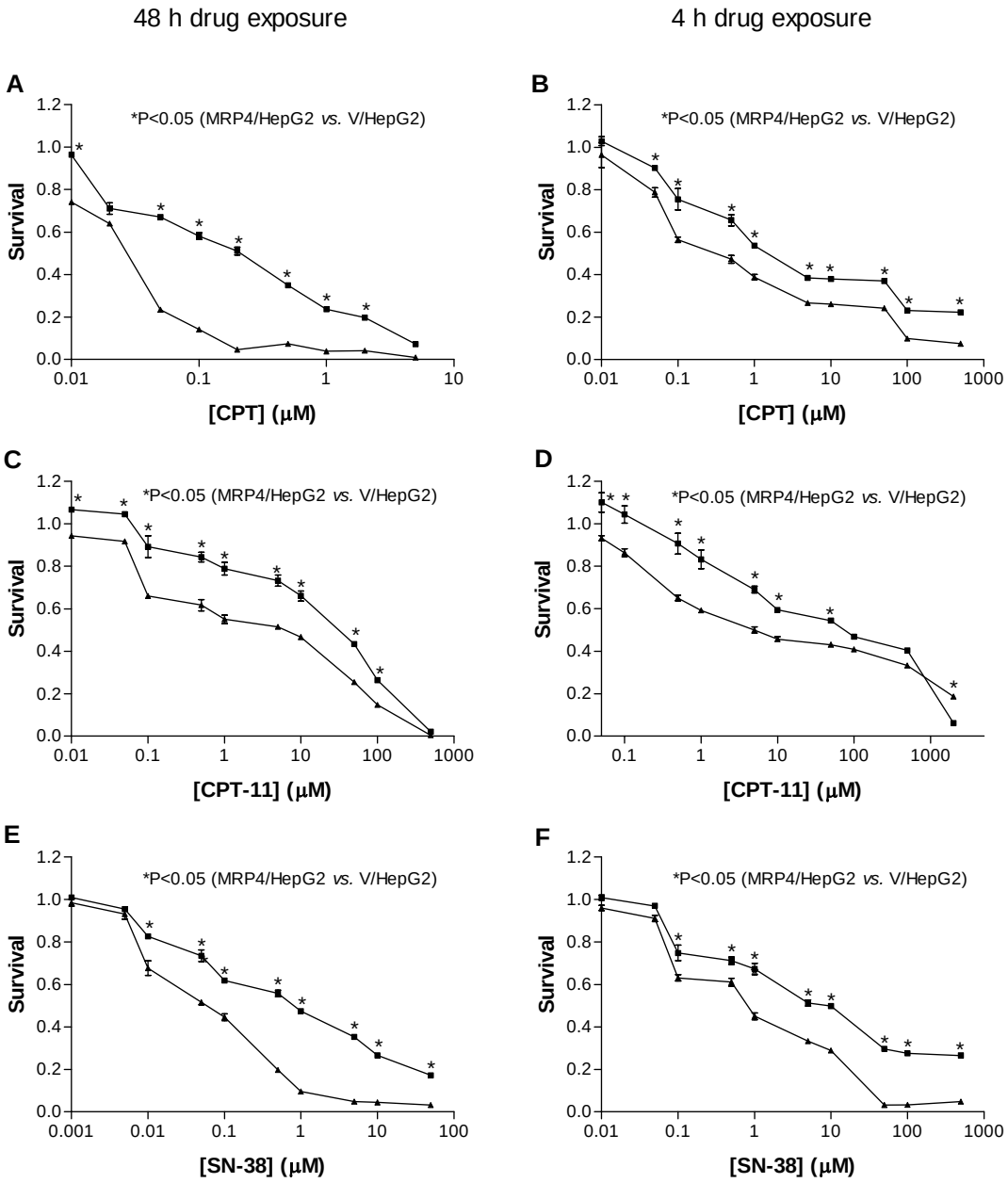


Figure 4-3. Representative cytotoxicity profiles of lactone form of CPT, CPT-11 and SN-38 when the cells were treated with the drug for 48 h (A, C & E) or 4 h (B, D & F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. **P* < 0.05.

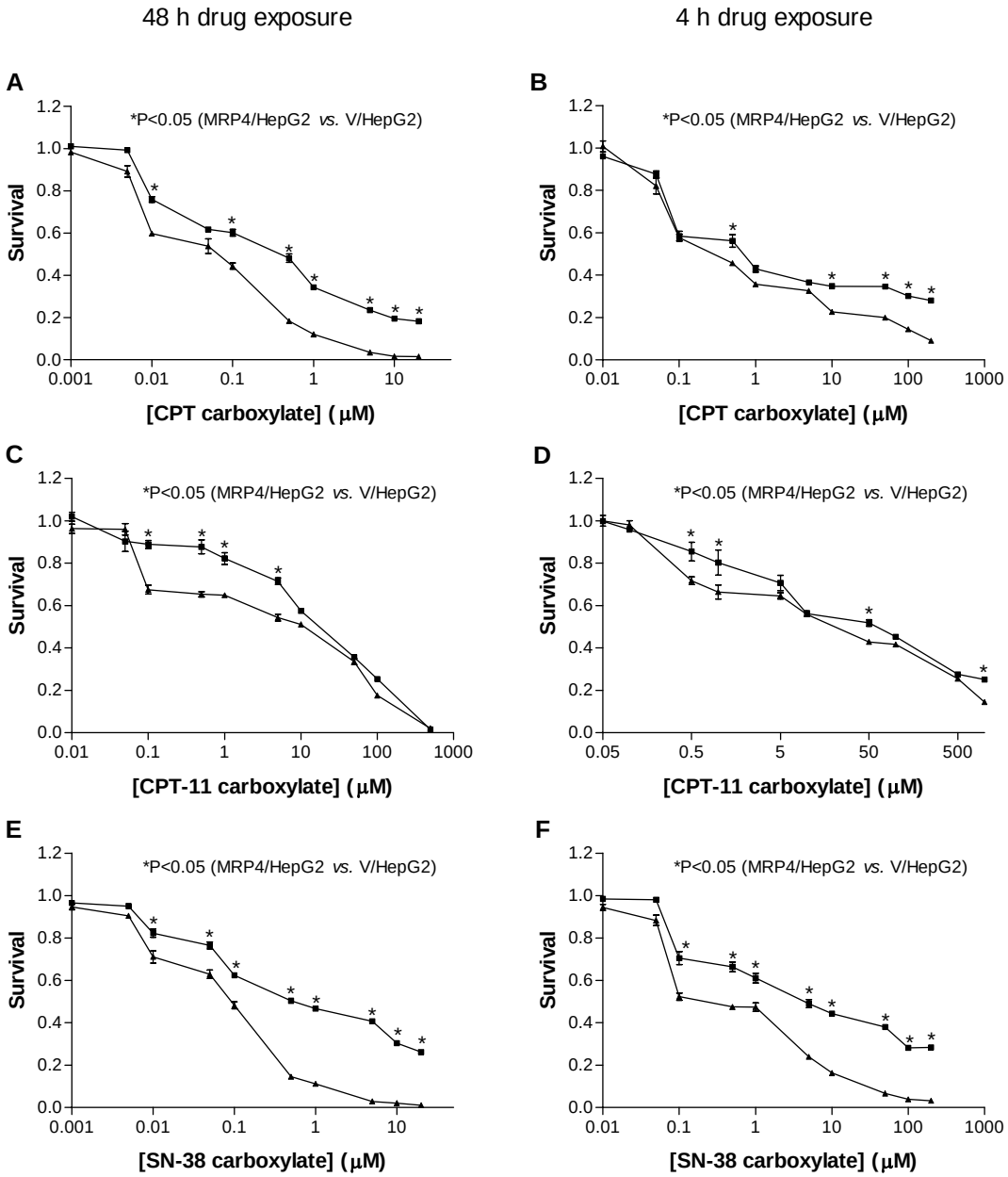


Figure 4-4. Representative cytotoxicity profiles of carboxylate form of CPT, CPT-11 and SN-38 when the cells were treated with the drug for 48 h (A, C & E) or 4 h (B, D & F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. **P* < 0.05.

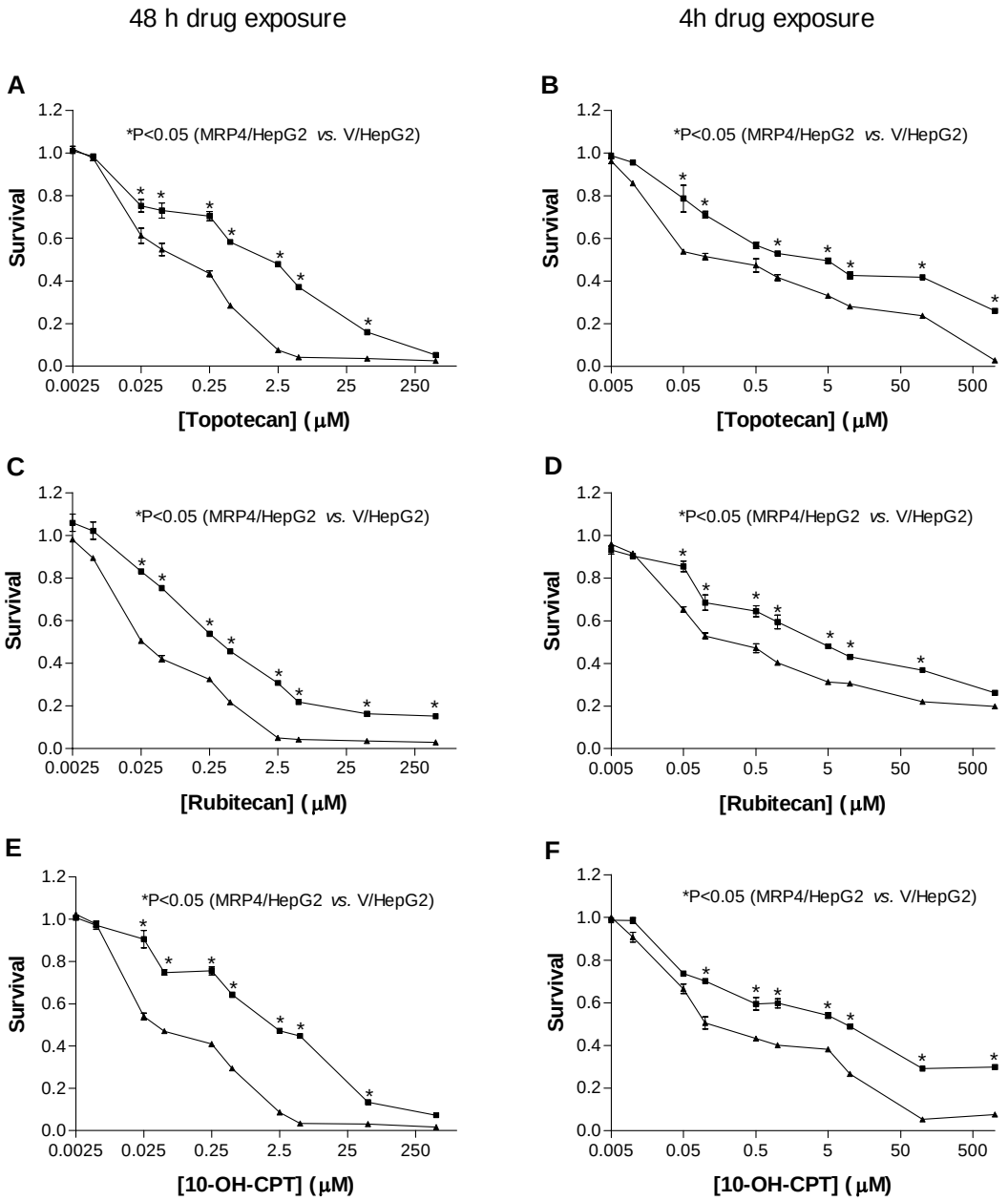


Figure 4-5. Representative cytotoxicity profiles of lactone form of topotecan, rubitecan and 10-OH-CPT when the cells were treated with the drug for 48 h (A, C & E) or 4 h (B, D & F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. **P* < 0.05.

4.1.3. Effect of BSO on MRP4-mediated resistance to CPTs

The observation that MRP4 is able to transport its established substrates (bis-POM-PMEA and MTX) and CPT derivatives indicates that the substrate range of this pump

is quite diverse. The effects of the GSH synthesis inhibitor BSO on the cytotoxicity of CPT and its analogs in V/HepG2 and MRP4-expressing cells were investigated (**Table 4-2** & **Table 4-3**). The addition of BSO significantly decreased the IC_{50} values of CPT lactone (by 52.4% and 62.6% when the cells were exposed to CPT lactone for 48 h and 4 h, respectively, **Figure 4-8**), CPT-11 lactone (by 64.1% and 68.0% when the cells were exposed to CPT-11 lactone for 48 h and 4 h, respectively, **Figure 4-6**) and SN-38 lactone (by 17.2% and 72.06% when the cells were exposed to SN-38 lactone for 48 h and 4 h, respectively, **Figure 4-7**) in MRP4/HepG2 cells (**Table 4-2**). BSO also significantly reduced the IC_{50} values of topotecan (by 65.2% and 82.3% when the cells were exposed to topotecan for 48 h and 4 h, respectively , rubitecan (by 80.1% and 71.4% when the cells were exposed to rubitecan for 48 h 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 h and 4 h, respectively) in HepG2 cell expressing MRP4 (**Table 4-3** & **Figure 4-8**). The pretreatment of HepG2 cells expressing MRP4 with BSO for 24 h resulted in cytotoxicity profiles for CPT, CPT-11, SN-38, topotecan, 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, topotecan, 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 133% to 159% in IC_{50} values in V/HepG2 cells when the cells were exposed to CPT and SN-38 for 4 h and 48 h (**Table 4-2**). This is consistent with our 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 h and 48 h. It appeared that BSO had a confounding

effect on the cytotoxicity of CPT and SN-38 lactone in V/HepG2 cells (**Table 4-2** & **Table 4-3**). The reduced cytotoxicity may be due to intracellular GSH level depletion caused by BSO, leading to functional modification of transporters other than MRP4 that play an important role in the accumlation of CPTs.

4.1.4. Effects of other inhibitors on MRP4-mediated resistance to CPT -11 & SN-38

To gain insight into the effects of other known inhibitors including MK571 (Chen et al., 2001; van Aubel et al., 2002), celecoxib, diclofenac (Reid et al., 2003b), on MRP4-mediated resistance to the CPT analogs, both V/HepG2 and MRP4/HepG2 cells were pre-incubated with celecoxib, diclofenac, or MK571 for 2 h before treatment with CPT-11 and SN-38. We found that the pretreatment of these inhibitors significantly decreased the IC_{50} values of CPT-11 and SN-38 lactone in MRP4/HepG2 cells (**Table 4-2**). Celecoxib significantly decreased the resistance fold by 76.6-78.0% for CPT-11 lactone, and 52.9-58.9% for SN-38 lactone (**Figure 4-6** & **Figure 4-7**). Addition of diclofenac resulted in significantly decreased IC_{50} of CPT-11 lactone by 40.7-71.8%, and SN-38 lactone by 50.6-56.3% (**Figure 4-6** & **Figure 4-7**). Furthermore, MK-571 significantly reduced the IC_{50} of CPT-11 lactone by 53.2-73.6% and SN-38 lactone by 50.5-58.6% (**Figure 4-6** & **Figure 4-7**). In contrast, all inhibitors used had little effect on the cytotoxicity of CPT-11, SN-38 in V/HepG2 cells (**Table 4-2**).

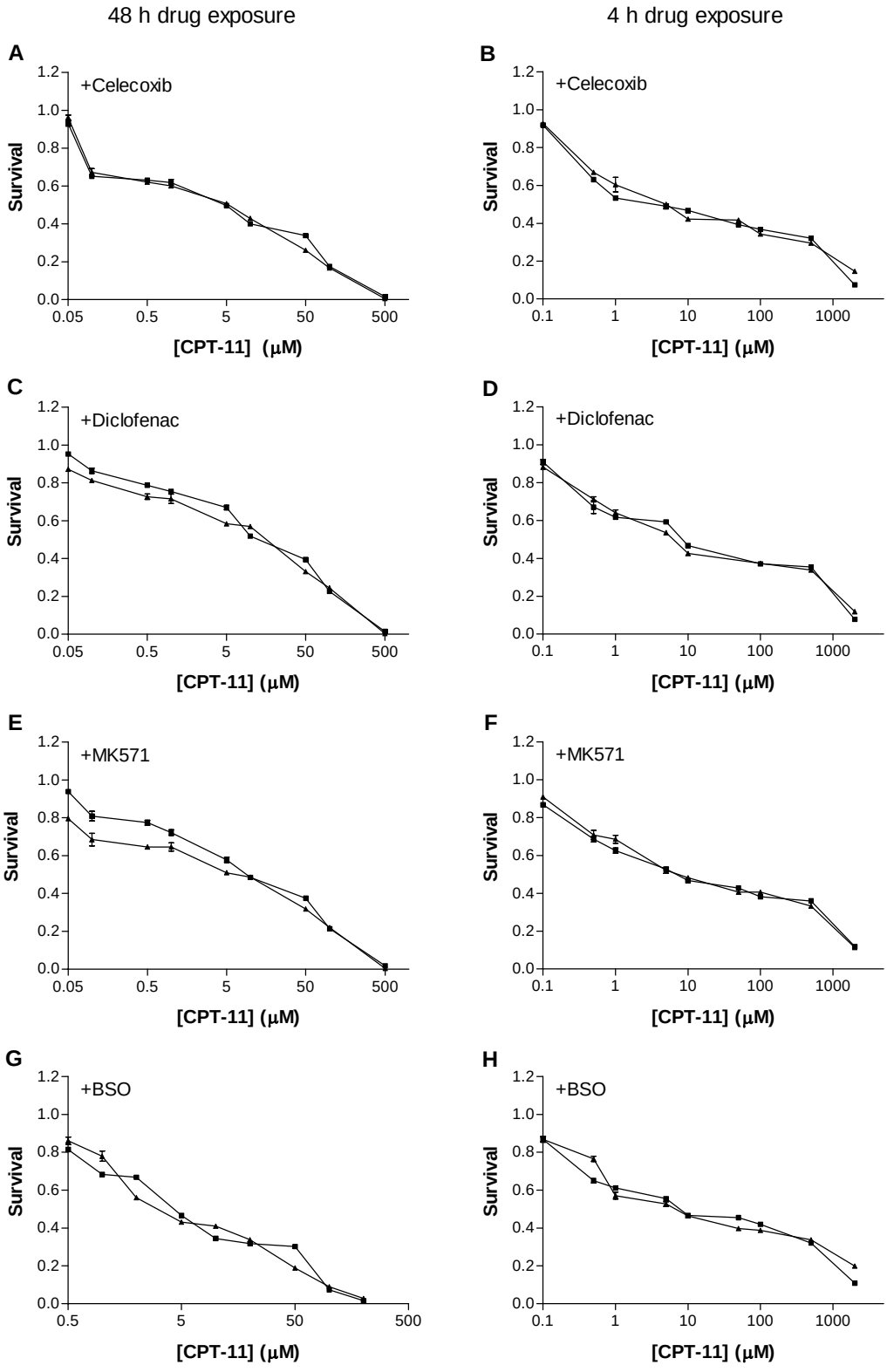


Figure 4-6. Effects of preincubation of cells with BSO at 200 μM, celecoxib at 50 μM, or MK571 at 100 μM on the cytotoxicity profiles of CPT-11 lactone when the cells were treated with CPT-11 for 48 h (A, C, E & G) or 4 h (B, D, F & H) in V/HepG2 (▲) and MRP4/HepG2 (■) cells.

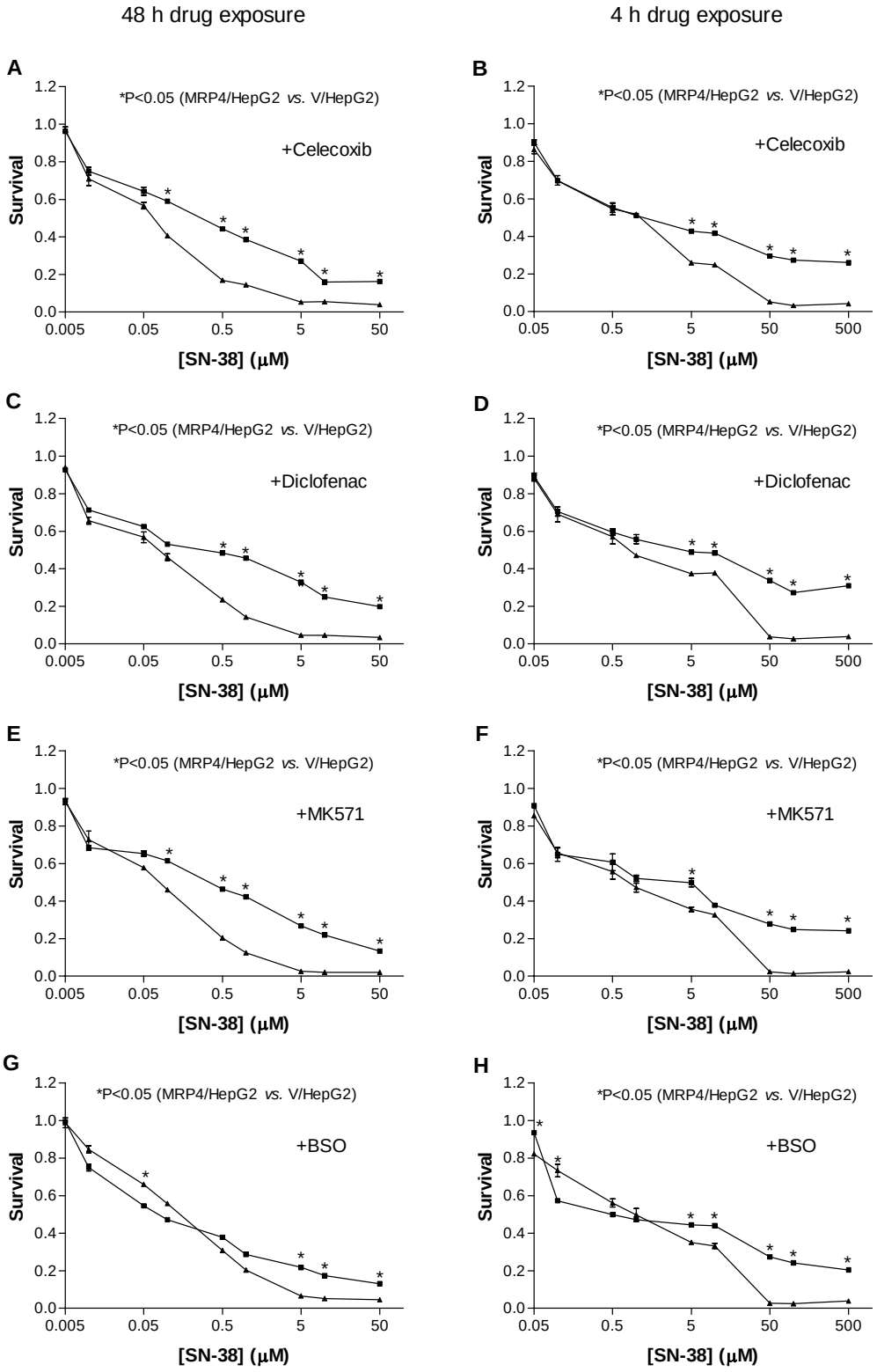


Figure 4-7. Effects of preincubation of cells with BSO at 200 μM, celecoxib at 50 μM, or MK571 at 100 μM on the cytotoxicity profiles of SN-38 lactone when the cells were treated with SN-38 for 48 h (A, C, E & G) or 4 h (B, D, F & H) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. *$P < 0.05$.

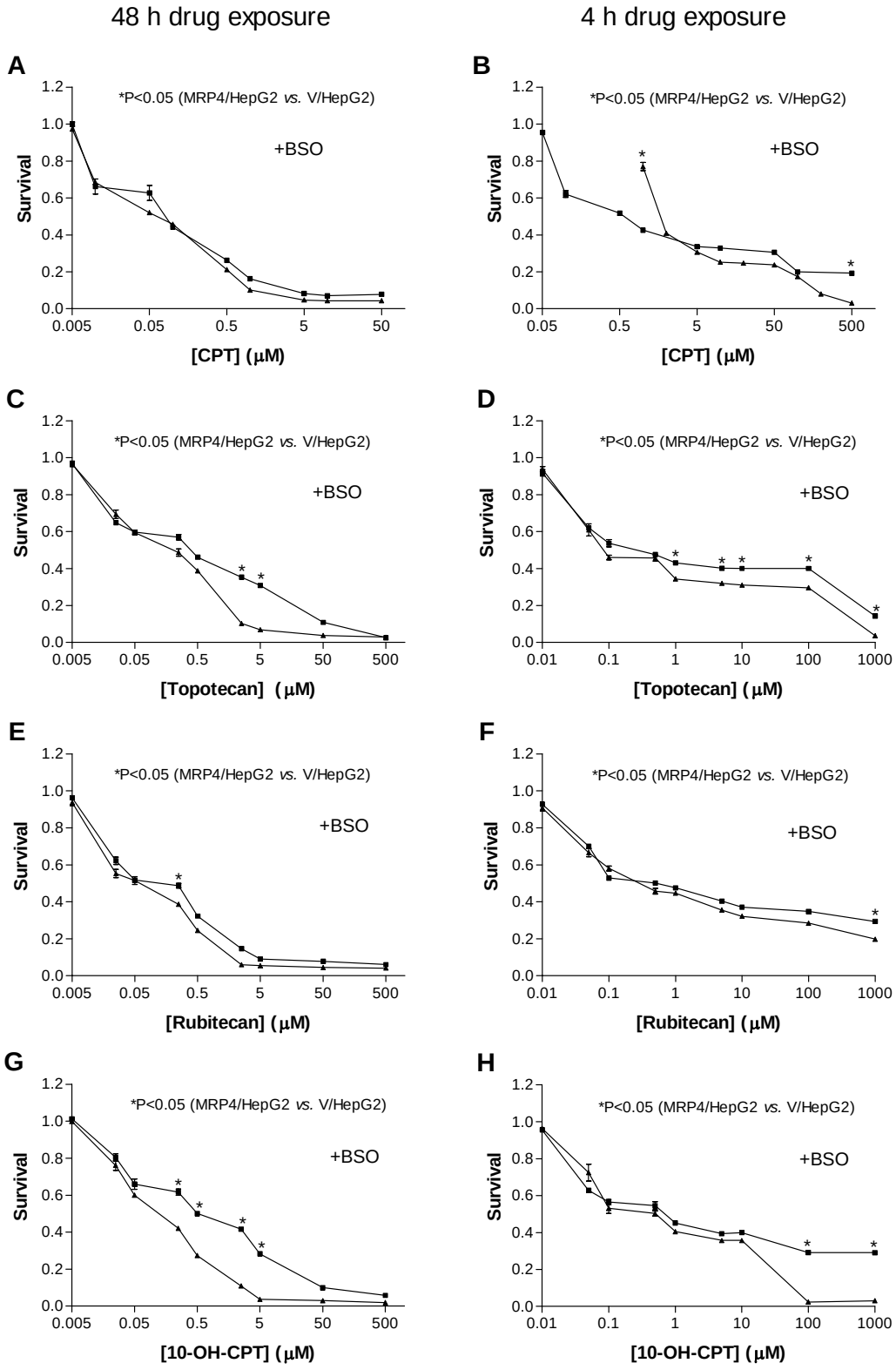


Figure 4-8. Effects of preincubation of cells with BSO at 200 μM on the cytotoxicity profiles of CPT, topotecan, rubitecan, and 10-OH-CPT lactone when the cells were treated with drugs for 48 h (A, C, E & G) or 4 h (B, D, F & H) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. **P* < 0.05.

4.1.5. Effect of MRP4 on the cytotoxicity of various anticancer drugs

To examine the specificity of our cell model system, the cytotoxicity of a panel of anticancer agents which are established substrates for various ABC transporters was examined in MRP4/HepG2 and V/HepG2 cells. The IC_{50} values are listed in **Table 4-4**. In summary, MRP4 did not exhibit any significant resistance to vinblastine, vincristine, etoposide, carboplatin, norcantharidin, 5-fluorouracil, and paclitaxel, as indicated by similar IC_{50} values in both MRP4/HepG2 and V/HepG2 cells (**Figure 4-9** & **Figure 4-10** & **Figure 4-11**), indicating MRP4 has no interaction with these drugs. There was also no significant difference in the cytotoxicity of cyclosporin A, a known PgP substrate (Augustijns et al., 1993; Gan et al., 1996), in the two strains of cells (**Table 4-4**), suggesting that PgP can be excluded as a transporter for CPT and its analogs resistance in this study. Vincristine, vinblastine and etoposide (**Table 4-4**) are typical substrates of PgP and MRP1-3 (Zeng et al., 1999; Borst et al., 2000). In NIH3T3 cells transfected with MRP4, no significant resistance is observed to etoposide, vincristine, and paclitaxel, which is consistent with our drug screening results (Lee et al., 2000). The possibility of other transporters instead of MRP4 playing a role in the resistance to CPTs may thus be excluded. However, MRP4 conferred 4.0- and 3.2-fold resistance to cyclophosphamide in cytotoxicity assays with 48 h and 4 h drug exposure time, respectively (**Figure 4-9**). A 2-way ANOVA analysis indicated that the cytotoxicity of cyclophosphamide to V/HepG2 and MRP4/HepG2 cells was drug concentration-dependent and there was significantly different cytotoxic profile between MRP4/HepG2 and V/HepG2 cells when incubated with cyclophosphamide. These initial studies indicate that cyclophosphamide may be a newly-characterized MRP4 substrate but further studies are warranted to elucidate this relationship.

Table 4-4. Cytotoxicity of various anticancer drugs in HepG2 cells expressing MRP4 and empty vector.

Drug	Drug exposure time (h)	IC_{50} (μM)*		Fold resistance
		V/HepG2	MRP4/HepG2	
Etoposide	48	0.81 ± 0.16	0.94 ± 0.18	1.2
	4	2.50 ± 0.48	2.83 ± 0.14	1.1
Cylophosphamide	48	10.4 ± 1.1	41.7 ± 7.3[a]	4.0
	4	38.46 ± 0.39	121.84 ± 0.24[a]	3.2
5-Fluorouracil	48	1.027 ± 0.056	1.07 ± 0.16	1.1
	4	6.135 ± 0.044	6.701 ± 0.090	1.1
Norcantharidin	48	14.9 ± 1.4	15.50 ± 0.42	1.0
	4	24.4 ± 1.9	24.98 ± 0.48	1.0
Carboplatin	48	5.79 ± 0.25	6.01 ± 0.56	1.0
	4	59.5 ± 2.7	58.49 ± 2.44	0.98
Vincristine	48	0.77 ± 0.14	0.75 ± 0.18	0.97
	4	1.73 ± 0.40	1.80 ± 0.24	1.0
Vinblastine	48	0.232 ± 0.025	0.288 ± 0.005	1.2
	4	2.13 ± 0.28	2.07 ± 0.14	0.97
Paclitaxel	48	3.548 ± 0.044	3.753 ± 0.091	1.1
	4	21.8 ± 1.3	21.27 ± 0.56	0.98
Cyclosporin A**	48	0.699 ± 0.144	0.754 ± 0.037	1.08
	4	6.99 ± 0.34	7.56 ± 0.55	1.1

Data are the means ± SD. Fold resistance is calculated as IC_{50} in MRP4/HepG2 cells over that in V/HepG2 cells. Each experiment was performed independently at least 3 times.

*IC_{50} was in μM for all drugs, except vincristine, vinblastine and paclitaxel for which nM was used.

** It is not an anticancer agent.

[a]$P < 0.05$ by Student's *t* test, MRP4/HepG2 *vs.* V/HepG2.

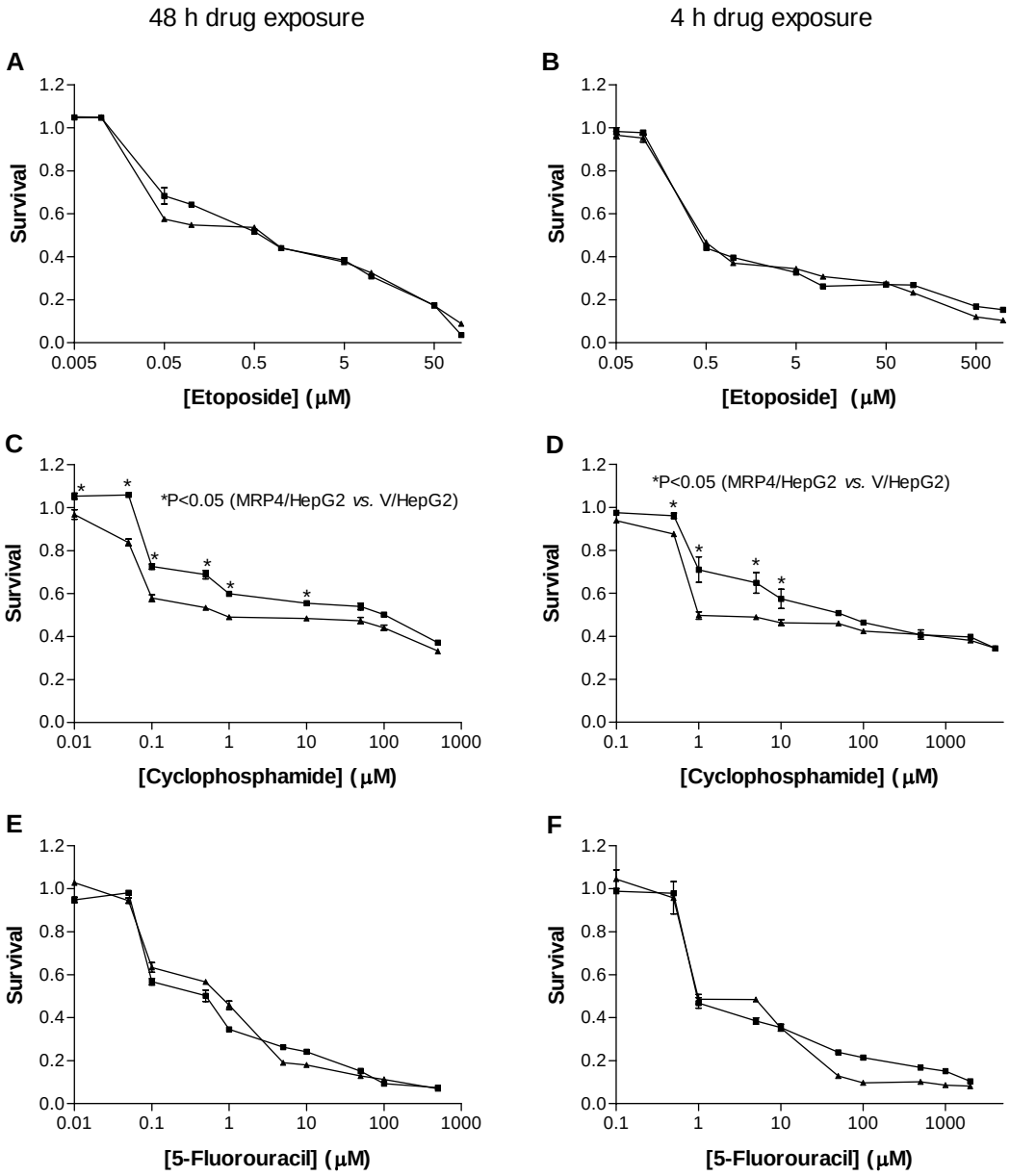


Figure 4-9. Representative cytotoxicity profiles of etoposide, cyclophosphamide and 5-fluorouracil when the cells were treated with the drug for 48 h (A, C & E) or 4 h (B, D & F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells. Student's *t* test was used. **P* < 0.05.

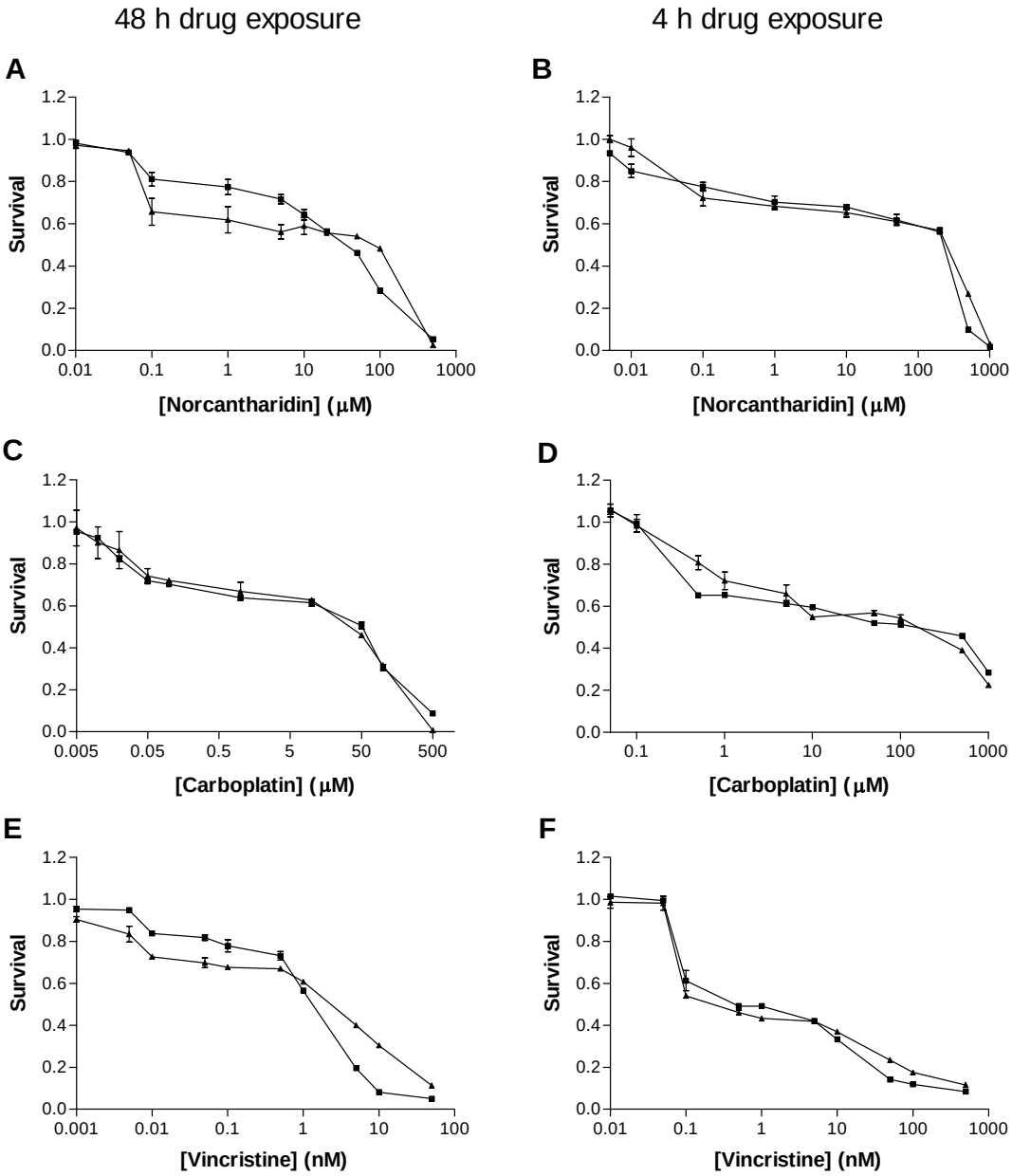


Figure 4-10. Representative cytotoxicity profiles of norcantharidin, carboplatin and vincristine when the cells were treated with the drug for 48 h (A, C & E) or 4 h (B, D & F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells.

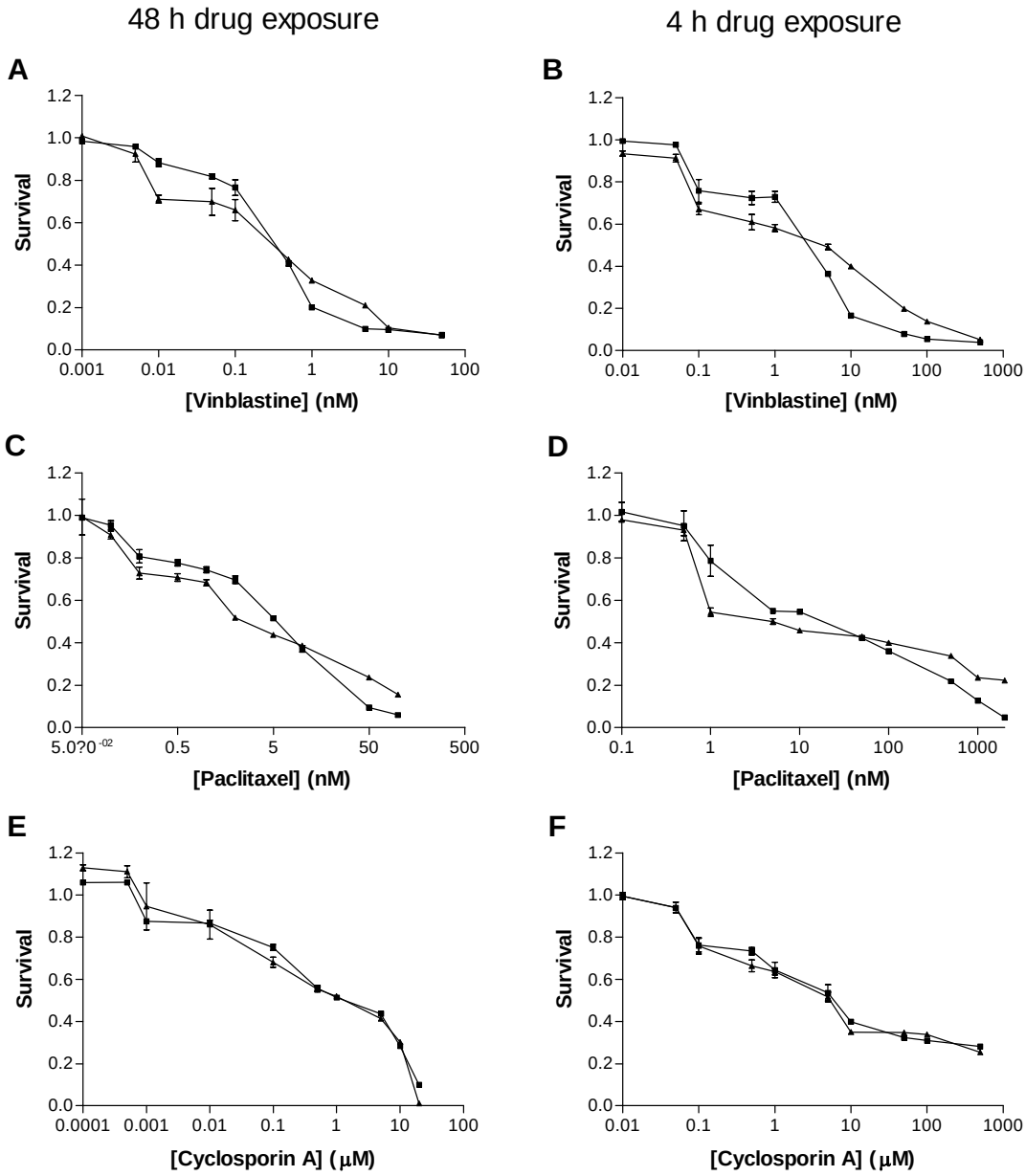


Figure 4-11. Representative cytotoxicity profiles of vinblastine, paclitaxel and cyclosporin A when the cells were treated with the drug for 48 h (A, C & E) or 4 h (B, D & F) in V/HepG2 (▲) and MRP4/HepG2 (■) cells.

4.1.6. Western blot analysis of related ABC transporters expression

We also verified the protein expression of MRP4 and some related ABC transporters using western blot in V/HepG2 and MRP4/HepG2 cells. The protein extracted from a series of MDCKII cell lines with overexpression of PgP, MRP1, MRP2, MRP3 and

MRP5 was used as the positive control of related transporters respectively (Bakos et al., 1998; Evers et al., 1998; Kool et al., 1999; Scheffer et al., 2000; Wijnholds et al., 2000). The protein from MCF/MX cells was used as the positive control of BCRP (Suzuki et al., 2003). The western blot results showed that MRP4 was overexpressed in MRP4/HepG2, and was hard to be detected in V/HepG2 (**Figure 4-12**). No detectable protein expression of other related ABC transporters, including PgP, MRP1, MRP2, MRP3, MRP5 and BCRP (**Figure 4-12**), was observed suggesting the exclusion of their involvements in the response difference to CPTs in the two cell lines. The difference of cytotoxicity of CPTs between V/HepG2 and MRP4/HepG2 is mainly due to the high expression level of MRP4.

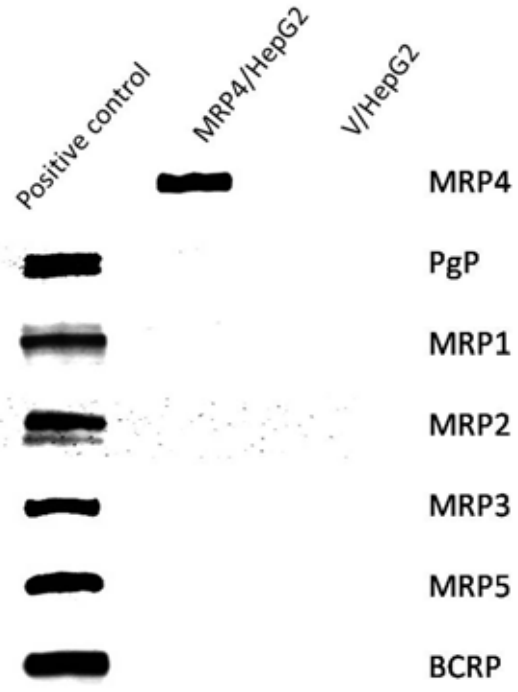


Figure 4-12. MRP4 and some related ABC transporters expression in V/HepG2 and MRP4/HepG2 cells. A series of MDCKII cell lines with the overexpression of PgP, MRP1, MRP2, MRP3 and MRP5 were used as positive controls of corresponding transporters. Each transporter was specifically recognized by its corresponding monoclonal antibody. Blots were developed with chemiluminescence and exposed to Alpha Innotech's FluorChem™.

4.2. UPTAKE AND TRANSPORT OF CPT ANALOGS BY MRP4

We have shown in the previous section that MRP4 overexpression conferred significant resistance to a panel of CPTs (in both lactone and carboxylate forms), which could be reversed by known inhibitors using cell proliferation assays. Clearly, the resistance magnitude of MRP4 to some CPTs, including CPT, CPT-11, and SN-38, is different in both the lactone and carboxylate form. This may possibly be due to different affinity and transport capacity of MRP4 to both forms. As this may have important clinical implications in the transport and disposition of CPTs given that CPTs undergo rapid interconversion *in vivo*, we thus set our second aim to characterize MRP4-mediated uptake, accumulation, and transport of CPT analogs.

4.2.1. Time-dependent intracellular accumulation of CPT analogs

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 **Figure 4-13** A & 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 cells 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. V/HepG2 cells accumulated 1.3- to 2.7-fold more CPT-11 than MRP4/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 5 min in MRP4/HepG2 and V/HepG2 cells; and then declined over the rest of the time. A similar maximum cellular concentration profile has been found in other cell lines, including HCT116,

HT29 and A2780 ovarian cancer cells, whose peak intracellular drug concentrations were detected at the earliest 5 min time point (Cummings et al., 2002b). Overall, MRP4/HepG2 cells accumulated 2.5- to 6-fold less SN-38 than V/HepG2 cells.

Cellular Accumulation of Topotecan

As shown in **Figure 4-13** C, the intracellular accumulation of topotecan in MRP4/HepG2 cells over 120 min was significantly lower than in V/HepG2 cells ($P < 0.05$). V/HepG2 cells accumulated 1.3- to 3.0-fold more topotecan than MRP4/HepG2 cells. The uptake of topotecan by V/HepG2 cells achieved the peak concentration by 10 min (1.46 ± 0.14 ng/10^6 cells), and then declined up to 120 min. Notably, the uptake profile of topotecan in MRP4/HepG2 cells was significantly different from that in V/HepG2 cells. Maximal topotecan accumulation was rapidly achieved within 5-10 min in MRP4/HepG2 cells and remained stable over the rest of the time (0.463 ± 0.011 - 0.487 ± 0.02 ng/10^6 cells).

Cellular Accumulation of Rubitecan

As shown in **Figure 4-13** D, the intracellular accumulation of rubitecan in MRP4/HepG2 cells over 120 min was significantly lower than in V/HepG2 cells ($P < 0.05$). V/HepG2 cells accumulated 1.3- to 2.7-fold more rubitecan than MRP4/HepG2 cells. A similar uptake manner of rubitecan was observed in V/HepG2 and MRP4/HepG2 cells with the highest intracellular concentration being detected at the earlist time point studied (2 min). After 60 min, intracellular concentration stopped declining and stabilized at a level that was maintained till 120 min.

Cellular Accumulation of 10-OH-CPT

As shown in **Figure 4-13** E, the intracellular accumulation of 10-OH-CPT in V/HepG2 cells over 120 min was significantly lower than in MRP4/HepG2 cells ($P < 0.05$). This result was not in agreement with the results of other CPTs (CPT-11, SN-38, topotecan, and rubitecan). In 48 h and 4 h MTT assay, MRP4 conferred 12- to 14-fold resistance to 10-OH-CPT in MRP4/HepG2 cells (**Table 4-3**). The time-dependent cellular accumulation data did not achieve the consistent conclusion with the cytotoxicity profile of 10-OH-CPT. Thus, the resistance of MRP4/HepG2 to 10-OH-CPT might not be directly caused by reduced cellular accumulation.

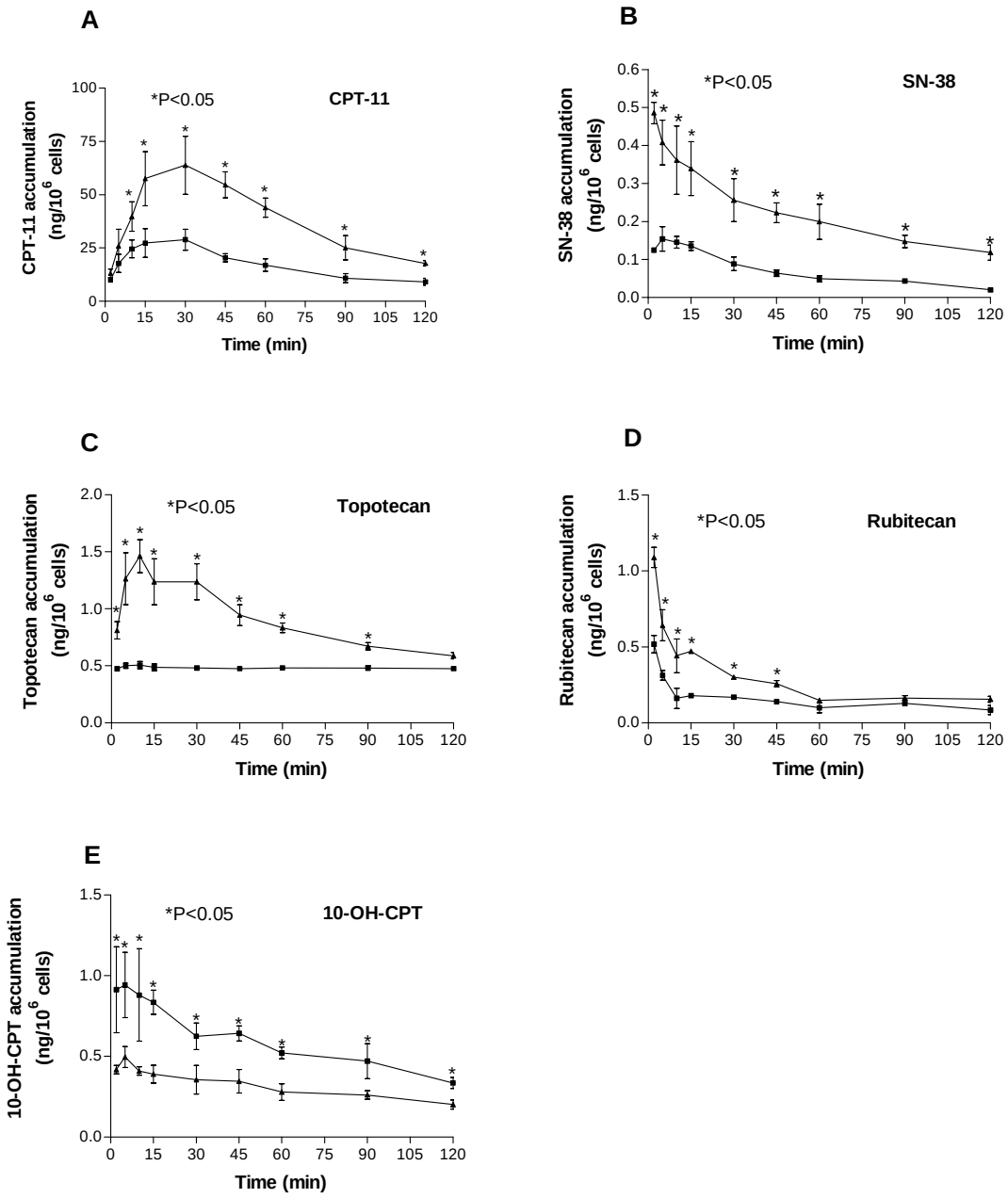


Figure 4-13. Time-dependent intracellular accumulation of CPT-11 (A), SN-38 (B), topotecan (C), rubitecan (D), and 10-OH-CPT (E) lactone over 120 min in V/HepG2 (▲) and MRP4/HepG2 (■) cells. About 10^7 cells were exposed to CPT analogs (5 μM CPT-11 or 1 μM SN-38, or 1 μM topotecan, or 5 μM rubitecan, or 1 μM 10-OH-CPT lactone) at 37°C. At indicated time, cells were rinsed with ice-cold PBS. Cells were then harvested, sonicated and acidified. The concentration of CPT analogs was determined by validated HPLC with fluorescence or UV detection. Data represent means ± SD. Student's *t* test was used. **P* < 0.05.

4.2.2. Concentration-dependent intracellular accumulation of CPT analogs

The concentration-dependent uptake of CPT analogs (0.1-10 μM CPT-11 for 30 min; 0.05-5 μM SN-38 or 10-OH-CPT for 5 min; 0.05-5 μM topotecan for 10 min; 1-25 μM rubitecan for 5 min) by V/HepG2 and MRP4/HepG2 was examined. The optimal time point for each drug was the time with the highest intracellular concentration of CPT analogs, which was chosen according to results of the time-dependent uptake assay (**Figure 4-13**). As shown in **Figure 4-14**, the uptake of CPT analogs in both cell lines increased rapidly in a concentration-dependent manner. The intracellular accumulation rate of CPT analogs in V/HepG2 increased faster than in MRP4/HepG2, with the exception of 10-OH-CPT. Overall, the accumulation rate of CPT-11 and SN-38 in V/HepG2 cells was 1.2-3.0 folds and 2.1-3.1 folds higher than that in MRP4/HepG2 cells, respectively. The accumulation rate of topotecan and rubitecan in V/HepG2 cells was 1.5-8.4 folds and 2.7-6.7 folds higher than that in MRP4/HepG2 cells, respectively. In contrast, the accumulation rate of 10-OH-CPT in V/HepG2 cells was lower than that in MRP4/HepG2 cells.

MRP4-mediated efflux of CPTs was estimated by fitting the data obtained from subtracting the accumulation rate of CPT analogs in MRP4/HepG2 cells from that in V/HepG2 cells (**Figure 4-15**). One-binding site model was found to give the best fit for the resultant data. Following the Michaelis-Menten equation, the estimated kinetic parameters of CPT-11, SN-38, topotecan, and rubitecan were summarized in **Table 4-5**. In contrast, MRP4 exhibited a much higher affinity (or a lower K_m value) toward topotecan than other CPTs examined. The highest V_{max} was observed for CPT-11, which was about two times of that of SN-38, and the lowest V_{max} was for topotecan. If the efflux activity (V_{max}/K_m) is considered (Chu et al., 1997b), the contribution of

MRP4 to the efflux of CPT-11 was approximately three times higher than that of SN-38. Topotecan efflux mediated by MRP4 was about two times of that of SN-38. MRP4 had lowest efflux activity for rubitecan.

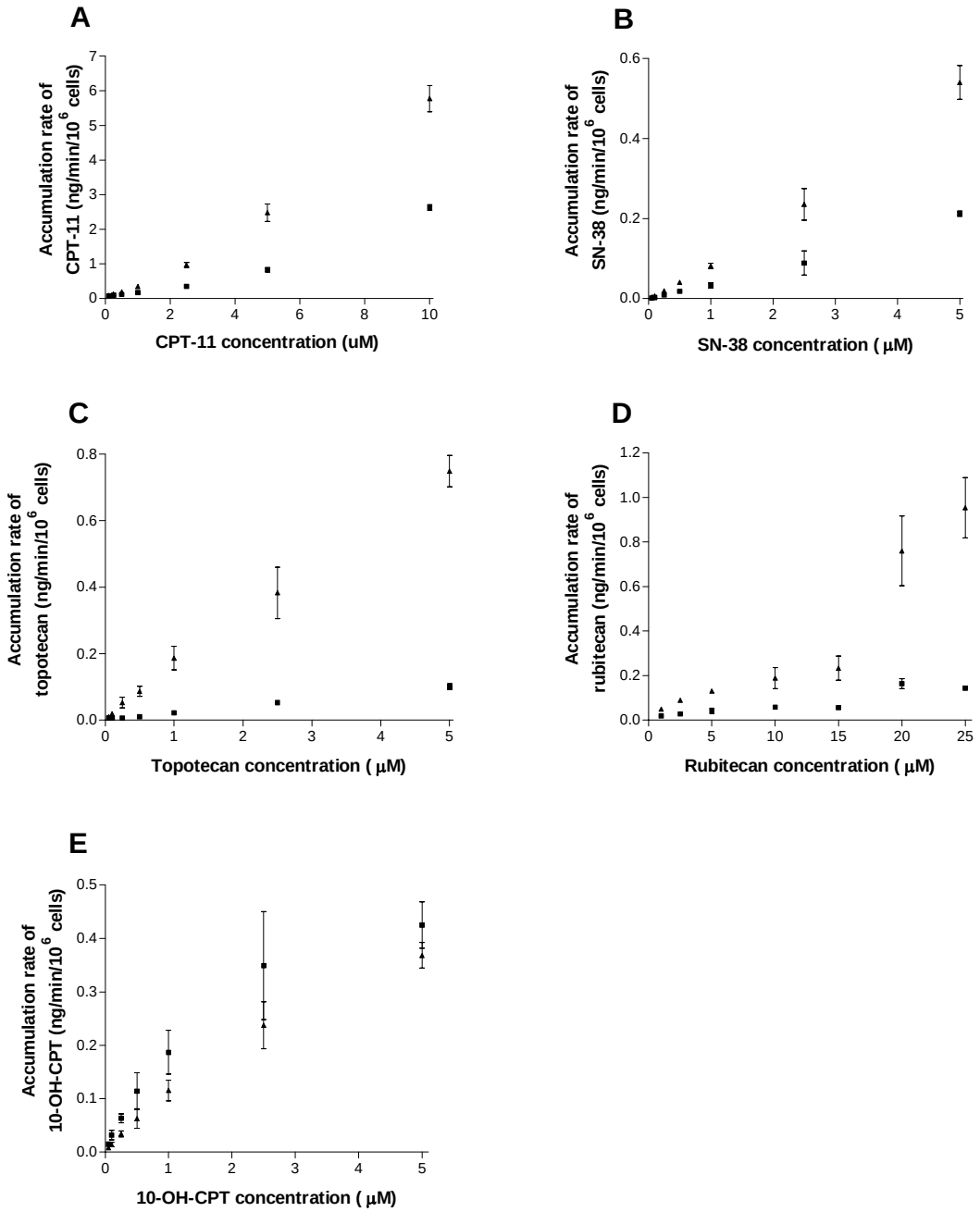


Figure 4-14. Concentration-dependent intracellular accumulation of CPT-11 (A), SN-38 (B), topotecan (C), rubitecan (D), and 10-OH-CPT (E) lactone in V/HepG2 (▲) and MRP4/HepG2 (■) cells. About 10^7 cells were exposed to different concentrations of CPT analogs at 37°C. After the fixed incubation time (30 min for CPT-11; 10 min for topotecan; 5 min for SN-38, rubitecan, and 10-OH-CPT), cells were rinsed with ice-cold PBS. Cells were then harvested, sonicated and acidified. The concentrations of CPT analogs were determined by validated HPLC with fluorescence or UV detection. Data represent means ± SD.

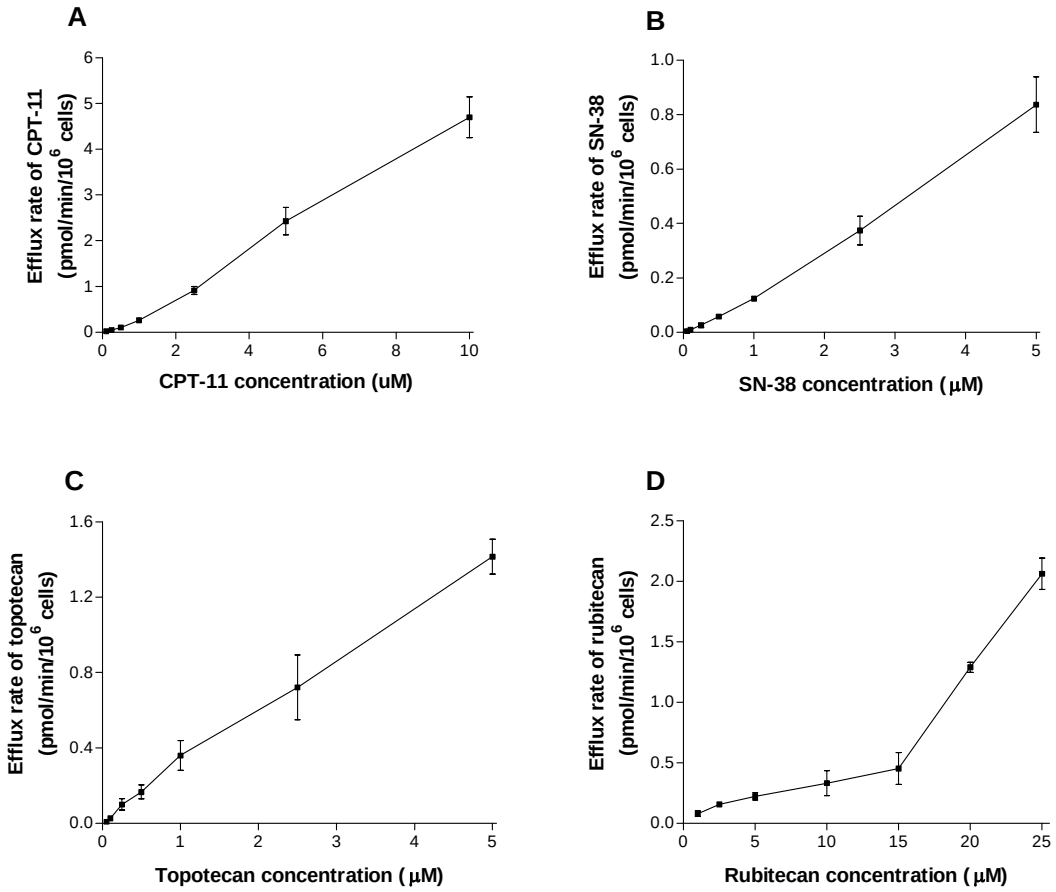


Figure 4-15. The MRP4-mediated efflux of CPT analogs by fitting the data resulting form subtracting the accumulation of CPT analogs (CPT-11 (A), SN-38 (B), topotecan (C), and rubitecan (D) lactone) in MRP4/HepG2 cells from that in V/HepG2 cells. Data represent means ± SD.

Table 4-5. Estimated kinetic parameters for the concentration-dependent efflux of CPT analogs by MRP4.

Substrates	K_m (mM)	V max (nmol/min/10^6 cells)	V_{max}/K_m ratio
CPT-11	1166	543.3	0.466
SN-38	1794	290.7	0.162
Topotecan	0.0309	0.0101	0.327
Rubitecan	906	58.36	0.0644

4.2.3. Effects of inhibitors on the intracellular accumulation of CPT analogs

The effects of inhibitors on the intracellular accumulation of CPT analogs were examined. The data for the intracellular accumulation of CPT analogs in lactone form in MRP4/HepG2 cells, with the preincubation with 200 μM BSO, 50 μM celecoxib or 100 μM MK-571, are shown in **Figure 4-16**. Pretreatment of the MRP4/HepG2 cells with 200 μM BSO for 24 h resulted in significantly ($P < 0.05$) increased accumulation of CPT-11 lactone over 30 min by 15%. Preincubation of MRP4/HepG2 cells with celecoxib (50 μM) or MK-571 (100 μM) for 2 h also significantly increased the accumulation of CPT-11 lactone by 16% and 13% ($P < 0.05$) over 30 min, respectively (**Figure 4-16** A). Similarly, preincubation of BSO, celecoxib or MK-571 significantly ($P < 0.05$) increased the accumulation of SN-38 lactone over 5 min in MRP4/HepG2 cells by 22 %, 47.0% and 86%, respectively (**Figure 4-16** B). The accumulation of topotecan over 10 min in MRP4/HepG2 was also slightly but significantly increased by 11% by the preincubation of BSO. Celecoxib or MK-571 significantly increased the accumulation of topotecan over 10 min by 72%, and 68% ($P < 0.05$), respectively (**Figure 4-16** C). In contrast, BSO, celecoxib and MK-571 showed insignificant effect on the accumulation of these three CPT analogs in V/HepG2 cells (99%-105%, compared with control group), and this may provide partial explanation for the negligible effect of inhibitors on the cytotoxicity of CPT-11, SN-38 and topotecan in V/HepG2 cells. It is also indicated that the possible involement of MRP4 or other transporters, such as MRP1-3, in the cellular uptake of CPT-11, SN-38 and topotecan might be excluded in V/HepG2 cells.

For rubitecan and 10-OH-CPT, BSO had no significant effect on their accumulation over 5 min in both V/HepG2 and MRP4/HepG2 cells (**Figure 4-16** D & E). Celecoxib

or MK-571 significantly increased the accumulation of rubitecan over 5 min by 33%, and 18% ($P < 0.05$), respectively (**Figure 4-16** D). In contrast, the accumulation of 10-OH-CPT in MRP4/HepG2 cells was significantly decreased 62% and 47% by celecoxib and MK571, respectively (**Figure 4-16** E). Interestingly, celecoxib and MK571 decreased the accumulation of rubitecan (27-49%), and 10-OH-CPT (44-52%) in V/HepG2, respectively. There may be a competition between drugs (10-OH-CPT and rubitecan) and inhibitors for the binding sites on cellular proteins. The fewer available binding sites on cellular proteins possibly led to the apparent decreased accumulation of the test drugs in cells.

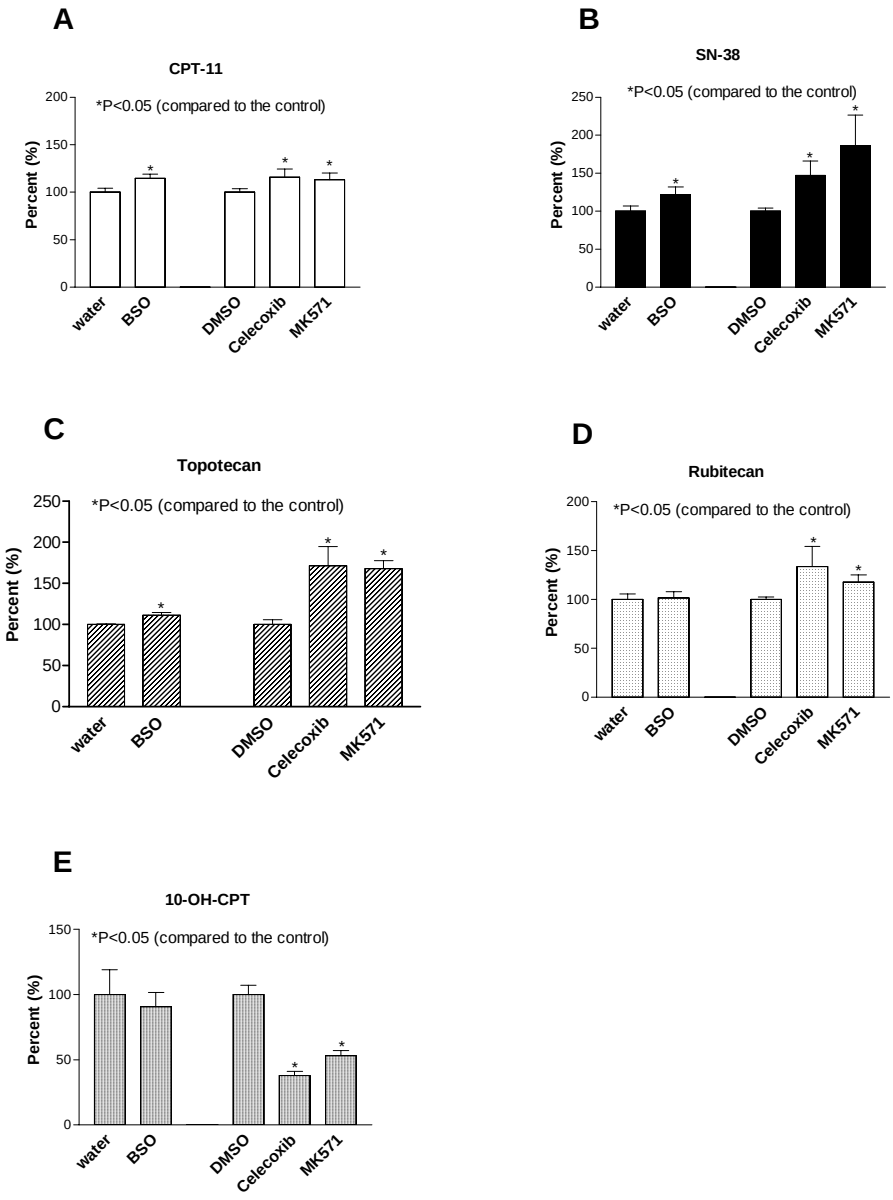


Figure 4-16. Effects of of preincubation of MRP4/HepG2 cells with BSO at 200 μM, celecoxib at 50 μM, or MK-571 at 100 μM on the accumulation of CPT analogs (CPT-11 (A), SN-38 (B), topotecan (C), rubitecan (D), and 10-OH-CPT (E) lactone). Both celecoxib and MK-571 were prepared by dissolution in DMSO, while BSO was dissolved in water. Celecoxib or MK-571 was preincubated with cells for 2 h, while BSO was preincubated for 24 h. Thereafter, cells were washed with warm PBS buffer for 4 times. After continued incubation for a fixed time point for CPT analogs (30 min for CPT-11; 10 min for topotecan; 5 min for SN-38, rubitecan, and 10-OH-CPT) treated cells were washed with ice-cold PBS. The cells were then harvested, lysed by sonication and extracted. The concentration of CPT analogs was determined by validated HPLC with fluorescence or UV detetion. Data represent means ± SD. One-way ANOVA test was used. *$P < 0.05$.

4.2.4. Intracellular accumulation of CPT-11 and SN-38 in MRP4 mutants

Many factors are involved in the substrate specificity of the transporters. It is of great interest to examine the relationship between the alterations of amino acid sequences in the transporters and their substrate specificity. Only limited information is available to date regarding the effect of amino acids position on the function properties of MRP family members (Deeley et al., 2006). Moreover, the relationship between amino acid structure and the specific transport capability of MRP4 is not clear (Russel et al., 2008). We collaborated with Dr. Theresa Tan of Department of Biochemistry (National University of Singapore, Singapore) to examine the effect of amino acid residue change on the MRP4 function. Since MRP1 is the only member in MRPs family which is under extensive investigation on the structure-function relationship (Deeley et al., 2006), the mutants of MRP4 were designed according to the related information obtained from MRP1. Trp residues in different regions of MRP1 (position 1246, 459 and 553) have been shown to affect substrate specificity and transport efficiency (Ito et al., 2001; Koike et al., 2002; Zhang et al., 2006). Trp residues are present at position 230 and 995 of MRP4 (analogous to Trp 459, and Trp 1246 in MRP1) and Phe is present at position 324 (analogous to Trp 553 in MRP1). Trp (W) residues at position 230, 216 and 995 of MRP4 molecule were replaced by Phe (F), Ala (A), and Cys (C), respectively. Phe (F) at position 324 of MRP4 was substituted by Ala (A) and Trp (W), respectively. Plasmid constructed encoding specific mutated amino acids were stably transfected into HepG2 cells. Seven different HepG2 cell lines with tranfection of MRP4 mutants (F324A, F324W, W230A, W230F, W216A, W995C, and W995F) were used to detect their intracellular accumulation of CPT-11 and SN-38, and their cytotoxicity response to CPT-11 and MTX.

The results of intracellular accumulation of CPT-11 and SN-38 in lactone form in MRP4 mutants were shown in **Figure 4-17**. Most mutant cell lines displayed little effect on intracellular accumulation of CPT-11, with the exception of W995C. Cellular accumulation of CPT-11 (5 μM CPT-11 over 5 min and 30 min, 1 μM CPT-11 for 30 min) was significantly increased by 21-34% in W995C cells, compared with that in MRP4/HepG2 cells (**Figure 4-17** A & C & E). However, all mutant cell lines could increase the intracellular accumulation of SN-38, compared with MRP4/HepG2. When cells were exposed to 1 μM SN-38 for 5 min, the intracellular accumulation of SN-38 was significantly increased by 85-259% in nearly all mutant cell lines except F324W cells (**Figure 4-17** B). If the time of incubation with 1 μM SN-38 was extended to 30 min, four mutant cell lines, including F324A, W230A, W216A, and W995C, showed 111-277% significantly higher accumulation of SN-38 than MRP4/HepG2 (**Figure 4-17** D). When cells were exposed to 0.2 μM SN-38 for 5 min, SN-38 accumulation was significantly increased in most mutant cell lines, excluding W995F (**Figure 4-17** F). These findings suggest that mutant cell lines display different effects on drug cellular accumulation contingent on the chemical structure, dose, and the incubation time of the drugs.

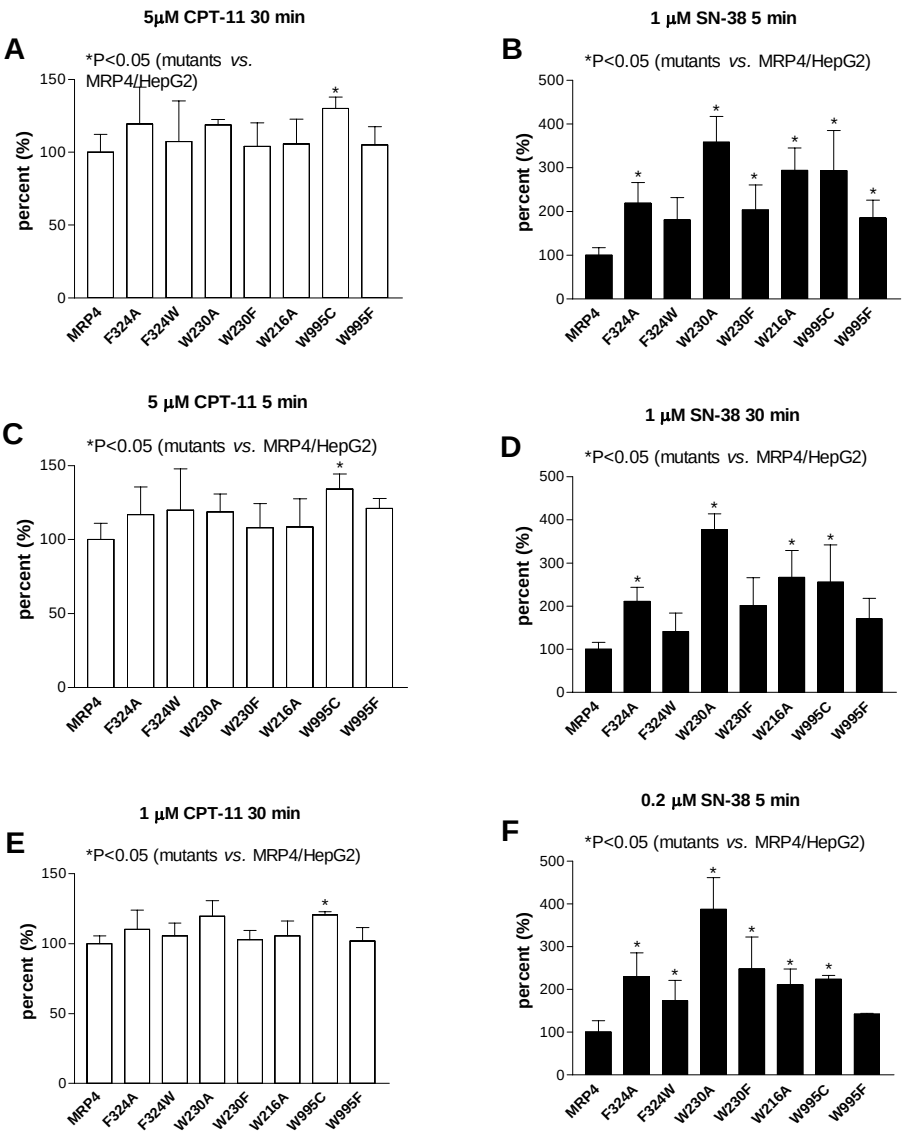


Figure 4-17. Relative intracellular accumulation of CPT-11 and SN-38 in MRP4 mutants. The concentration of CPT-11 and SN-38 was determined by a validated HPLC method with fluorescence detection. Data represent means ± SD. One-way ANOVA test was used. *P < 0.05.

The relative cytotoxicity results of CPT-11 lactone for 48 h exposure and MTX for 4 h exposure in MRP4 mutant cells (with exception of W216A cells) were shown in **Figure 4-18**. For cytotoxicity of CPT-11 in 48 h exposure, the IC_{50} values in most mutant cell lines were significantly decreased by 30-57% compared with that in

MRP4/HepG2 cells, suggesting that these MRP4 mutants have significantly lower resistance to CPT-11 in comparison to MRP4 (**Figure 4-18** A). However, W995C cell line had no significant difference in resistance to CPT-11 for 48 h exposure compared with MRP4. In MTX 4 h exposure assay, the IC_{50} value in W230A cell line was significantly decreased by 31% of that in MRP4/HepG2 cells, whereas in F324A cells it was not significantly changed (**Figure 4-18** B). In other MRP4 mutants, F230W, W230F, W995C, and W995F, their MTX IC_{50} values were greatly increased to 136-395% of that in MRP4/HepG2 cells, suggesting these mutants have significantly higher resistance ability than MRP4 in 4 h exposure to MTX (**Figure 4-18** B). Because CPT-11 is converted to the active metabolite, SN-38, under long-term exposure, it is more accurate to compare our cytotoxicity results with the accumulation data of SN-38 instead of CPT-11. The lower resistance to CPT-11 displayed in some mutants including F324A, W230A, and W230F, is considered consistent with higher intracellular SN-38 concentration in these mutants (**Figure 4-17** & **Figure 4-18** A).

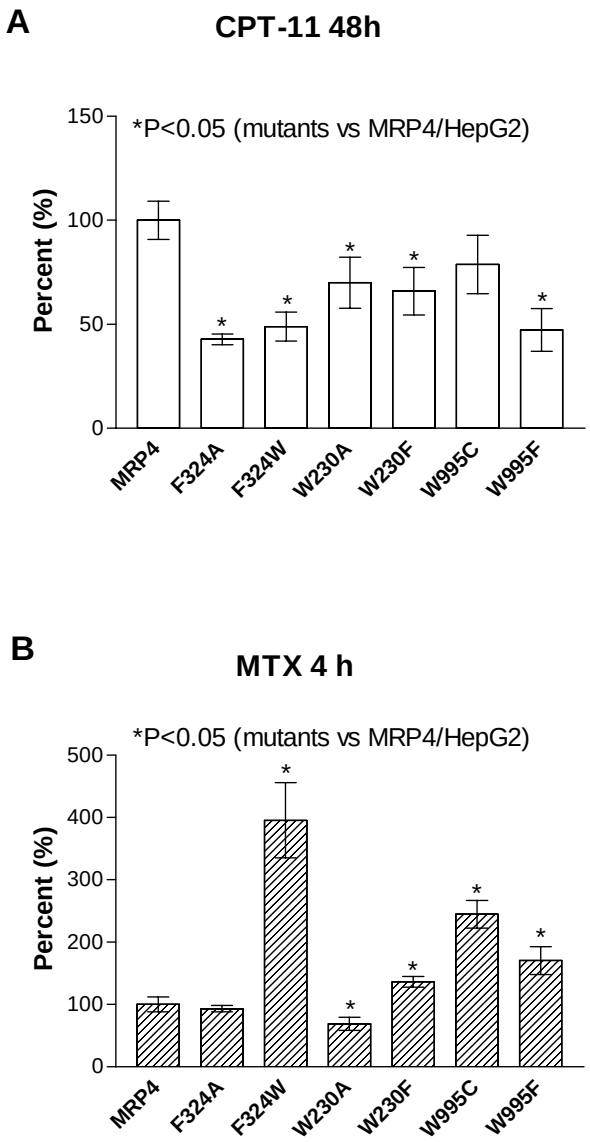


Figure 4-18. Relative cytotoxicity profiles in MRP4 mutants when the cells were treated with CPT-11 for 48 h (A) or MTX for 4 h (B). The relative cytotoxicity percentage was calculated by taking the ratio of the IC_{50} value of drugs in MRP4/HepG2 to that in MRP4 mutant cell lines and multiplying by 100%. Data represent means ± SD. Each experiment was carried out independently at least 3 times. One-way ANOVA test was used. *$P < 0.05$.

4.3. EFFECT OF CPT ANALOGS ON MRP4-MEDIATED EFFLUX OF GSH

The third aim of our study was to examine the effect of CPT analogs on MRP4-mediated GSH efflux. CPT analogs have been identified as new substrates for MRP4.

GSH is a critical component in MRP4-mediated efflux and its level can greatly influence MRP4 transport function. GSH also plays important roles in non-MRPs-related resistance to CPT analogs. These studies will be useful in clarifying the multifunction of GSH in the resistance to CPT analogs.

4.3.1. Export of bimane-GS by MRP4

The bimanes are a series of heterocyclic compounds that produce fluorescent adduct with thiols. MCB has been shown to form a fluorescent adduct with GSH specifically and preferentially over other thiols (Fernandez-Checa and Kaplowitz, 1990). MCB enters the cell *via* passive diffusion but within the cell, MCB is conjugated with GSH by cellular GST. The product hydrophilic bimane-GS product can only leave the cell through carrier-mediated transport (Ishikawa et al., 1994; Zhang and Wong, 1996; Terlouw et al., 2001). In this study, the formation and efflux of fluorescent bimane-GS adduct was examined in MRP4/HepG2. Cells were first incubated with 100 μM MCB at 10°C to ensure that active efflux of the bimane-GS was totally inhibited. The MCB-containing medium was then replaced with HBSS containing glucose and upon warming to 37°C, cells preincubated with MCB showed a time-dependent efflux of bimane-GS.

The percent of bimane-GS present in the incubation buffer of MRP4/HepG2 increased to 85% in 15 min (**Figure 4-19**). This is accompanied by a corresponding decrease in cellular bimane-GS levels. The efflux of bimane-GS from MRP4/HepG2 cells is rapid and tapered off after 10 min as intracellular bimane-GS level decreases and becomes limiting. Subsequent export assays were thus carried out after 10 min of incubation at 37°C. In contrast, 57% of bimane-GS was exported from V/HepG2 cells after 15 min, which increased more gradually (**Figure 4-19**). Efflux of bimane-GS from V/HepG2

cells may possibly be mediated by other transporter proteins that are endogenously expressed in HepG2 cells (Roelofsen et al., 1997; Lee et al., 2000; Lee et al., 2001; Norris et al., 2005). Although our western blot analysis in Section 4.1.6 (**Figure 4-12**) showed that the expression level of some related ABC transporters (PgP, MRP1-3, MRP5 and BCRP) was undetectable V/HepG2 and MRP4/HepG2 cells, this list of transporters is not exhaustive and we may not exclude the presence of other transporters not included in this list, such as transporters of the organic anion transporting polypeptide (OATP) family (Ballatori et al., 2005). At all time points beyond 0 min, efflux of bimane-GS from MRP4/HepG2 cells was significantly higher than that from V/HepG2 cells (**Figure 4-19**).

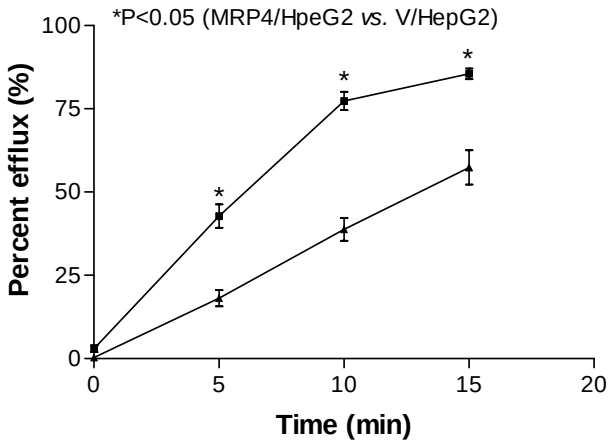


Figure 4-19. Efflux of bimane-GS in V/HepG2 (▲) and MRP4/HepG2 (■). Cells were preincubated with medium containing 100 μM MCB. The medium was then removed and replaced with HBSS containing 5.6 mM glucose and incubated at 37°C. The percent efflux was calculated by taking the ratio of the amount of bimane-GS in the incubation buffer to that of the total bimane-GS (sum of bimane-GS in incubation buffer and cell lysate) and multiplying by 100%. All points for MRP4/HepG2 cells with the exception of that at 0 min were significantly different from that observed for V/HepG2 cels (*$P < 0.05$). Data shown are means ± SD.

4.3.2. Time-dependent effects of CPT analogs and BSO on bimane-GS export

A series of CPT analogs (5 μM of CPT-11, 1 μM of SN-38, topotecan, rubitecan and 10-OH-CPT) were used to examine their effects on bimane-GS efflux in V/HepG2 and MRP4/HepG2. The assay for control group was carried out in the presence with 1% DMSO (the solvent used for CPT analogs); whose bimane-GS efflux values in both cell lines were very close to those corresponding untreated cells (**Figure 4-20**). Compared with the control group (1% DMSO), the presence of CPT analogs displayed no significant effect on the bimane-GS efflux in both cell lines during a period of 15 min tested and the range was between 90-110% of the control group value (**Figure 4-21**).

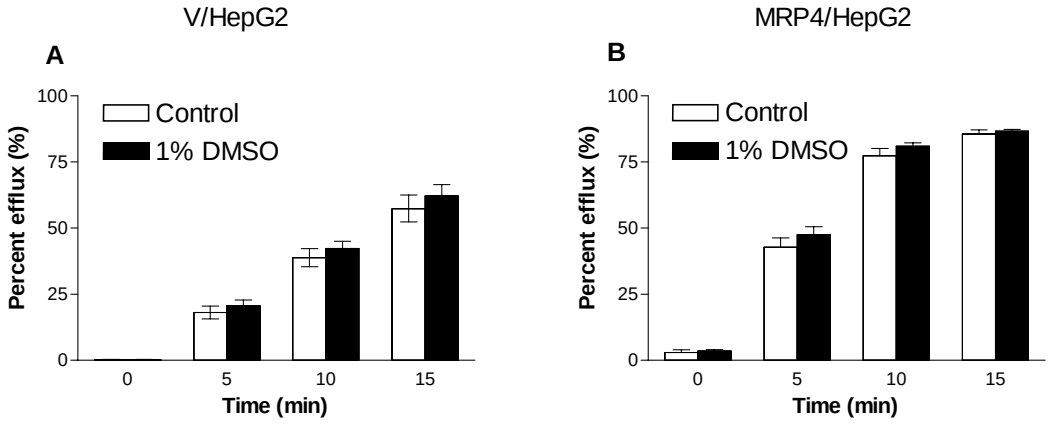


Figure 4-20. Time-dependent effects of 1% DMSO on bimane-GS efflux in V/HepG2 (A) and MRP4/HepG2 (B). Cells were incubated in 1 ml DMEM containing 100 μM MCB at 10°C for 1 h together with 1% DMSO. The medium was then removed and efflux was carried out in HBSS/5.6 mM glucose with 1% DMSO at 37°C. Percent efflux was calculated by taking the ratio of the amount of bimane-GS in the incubation buffer to that of the total bimane-GS (sum of bimane-GS in incubation buffer and cell lysate) and multiplying by 100%. Data shown are means ± SD.

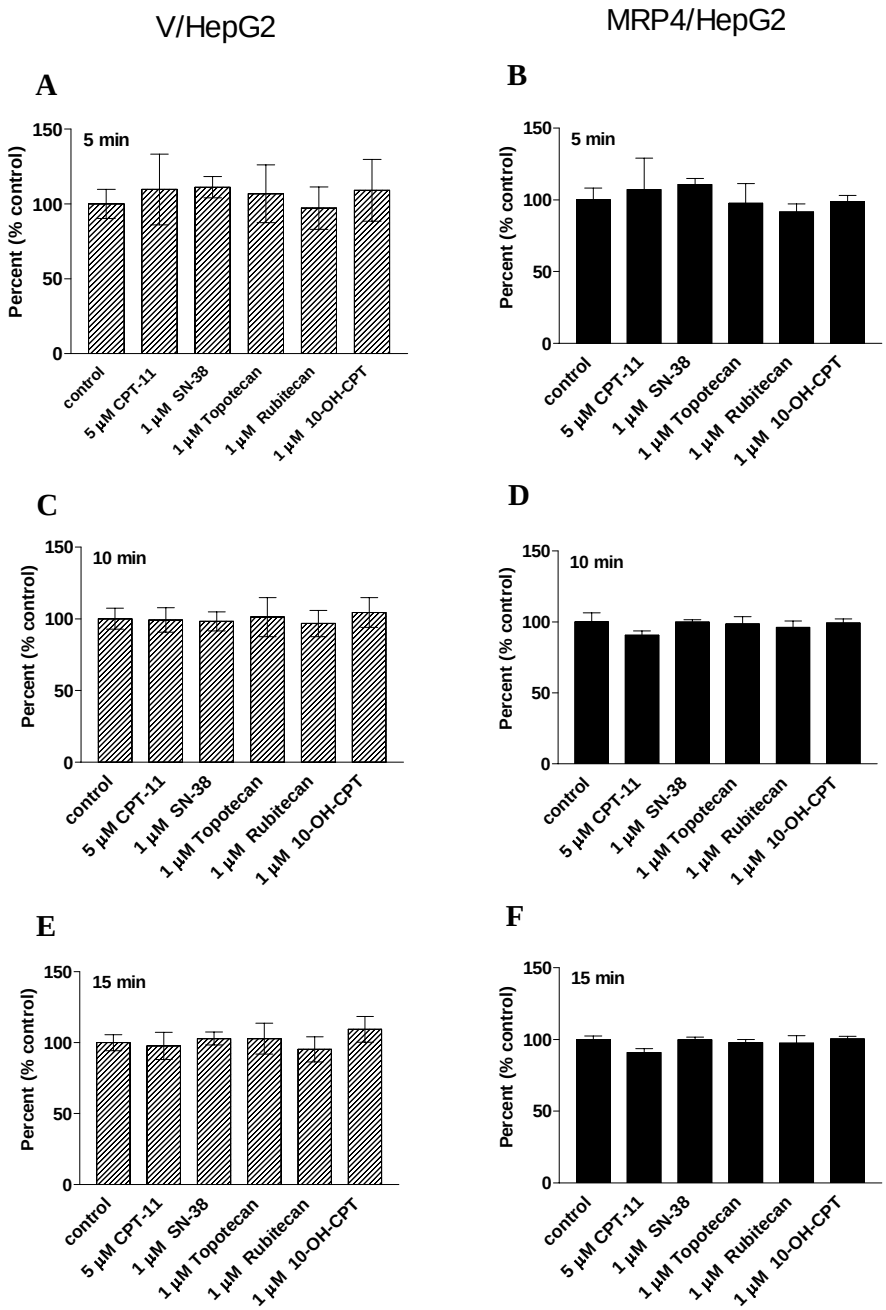


Figure 4-21. Time-dependent effects of CPT analogs on bimane-GS efflux in V/HepG2 (A, C, & E) and MRP4/HepG2 (B, D, & F). Cells were incubated in 1 ml DMEM containing 100 µM MCB at 10°C for 1 h together with CPT analogs. The medium was then removed and efflux was carried out in HBSS/5.6 mM glucose with CPT analogs at 37°C. The 100% value represents bimane-GS efflux in the absence of CPT analogs. The slanting striped bars show the values in V/HepG2, and the black bars show the values in the MRP4/HepG2 cells. Values shown are means ± SD.

As a known GSH synthesis inhibitor, BSO could lead to the decrease of the intracellular GSH in HepG2 cells (Zhang et al., 2001). In this study, the effect of BSO on the efflux of bimane-GS efflux was also examined. The assay for control group was carried out in the presence with 1% water (the solvent used for BSO). The presence of BSO led to significant decrease in bimane-GS efflux from MRP4/HepG2 cells (**Figure 4-22**). At 5 min, the exported bimane-GS in BSO-treated MRP4/HepG2 cells was only 52% of that in control group. At 10 min and 15 min, BSO decreased bimane-GS efflux in MRP4/HepG2 by about 24% of control group values (**Figure 4-22**). However, BSO showed little effect on the bimane-GS efflux in V/HepG2, which suggests that BSO might have specific interaction with MRP4. Correspondingly, our cytotoxicity assays showed that BSO could increase the sensitivity of MRP4/HepG2 cells to known MRP4 substrates (bis-POM-PMEA, MTX, **Table 4-1**), CPT and its analogs (**Table 4-2 & Table 4-3**).

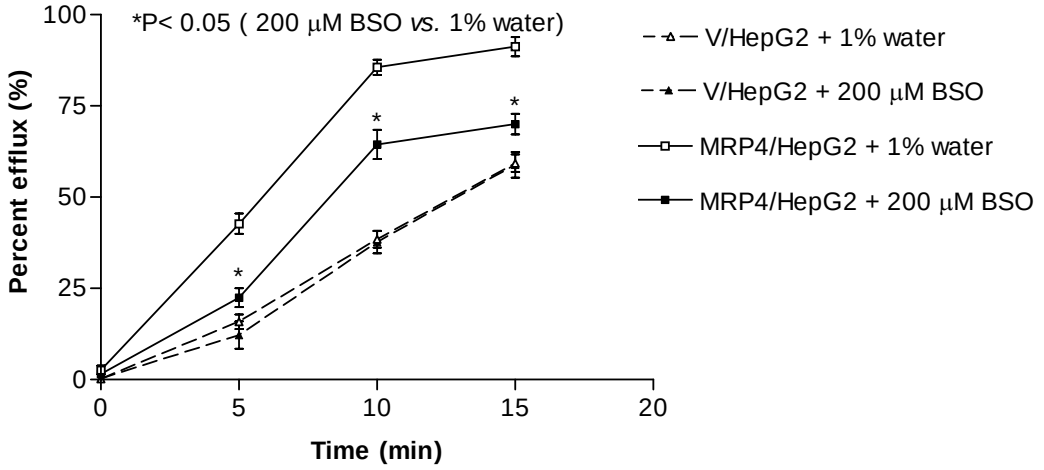


Figure 4-22. Time-dependent effect of BSO on bimane-GS efflux in V/HepG2 and MRP4/HepG2. Cells were seeded with the presence of 200 μM BSO. After 24 h for attachment, cells were preincubated with medium containing 100 μM MCB and 200 μM BSO. The medium was then removed and efflux was carried out in HBSS/5.6 mM glucose containing 200 μM BSO at 37°C. Control group consisted of cells incubated with 1% water (solvent used for BSO). The percent efflux was calculated by taking the ratio of the amount of bimane-GS in the incubation buffer to that of the total bimane-GS (sum of bimane-GS in incubation buffer and cell lysate) and multiplying by 100%. Data shown are means ± SD. **P* < 0.05.

4.3.3. Concentration-dependent effects of CPT analogs on bimane-GS export

The concentration-dependent effects of CPT analogs on bimane-GS efflux in V/HepG2 and MRP4/HepG2 were examined at time point of 10 min. Compared with the control group (1% DMSO), CPT-11 at high concentration (10 μM) led to significant decrease by 20% in bimane-GS in MRP4/HepG2 cells, while no significant decrease in bimane-GS efflux was observed in V/HepG2 cells (**Figure 4-23** A & B). SN-38 and topotecan at low concentrations (0.05 and 1 μM) had little effect on the bimane-GS efflux in both in V/HepG2 and MRP4/HepG2 cells. With the presence of 5 μM SN-38 and topotecan, MRP4/HepG2 cells also displayed no significant change of bimane-GS efflux (93% -103% of control group value, **Figure 4-23** D & F). However, V/HepG2 cells showed significant decrease in bimane-GS efflux by 15% and 22% with the presence of 5 μM SN-38 and topotecan respectively (**Figure 4-23** C & E). The presence of 0.01-5 μM rubitecan and 10-OH-CPT had no significant effect on bimane-GS efflux in both cell lines at 10 min tested and the range was between 94%-104% of the control group value (**Figure 4-24**).

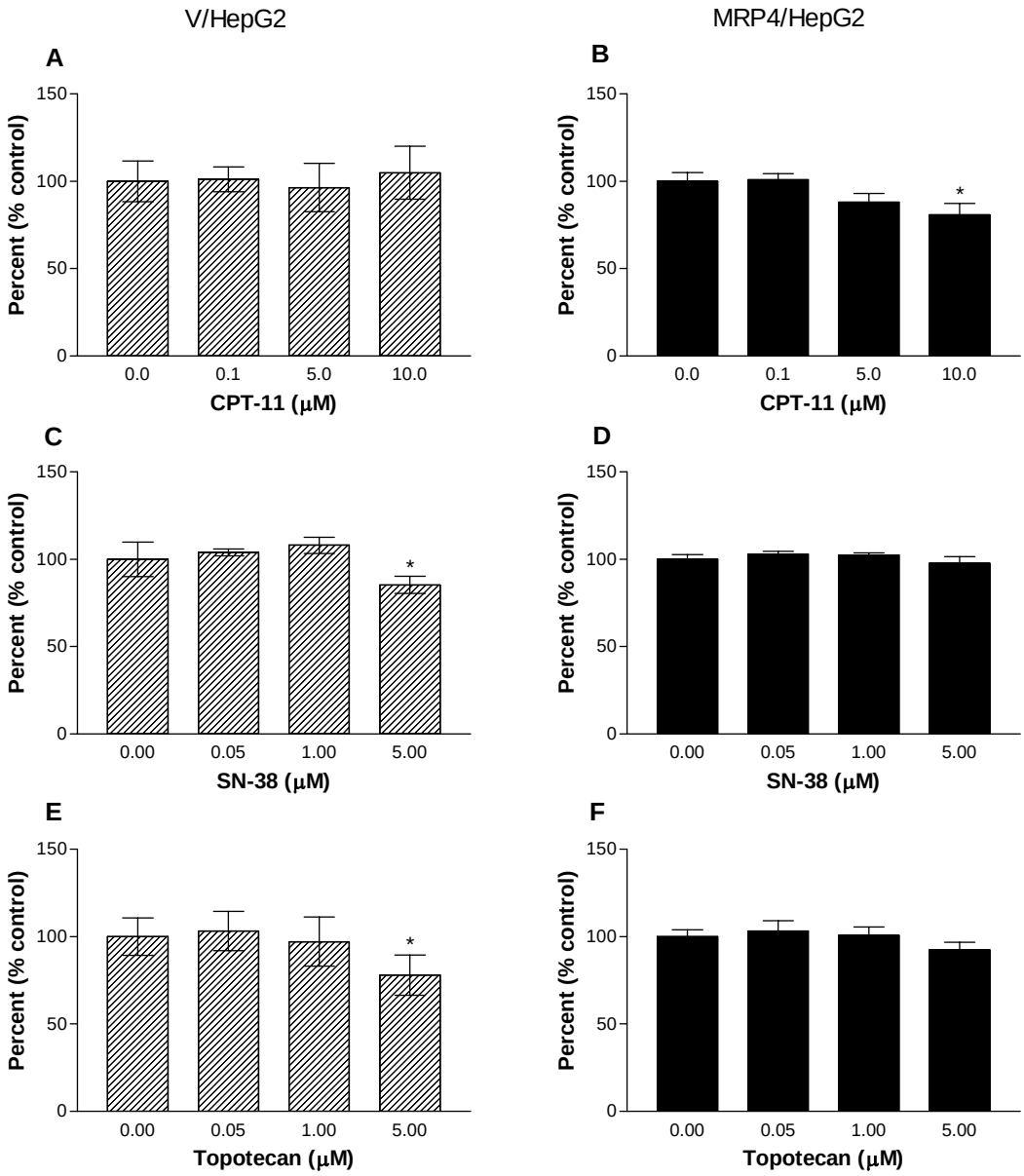


Figure 4-23. Concentration-dependent effects of CPT-11 (A & B), SN-38 (C & D) and topotecan (E & F) on bimane-GS efflux in V/HepG2 and MRP4/HepG2. Cells were incubated in 1 ml DMEM containing 100 μM MCB at 10°C for 1 h together with CPT analogs at different concentrations. The medium was then removed and efflux was carried out in HBSS/5.6 mM glucose with CPT analogs at 37°C for 10 min. The 100% value represents bimane-GS efflux in the absence of CPT analogs. The slanting striped bars show the values in V/HepG2, and the black bars show the values in the MRP4/HepG2 cells. Data shown are means ± SD. *$P < 0.05$.

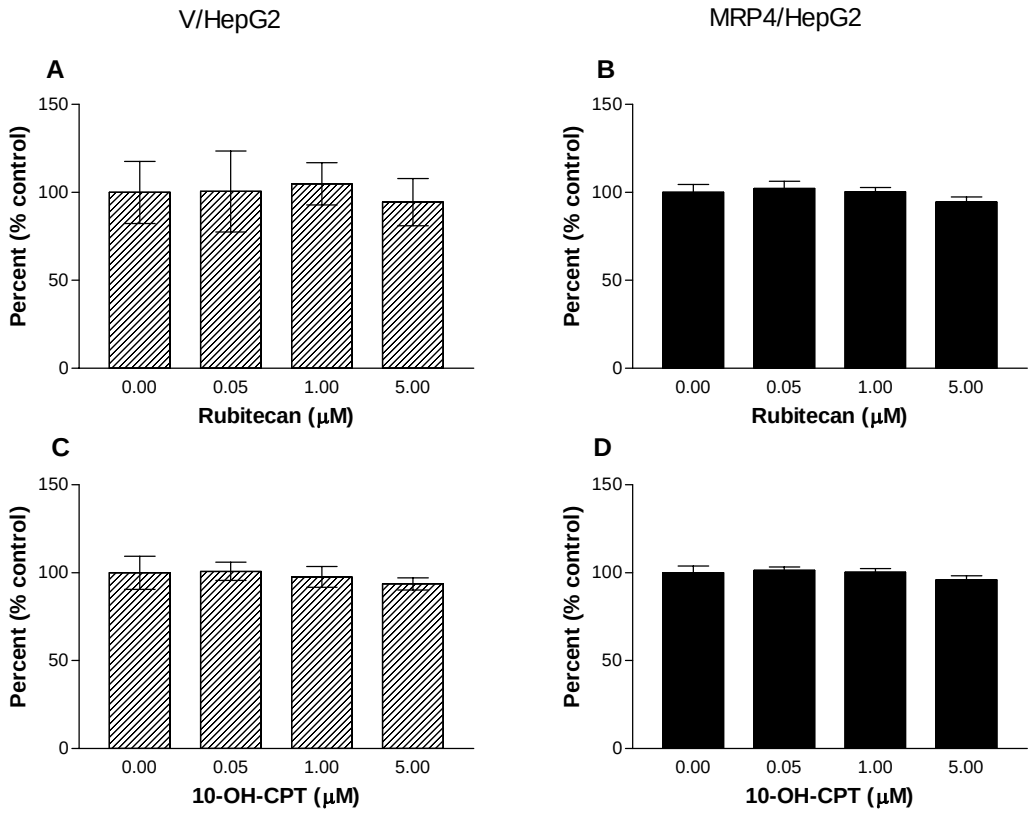


Figure 4-24. Concentration-dependent effects of rubitecan (A & B) and 10-OH-CPT (C & D) on bimane-GS efflux in V/HepG2 and MRP4/HepG2. Cells were incubated in 1 ml DMEM containing 100 μM MCB at 10°C for 1 h together with CPT analogs at different concentrations. The medium was then removed and efflux was carried out in HBSS/5.6 mM glucose with CPT analogs at 37°C for 10 min. The 100% value represents bimane-GS efflux in the absence of CPT analogs. The slanting striped bars show the values in V/HepG2, and the black bars show the values in the MRP4/HepG2 cells. Data shown are means ± SD.

CHAPTER 5 DISCUSSION, CONCLUSION & FUTURE WORK

5.1. DISCUSSION

5.1.1. Resistance to CPTs by MRP4

In this study, we first validated the functionality of MRP4 in the stably transfected HepG2 cells using bis-POM-PMEA and MTX, which are known substrates for MRP4 (Lee et al., 2000; Dallas et al., 2004). Bis-POM-PMEA was chosen in this study as it first spontaneously hydrolyzed to mono-POM-PMEA (an intermediate compound formed during the conversion of bis-POM-PMEA to PMEA) (Hatse et al., 1998; Dallas et al., 2004), which is then rapidly converted to PMEA by cellular esterases (Srinivas et al., 1993). PMEA is also a substrate for MRP4 (Dallas et al., 2004). The established HepG2 cells expressing human MRP4 conferred significant resistance to bis-POM-PMEA when the cells were treated with bis-POM-PMEA for 4 h and 48 h and the presence of the GSH synthesis inhibitor BSO partially reversed the resistance in the present study (**Table 4-1**). As reported, intracellular conversion efficiency of bis-POM-PMEA *in vitro* to PMEA is in the order of 80-90% for initial substrate concentrations of 0.125-1.0 μM (Robbins et al., 1995; Wijnholds et al., 2000), and we presume that the observed cytotoxicity for bis-POM-PMEA is due to the action of PMEA. Since PMEA does not appear to be a substrate for PgP, MRP1 or BCRP (Reid et al., 2003a; Dallas et al., 2004), nor does it interact with typical substrates for MRP1-3 (McAleer et al., 1999; Lee et al., 2000), the observed resistance to bis-POM-PMEA by MRP4/HepG2 cells is thus mediated by MRP4.

As for MTX, MRP4 displayed resistance which was reversed by BSO when the drug exposure time was 4 h only, but not when the drug exposure time was prolonged to 48

h (**Table 4-1**). The resistance level of MRP1-4 to MTX seemed to be strictly time-dependent, and associated with the schedule of MTX exposure. Various studies have shown that MRP1-4 had high resistance capacity to MTX when the drug exposure was limited to 1-4 h of a 3-day growth assay, whereas little resistance to MTX was conferred in a standard continuous exposure assay (Hooijberg et al., 1999; Kool et al., 1999; Zeng et al., 1999; Lee et al., 2000; Zeng et al., 2001; Chen et al., 2002). This may be explained by the unique metabolic processes that MTX undergoes in the cells. MTX is a monoglutamate and enters the cells through the reduced folate carrier (RFC) (Dixon et al., 1994). Thereafter, up to six γ-linked glutamate residues is enzymatically added by folylpolyglutamate synthetase (FPGS), converting MTX to its polyglutamylated forms inside the cells, which results in its therapeutic action of inhibiting the enzyme dihydrofolate reductase (DHFR) (Hooijberg et al., 1999; Kool et al., 1999). Notably, the polyglutamated forms of MTX are not transported by MTX efflux pumps, though monoglutamated forms have been shown to be substrates. For instance, MRP1 and MRP3 have been shown to be high capacity, low affinity MTX efflux pumps, which have little or no activity to MTX polyglutamates (Zeng et al., 2001). Similarly, MRP2 and MRP4 are also unable to transport MTX polyglutamates out of cells (Chen et al., 2002). Due to the slow polyglutamylation of MTX, cells with overexpression of one of these MTX efflux pumps can transport MTX monoglutamate out of cells after short-term exposure to MTX. The rest of MTX accumulates in cells in its long-chain polyglutamylated forms, whose concentration is low because the short-term MTX exposure schedule doses not provide sufficient conversion time. However, with longer duration of drug exposure, polyglutamylated metabolites of MTX accumulate to concentrations sufficient to kill cells. This essentially explains our observations with MTX.

Our results with bis-POM-PMEA and MTX in MRP4/HepG2 cells are consistent with those reported in other cell systems with the exogenous overexpression of MRP4 (Schuetz et al., 1999; Lee et al., 2000; Reid et al., 2003a). To name a few examples, resistance to PMEA by human T-lymphoid selected cell line CEM-r1 has been identified resulting from ATP-dependent PMEA efflux mediated by MRP4 which was overexpressed in cells (Schuetz et al., 1999). The time required for the intracellular concentration of the PMEA to decrease to half of its initial values ($t_{1/2}$) in CEMss cells was about 5.9-fold longer than that in CEM-r1 cells, after both cell lines have been incubated with 1 μM bis-POM-PMEA for 1 h. Moreover, the rate of PMEA efflux in CEM-r1 cells was significantly decreased under ATP-depleting condition, but not in CEMss. In cytotoxicity assay, CEM-r1 cells showed 250-fold higher resistance to PMEA than CEMss cells (Schuetz et al., 1999). In another report, MRP4-transfected NIH3T3 cells displayed a modest higher resistance (2.3-fold) to PMEA than parental vector-transfected NIH3T3 cells (Lee et al., 2000). Similarly, MRP4 overexpression in HEK293 cells conferred significant resistance to bis-POM-PMEA and PMEA in cytotoxicity assay (Reid et al., 2003a). In all these reports, MRP4 was verified to transport PMEA in its unchanged form. MRP4 inhibitors, including MK571, dipyridamole and dilazep, could inhibit MRP4-mediated PMEA efflux (Reid et al., 2003a). Similarly for MTX, MRP4-mediated resistance has been studied in a variety of cell lines. Transfection of MRP4 increased the resistance of NIH3T3 cells to MTX by 5.5-fold in short-term drug exposure assays and decreased intracellular accumulation of MTX at 4 h and 24 h (Lee et al., 2000). In general, MTX is subject to high capacity and low affinity transport by MRP4 (Chen et al., 2002).

Besides MRP4, other ABC transporters are also components of bis-POM-PMEA, PMEA and MTX efflux system. As another efflux pump for PMEA, MRP5 is able to

transport PMEA in an unmodified form (Wijnholds et al., 2000). PgP, MRP1-3 and BCRP are all involved in resistance to MTX (Norris et al., 1996; Hooijberg et al., 1999; Kool et al., 1999; Volk et al., 2000). In contrast to MRP1-4, BCRP is able to mediate the transport of MTX polyglutamates (Volk and Schneider, 2003). Both MTX-Glu(2) MTX-Glu(3) are substrates for BCRP, although the decreased efflux correlates with the increased polyglutamate chain length (Volk and Schneider, 2003). The capacity to transport MTX-polyglutamates may account for the high levels (> 100-fold) of BCRP-mediated resistance to MTX in the long-term assay (7-day) (Volk et al., 2000). Thus, similar MTX cytotoxicity values in V/HepG2 and MRP4/HepG2 cell at 48 h exposure suggest that BCRP may not be involved in the MTX resistance. Moreover, expression levels of these ABC transporters in model cell lines, V/HepG2 and MRP4/HepG2, are too low to be detected in western blot analysis, which further exclude their effects in resistance to bis-POM-PMEA and MTX. On the other hand, the expression of MRP4 is very high in MRP4/HepG2 cells, but undetectable in V/HepG2 cells, according to our western blot analysis result (**Figure 4-12**). The resistance to bis-POM-PMEA and MTX observed in MRP4/HepG2 can thus be attributed to overexpression of MRP4, suggesting MRP4/HepG2 has normal MRP4 transport function.

CPTs clearly represent an 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 CPTs is a major clinical problem often resulting in therapeutic failure. Detailed investigations aimed at identification of resistant proteins and approaches circumventing intrinsic drug resistance are thus warranted. Our cytotoxicity data is novel evidence that MRP4 conferred significant resistance to

various CPTs including both lactone and carboxylate forms of CPT, CPT-11 and SN-38, and the lactone form of topotecan, rubitecan and 10-OH-CPT (**Table 4-2** & **Table 4-3**). 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 celecoxib, diclofenac, and MK-571, also significantly reduced the resistance to CPT-11 and SN-38 in MRP4/HepG2 cells. Estimated from the resistance folds for the MTT assay with 48 h drug exposure time of the test CPTs (**Table 4-2** & **Table 4-3**), the substrate affinity of MRP4 appears to decrease from 10-OH-CPT lactone (14.18)>SN-38 carboxylate (9.70)>rubitecan lactone (9.05)> SN-38 lactone (8.91)>CPT lactone (7.22)>topotecan lactone (6.86)>CPT-11 lactone (5.64)> CPT carboxylate (4.29)>CPT-11 carboxylate (2.68). 10-OH-CPT lactone seems to have the highest affinity to MRP4, whereas CPT-11 carboxylate might have the lowest affinity. These observations have important implications in the clinical setting for drug combination therapy and administration route design. At the same time, there should be an awareness of the interaction with MRP4 in the further development of CPTs which should gear towards the synthesis of new CPT analogs without binding affinity to MRP4.

The resistance magnitude of MRP4 to CPT, CPT-11 and SN-38 in lactone and carboxylate form is different (**Table 4-2**). Thus, MRP4 is considered to have different affinity and transport capacity to their lactone and carboxylate form. This may have important implications in the transport and disposition of these CPT analogs given that CPTs undergo rapid interconversion *in vivo*. The half-life of CPT, CPT-11 and SN-38 lactone varies from 29-32 min at pH 7.3 and the equilibrium lactone content is approximately 15% to 23% (Chourpa et al., 1998). 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 versus time curve (Rivory et al., 1994; Sasaki et al., 1995). 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 being rapidly taken up than their carboxylate forms in HT29 cells, whereas their respective carboxylate forms were primarily transported *via* an ATP-dependent mechanism (Kobayashi et al., 1999). Similarly, the intestinal uptake of CPT-11 and SN-38 lactone is about 10 times greater than that of the carboxylate form (Kobayashi et al., 2001). Active uptake of the CPT-11 carboxylate instead of lactone was also observed in KB-C2 membrane vesicles (Chu et al., 1999b). MRP2 is responsible for the biliary excretion of the carboxylate form of CPT-11 and SN-38 as well as the lactone and carboxylate forms of SN-38 glucuronide in rats (Chu et al., 1997a; Chu et al., 1997b).

In our study, the lactone form of CPT, CPT-11 and SN-38 displayed different cytotoxicities compared with their corresponding carboxylate form (**Table 4-2**). V/HepG2 cells were more sensitive to CPT lactone form (IC_{50}: 0.032 ± 0.001 μM) than its carboxylate form (IC_{50}: 0.072 ± 0.005 μM) in 48 h drug exposure (**Table 4-2**). This may be attributed to the fact that CPT in carboxylate form has lower activity than in lactone form, and once bound with proteins in the cell culture medium, the carboxylate form could not easily convert back to the lactone form. With the exception of SN-38 and CPT-11, it has been found that CPT and most CPT analogs had a stronger binding ability to HSA in negatively charged carboxylate form than in lactone form (Burke and Mi, 1993; Mi and Burke, 1994b; Prijovich et al., 2003). Moreover, only the free drug (unbound to protein) is primarily responsible for drug activity. The structure of CPT carboxylate bound to protein will be much bigger than the free CPT carboxylate, leading to difficulty in transport through cell membrane by

passive diffusion. On the other hand, CPT in free lactone form enters cells easily and is able to exert higher cytotoxicity than CPT carboxylate, which needs additional conversion to the lactone form in cells. In V/HepG2 cells, the cytotoxicity difference between CPT lactone and CPT carboxylate in 48 h exposure was 2.3-fold, while the difference decreased to 1.0-fold in 4 h exposure. This may be explained by short drug exposure time which is not sufficient for the drug to fully exert its toxicity. For MRP4/HepG2 cells, CPT lactone displayed significantly lower cytotoxicity than CPT carboxylate in 4 h drug exposure, which may be explained by the fact that MRP4 showed higher resistance to CPT lactone (7.2) than to CPT carboxylate (4.3) (**Table 4-2**). The cytotoxicity difference in 48 h exposure between CPT lactone and carboxylate in V/HepG2 cells (2.3-fold) was much higher than that in MRP4/HepG2 (1.3-fold) (**Table 4-2**), which may be explained by the higher resistance to CPT lactone by MRP4 overexpression in MRP4/HepG2 cells.

Looking at the effect of SN-38, we noted a similar response to both the lactone form and carboxylate form in 48 h exposure assay in both V/HepG2 (IC_{50}: 0.083 ± 0.005 μM *vs.* 0.079 ± 0.007 μM) and MRP4/HepG2 (IC_{50}: 0.741 ± 0.004 μM *vs.* 0.766 ± 0.091 μM) (**Table 4-2**). In contrast, SN-38 lactone showed higher IC_{50} value than SN-38 carboxylate in both V/HepG2 (IC_{50}: 0.948 ± 0.009 μM *vs.* 0.674 ± 0.061 μM) and MRP4/HepG2 (IC_{50}: 7.70 ± 0.65 μM *vs.* 5.53 ± 0.63 μM) for 4 h drug exposure (**Table 4-2**), suggesting that SN-38 carboxylate is more potent than lactone. For V/HepG2, the concentration of unchanged SN-38 lactone in cell culture medium may have been decreased by its binding to protein, and less SN-38 molecules in lactone form are available to enter into cells. Although SN-38 lactone form has higher cytotoxicity than SN-38 carboxylate, SN-38 carboxylate in free form may enter cells and be transformed to active lactone form and display corresponding cytotoxicity.

This provides explanation for short duration of exposure, but for longer duration, the action of SN-38 carboxylate may not have been sustained because the equilibrium between lactone form and carboxylate form has sufficient time to shift according to the environment. This may also have been the case for MRP4/HepG2 cells, whereby a similar resistance level to SN-38 lactone (8.9) and SN-38 carboxylate (9.7) (**Table 4-2**) has been observed.

For CPT-11, its lactone form displayed higher cytotoxicity than its carboxylate form in V/HepG2 cells at 4 h and 48 h assay. CPT-11 lactone has a stronger binding ability to protein than CPT-11 carboxylate. Although CPT-11 carboxylate mainly exists as free form, it is still difficult to enter cells very quickly like SN-38 carboxylate, because CPT-11 structure is much bigger. Moreover, both lactone and carboxylate forms of CPT-11 are able to convert to corresponding lactone and carboxylate forms of SN-38. As the products of CPT-11 metabolism, SN-38 lactone has higher stability than SN-38 carboxylate in cell culture medium with the binding to protein. As such, the conversion of CPT-11 lactone to SN-38 lactone will be easier to proceed than the conversion of CPT-11 carboxylate to SN-38 carboxylate, leading to higher concentration of SN-38 lactone than that of SN-38 carboxylate. Besides its own activity, the final cytotoxicity of CPT-11 lactone showed in V/HepG2 cells is also attributed to SN-38 lactone produced by CPT-11 lactone metabolism. All these factors may contribute to the reason why CPT-11 lactone showed higher toxicity than CPT-11 carboxylate in V/HepG2 cells. As a result of being a more effective substrate, MRP4 showed higher fold resistance to CPT-11 lactone (5.6) than CPT-11 carboxylate (2.7) (**Table 4-2**).

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 co-transported with another substrate (Paulusma et al., 1999; Wijnholds et al., 2000; Lai and Tan, 2002). 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 (**Table 4-1** & **Table 4-2** & **Table 4-3**). When high concentration of BSO (500 μM) was used, MRP4-transfected HepG2 cells showed altered drug resistance in the presence of 200-400 μM PMEA (Lai and Tan, 2002). However, GSH does not appear to play a significant role in PMEA efflux in MRP4-overexpressing HEK293 (Wielinga et al., 2003) cells or rat microglial MLS-8 cells (Dallas et al., 2004). 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} of paclitaxel (a known PgP substrate) in MRP4/HepG2 and V/HepG2 cells (**Table 4-4**). This is further supported by the similar IC_{50} values in the two strains of cells with cyclosporin A, a known PgP substrate (Gan et al., 1996). Furthermore, our study did not find resistance of MRP4 to vincristine, vinblastine and etoposide (**Table 4-4**) which are typical substrates of PgP and MRP1-3 (Borst et al., 2000; Borst and Elferink, 2002). It has been reported that MRP4 does not interact with typical substrates of MRP1-3, including vincristine, etoposide, daunorubicin, and cisplatin (McAleer et al., 1999; Lee et al., 2000). Moreover, the involvement of some related ABC transporters, including PgP, MRP1, MRP2, MRP3, MRP5 and BCRP, could be excluded by their

undetectable expression level in both cell lines (**Figure 4-12**). The overexpression of MRP4 in MRP4/HepG2 cells (**Figure 4-12**) further supports that the resistance to CPTs observed in this study is mediated by MRP4 which is functionally distinct from other transporter proteins.

In conclusion, our results in the first part of our study indicate that CPTs are part of the MRP4 resistance profile. The findings from MTT assays highly indicate the possibility that CPTs used in this study are substrates for MRP4 transporter. This is supported by the following: a) MRP4/HepG2 cells had lesser sensitivity to both lactone and carboxylate forms of CPT, CPT-11 and SN-38, and the lactone form of topotecan, rubitecan and 10-OH-CPT than V/HepG2 cells; b) the resistance to CPTs in MRP4/HepG2 cells was inhibited by BSO; c) the resistance to CPT-11 and SN-38 in MRP4/HepG2 cells was inhibited by celecoxib, diclofenac and MK-571; d) MRP4 was only highly expressed in MRP4/HepG2 cells; and e) some ABC transporters (PgP, MRP1, MRP2, MRP3, MRP5 and BCRP) which showed resistance to CPTs were not detected in both V/HepG2 and MRP4/HepG2 cells by western blot analysis. Our new finding that MRP4 may interfere with the therapeutic efficacy of CPTs creates a new awareness in the successful management of patients undergoing CPT-related anticancer therapies.

5.1.2. Uptake and transport of CPT analogs

Targeting Top1 to kill tumor cells requires high enough intracellular accumulation of the active lactone form of CPTs. Inadequate accumulation of CPTs in tumor cells is considered an important reason for their resistance. The mechanism for the uptake of CPTs 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 (Gupta et al., 2000), while the influx of topotecan and SN-38 by ovarian tumor cells requires active transporters and disrupted influx results in drug resistance (Ma et al., 1998). To date, CPTs have been shown to be substrates for various transporters including PgP, BCRP, OATP1B1, MRP1 and MRP2 (Yang et al., 1995; Chu et al., 1997b; Luo et al., 2002; Nozawa et al., 2005). Its relationship with MRP4 has also now been reported herein. The different accumulation of CPT analogs in MRP4/HepG2 and V/HepG2 cells (**Figure 4-13**) provides partial explanation for the different cytotoxicities observed and contributes to the acquisition of resistance *in vitro*. The undetectable expression of some related ABC-transporters, including PgP, MRP1, MRP2, MRP3, MRP5 and BCRP, excluded their interaction with CPT analogs. The different intracellular concentrations of CPT analogs in V/HepG2 and MRP4/HepG2 cells can be mainly attributable to the high expression level of MRP4 in MRP4/HepG2 cells.

The differential accumulation cannot be attributed to the damage of the plasma membrane, which in turn could lead to increased drug influx, given that the tumor cells remained viable during our drug accumulation studies over 120 min as measured using trypan blue exclusion. The differential uptake manner of various CPT analogs tested in this study are mainly due to differences in their lipophilicity, transport across cell membrane, steric hindrance of chemical structures, and binding affinity to MRP4. In this study, the detection of intracellular CPT analogs accumulation did not distinguish between the active lactone form (the parent drug) and the inactive carboxylate form (the chemical hydrolysis product of corresponding lactone form). Our time-dependent accumulation studies of CPT analogs in V/HepG2 and MRP4/HepG2 led us to conclude that a different set of dynamics operates for the compounds in cells. It appears that CPT analogs with small chemical structures (SN-

38, rubitecan and 10-OH-CPT) reached the peak intracellular level faster (within 5 min) than the analogs with bigger chemical structures (CPT-11 and topotecan, 10-30 min). Significant difference in intracellular accumulation was observed at the earliest time point studied (2 min) for SN-38, rubitecan and 10-OH-CPT, but not for CPT-11 and topotecan. The rate of chemical degradation of the parent lactone form to the carboxylate form in the extracellular culture medium appears to be one of major determinants behind intracellular accumulation, perhaps because only the lactone forms are being taken up into cells predominantly by passive diffusion, as previously reported for normal intestinal cells (Gupta et al., 2000; Cummings et al., 2002b). At physiological pH, the extracellular lactone concentration decreases rapidly over the first 30 min of incubation, as chemical equilibrium favours the carboxylate form (Fassberg and Stella, 1992; Cummings et al., 2002b). This may partly explain why peak intracellular drug concentrations of small CPT analogs (SN-38, rubitecan and 10-OH-CPT) were detected within the first few minutes of incubation in our cell lines. Similar accumulation profiles of SN-38 lactone were also reported in a number of cancer cell lines (Gabr et al., 1997; Kobayashi et al., 1999; Cummings et al., 2002b). For example, the highest intracellular SN-38 concentrations were observed at the earliest time point 5 min in three cell lines, HCT116 and HT29 colon cancer cells and A2780 human ovarian cancer cells (Cummings et al., 2002b). On the other hand, the observation may be attributable to the following: CPT-11 and topotecan tend to enter tumor cells at a moderate rate, and are then distributed within cells and bound by subcellular organelles, drug metabolizing enzymes located on endoplasmic reticulum and drug target in nucleus (Top 1), which in turn act as a store for the active drug. The drug is finally cleared from the cells by metabolism and transporter-mediated efflux. In contrast, small CPT analogs (SN-38, rubitecan and 10-OH-CPT) tend to enter cells

more rapidly and are also removed from the cells just as rapidly. This may be due to the steric hindrance difference between the small functional groups of SN-38, rubitecan and 10-OH-CPT and the bigger ones of CPT-11 and topotecan.

Moreover, differential lipophilicity of CPT analogs can result in different uptake, subcellular compartmentation, metabolism and efflux of drugs. For example, topotecan is mainly localized in mitochondria when incubated with HT29 cells, while gimatecan (ST1481, a water-insoluble CPT analog) is mainly distributed into lysosomes (Croce et al., 2004). Furthermore, it has been indicated that the lipid bilayer membrane affinities of the CPTs should be important determinants influencing cellular uptake and cytotoxic potency of CPTs, because a number of CPTs are accumulated in cancer cells by passive diffusion (Gabr et al., 1997). Increased lipophilic character of CPTs allows for more extensive membrane transport, thereby results in greater levels of accessibility and drug accumulation by cancer cells at the tumor site (Gabr et al., 1997). The difference in lipophilicity of CPTs might lead to the difference in the degree of penetration of a drug into the lipid bilayer domains of MRP4-containing membranes. Such a difference in lipid bilayer location may potentially, at least in part, explain the observed differences between the CPT analogs in their degrees of transport by the protein. For example, different uptake rate and extent of CPT-11 and SN-38 have been observed in intestinal and lung cancer cells (van Ark-Otte et al., 1998; Chu et al., 1999b). Metabolism is also considered an important determinant for the cellular accumulation of CPT-11 and SN-38. CPT-11 is hydrolyzed by cellular CEs, whereas SN-38 is readily conjugated by UGT1A1/1A9 which is associated with increased efflux of the drug from HT29 and HCT116 cells (Cummings et al., 2002a). The human colon cancer cell line HT29 also exhibited a 2-fold intrinsic resistance to SN-38 owing to enhanced drug clearance *via*

glucuronidation, compared with A2780 ovarian cancer cells (Cummings et al., 2003). The determination of the Michaelis-Menten parameters will be entirely dependent on the experimental conditions (Rivory et al., 1996a). The kinetics reported in this study was limited to the examination of CPT analogs in the mixture of lactone and carboxylate forms found at physiological pH, mostly existing as the carboxylate form. The carboxylate forms of CPTs carry a full negative charge and can hardly diffuse across the membrane, if there is no involvement of an active organic anion transporter (Kobayashi et al., 1999; Gupta et al., 2000). Thus, intracellularly carboxylate form of CPT analogs is likely to be derived from in situ hydrolysis of the corresponding lactone (Kobayashi et al., 1999).

It did not preclude the possibility that the lactone and carboxylate forms of CPTs compete for a single binding site and/or that there is a difference between the cleavage rates of the two forms. Due to the higher lipophilicity, the lactone form of CPTs is likely to enter the cells easier and faster than the corresponding carboxylate form. If the efflux rate obtained in the uptake assay is considered (Chu et al., 1997b), the contribution of MRP4 to the efflux of CPT-11 was approximately three times higher than that of SN-38. Topotecan efflux mediated by MRP4 was about two times of that of SN-38. MRP4 had lowest efflux activity for rubitecan. However, the sequence of substrate fold resistance obtained from the cytotoxicity in the 48 h drug (lactone form) exposure (**Table 4-2** & **Table 4-3**) was: rubitecan (9.1) > SN-38 (8.9) > topotecan (6.9) > CPT-11 (5.6). There is clearly discordance in data obtained from the cytotoxicity and uptake assays. This discrepancy may be attributed to the difference in the incubation periods. The cytotoxicity effect of CPT analogs was determined after a continuous drug exposure for 48 h, whereas the accumulation data were determined after an incubation of 30 min for CPT-11, 10 min for topotecan, and 5 min for SN-38

and rubitecan. It appears that the effect of CPT analogs in the accumulation assays occurred only in an acute manner but was not sustained in chronic periods of 48 h of cytotoxicity assays. In longer periods of drug exposure, factors such as equilibrium of lactone and carboxylate inside and outside the cells, interaction of CPTs with the target Top 1, and interaction of CPTs with cellular proteins come into play to influence our results (Beretta and Zunino, 2007). We also cannot rule out rapid cellular metabolism of the CPTs in question leading to limited effects over longer periods of time.

In this study, the accumulation of rubitecan in MRP4/HepG2 cells was increased by celecoxib and MK571 (**Figure 4-16**), which are known inhibitors of MRP4 that reversed drug resistance in cytotoxicity assays. Celecoxib and MK571 were able to directly block MRP4-mediated transport, leading to decreased efflux of rubitecan out of cells and consequent increase in drug accumulation. On the contrary, celecoxib and MK571 significantly decreased 10-OH-CPT accumulation in MRP4/HepG2 cells. Several reports have presented the assumption that MK571 is able to modulate MRP-mediated drug resistance *via* direct interaction with transporters and serves as competitor with substrates in protein binding (Jedlitschky et al., 1994; Leier et al., 1994; Renes et al., 1999). Moreover, MRP4 has been proposed to have multiple allosteric binding sites (Van Aubel et al., 2005). It is possibly because that celecoxib and MK571 might inhibit an uptake transporter on the cell membrane thereby inhibiting the entry of 10-OH-CPT into cells through the membrane. If the effect of celecoxib and MK571 is bigger on drug influx than on drug efflux, the intracellular concentration of drug may become lower. On the other hand, BSO showed no significant effect on rubitecan or 10-OH-CPT uptake in MRP4/HepG2 cells (**Figure 4-16**). BSO is able to reduce MRP4-mediated efflux by lowering GSH cellular level.

Although GSH is needed by a number of MRP4 substrates, it is not clear if the same applies for rubitecan or 10-OH-CPT. If GSH is not necessary component in their efflux mediated by MRP4, the final effect of a decrease of GSH level by BSO would not be as expected.

For other CPT analogs (CPT-11, SN-38 and topotecan), all inhibitors (celecoxib, MK571 and BSO) were able to increase their accumulation in MRP4/HepG2 cells. These findings strongly suggest that MRP4 overexpression significantly decreases the cellular accumulation of CPT analogs and it is involved in the resistance to CPTs. This is consistent with previous reports, which also showed that reduced accumulation and/or increased drug efflux is a component of resistance to CPT analogs, including CPT-11 and topotecan (Chen et al., 1991; Chen et al., 1999; Shiozawa et al., 2004). Moreover, the inhibitory effects of BSO indicate that GSH is an important component of the MRP4-mediated transport system.

Notably, there have been some reports on such inconsistency between accumulation and cytotoxicity data with some CPT analogs and the implication of MRP family transporters (Chen et al., 1999; Cummings et al., 2002b). In MTT assays, C-A120 and KB/MRP cells with the overexpression of MRP1 showed 6.5- to 29.6-fold resistance to CPT-11 and SN-38 compared to the parental KB-3-1 cells (Chen et al., 1999). Consistent with the cytotoxicity results, the accumulation of CPT-11 and SN-38 in C-A120 and KB/MRP was lower than in KB-3-1 cells at low dose exposure. However, when the doses of drugs were increased near to their IC_{50} values, the accumulation levels of CPT-11 and SN-38 in C-A120 became 3- to 5-fold higher than in KB-3-1 which did not correlate with the cytotoxicity data (Chen et al., 1999). Similarly, Cummings *et al* showed that SN-38 and CPT exhibited an unexpected cellular

accumulation phenomenon in HCT116 cells with the overexpression of MRP2 (Cummings et al., 2002b). SN-38 is an established MRP2 substrate (Chu et al., 1997a; Chu et al., 1997b; Koike et al., 1997) and the cellular accumulation of SN-38 is expected to be significantly lower in the control cell line, A2780 human ovarian cancer cells, which do not have any expression of MRP1, MRP2, or PgP. Interestingly, however, the cellular accumulation profile of SN-38 over 20 h was similar in both HCT116 cells and A2780 cells. At the 24 h time point, the HCT116 cells accumulated about 2 times more SN-38 than A2780 cells. It is concluded that the contribution of MRP2 expression to cellular accumulation of SN-38 is of less importance, although it is considered as a critical determinant (Cummings et al., 2002b).

In our context, the discrepancy between 10-OH-CPT accumulation and cytotoxicity may be attributed to the difference in drug incubation periods. The cytotoxic effect was determined after a continuous exposure for 48 h or a short exposure for 4 h with another 44 h of growth, whereas the accumulation data was determined after incubation for 2 min to 120 min. Although 10-OH-CPT accumulated to a greater extent in MRP4/HepG2 than V/HepG2 cells for a test period of up to 2 h, these effects may have occurred only in an acute manner and not sustained at more prolonged periods as those used for cytotoxicity assays, hence the retention of cellular resistance against 10-OH-CPT at 4 h and 48 h. Moreover, intracellular accumulation is mainly determined by the balance between the influx and efflux of the drug, and affected by binding to proteins and organelles. 10-OH-CPT, especially in carboxylate form, has been found to be highly bound to plasma and tissue protein (Li and Zhang, 1996). The higher intracellular accumulation of 10-OH-CPT in MRP4/HepG2 might partly be attributed to the specific binding between the carboxylate form of 10-OH-CPT and

MRP4 membrane protein. It is also possible that some unidentified influx-related factors that play a predominant role at early time points. Further experiments are required to fully elucidate the mechanisms responsible for these differences.

Last but not least, we have also performed uptake assays of CPT-11 and SN-38 in various MRP4 mutants. As mentioned in section 4.2.4, Trp residues in different region of MRP1 (position 1246, 459 and 553) have been shown to affect substrate specificity and transport efficiency (Ito et al., 2001; Koike et al., 2002; Zhang et al., 2006). Corresponding Trp residues in MRP4 were replaced by different amino acids and the resultant mutant protein evaluated for their function in drug cytotoxicity and accumulation. Our results showed that when cells were exposed to 1 μM SN-38 for 5 min, the intracellular accumulation of SN-38 was significantly increased by 85-259% in nearly all mutant cell lines except F324W cells. This indicates that like MRP1, Trp residues in MRP4 are also critical in substrate transport efficiency. All replacements of Trp (W) by Ala (A), Cys (C) and Phe (F) at position 230, 216, and 995 showed a significant effect on the uptake of 1 μM SN-38 for 5 min. There is no difference for F324W probably because that the difference between Trp (W) and Phe (F) in polarity is not very substantial and the replacement at position 324 may not have significant effect on MRP4 protein structure. In the four amino acids tested, Cys (C) has the highest polarity and Ala (A) has the smallest chemical structure. MRP4 mutant with Ala (A) at position 230 had the lowest efflux activity for SN-38, indicating that stereo-structure is a crucial component in determing protein function, besides polarity.

Interestingly, the accumulation profile for CPT-11 was entirely different from that for SN-38, which may be because CPT-11 has a larger chemical structure than SN-38, resulting in differences in their transport through cell membrane and in their

interactions with transporters. Only W995C showed significantly increased CPT-11 accumulation in the time-dependent and concentration-dependent assays, which may be attributed to the fact that Cys is a polar amino acid, while others (Ala, Phe and Trp) are nonpolar amino acids. The replacement of Trp (W) at position 995 by Cys (C) could increase the accumulation of SN-38 to a greater degree than by Phe (F).

In our studies, MRP4 displayed different response to CPT analogs in cytotoxicity assays and uptake assays. The effect of amino acid variances on MRP4-mediated resistance to CPT-11 and MTX in cytotoxicity assays was also studied. We found that variances in amino acids also displayed their effects on cytotoxicity assays by sensitizing cells with transfection of corresponding MRP4 mutants to CPT-11 at 48 h exposure. Most mutants decreased the IC_{50} of CPT-11 for 48 h exposure, which is different from their small effect on CPT-11 accumulation. This may be because most mutants were able to increase intracellular SN-38 accumulation, and the 48 h drug exposure afforded sufficient time for the metabolism of CPT-11 to SN-38. However, mutant W995C had little effect on cell sensitivity to 48 h CPT-11 exposure, although an increase in CPT-11 accumulation was observed. This discrepancy may be attributed to the difference assay system for cytotoxicity and uptake in incubation period, and related determining factors, such as equilibrium of lactone and carboxylate inside and outside cells, interaction of CPTs with the target Top 1, and interaction of CPTs with cellular proteins (Beretta and Zunino, 2007). The MRP4 function for efflux of SN-38 was moderately decreased by the replacement of Phe (F) at position 324 by Trp (W), or the replacement of Trp (W) at position 230 by Phe (F), which may contribute to the lower resistance to CPT-11 at 48 h exposure in cytotoxicity assay. For most MRP4 mutants, they showed totally different influences on resistance to CPT-11 at 48 h and to MTX at 4 h in cytotoxicity assay, suggesting

that the variance in amino acids may affect substrate selectivity of MRP4. Mutant W230A with Ala at position 230 significantly increased resistance to MTX, while mutant F324A with Ala (A) at position 324 showed no effect on MRP4-mediated resistance to MTX. The replacement of Trp (W) by Ala (A), Cys (C) and Phe (F) at position 230, 216, and 995 significantly increased cells sensitivity to MTX cytotoxicity, further supporting the importance of Trp (W) in MRP4 function.

In conclusion, MRP4 is able to mediate CPT analogs efflux. The increased resistance to CPT analogs in MRP4/HepG2 cells correlated with decreased accumulation, which can be attributable to the overexpression of MRP4. Similar to MRP1, Trp residues at critical positions of the MRP4 protein are important determinants of CPTs transport activity. MRP4-mediated resistance to CPTs and identification of CPTs as MRP4 substrates has important clinical implications. Firstly, 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 (Norris et al., 2005). Secondly, 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. Thirdly, modulation of MRP4 may have important pharmacokinetic implications for CPTs. MRP4 is expressed at moderate levels in most organs such as liver, gut, lung, and brain, and substantial MRP4 levels are detected in the kidneys and prostate (Kool et

al., 1997). MRP4 may play an important role in CPTs excretion into bile and urine. 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. With the accumulation of information on 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 the development of novel CPT analogs that overcome MRP4-mediated resistance.

5.1.3. Effect of CPT analogs on GSH efflux

In our study, results of CPTs cytotoxicity assay and uptake detection suggest that GSH may play a significant role in the MRP4-mediated resistance to CPTs. When GSH level in MRP4/HepG2 cells was decreased by preincubation of cells with 200 μM BSO for 24 h, it was found that the resistance to CPTs was significantly reduced in cytotoxicity assay and intracellular accumulation of CPT analogs (CPT-11, SN-38, and topotecan) increased. All these results suggest that the change in GSH cellular level is able to greatly decrease MRP4-mediated resistance to CPTs, and GSH is very important component of transport of CPTs by MRP4.

The effect of GSH in the transport of substrates by MRP proteins has been well documented. As a GSH-*S*-conjugate export pump, MRP1 displayed decreased sensitivity to drugs that did not form GSH-*S*-conjugates, suggesting that MRP1 mediate some substrates transport in both ATP-dependent and GSH-dependent manner (Loe et al., 1998; Renes et al., 1999). The MRP1-mediated transport of some natural product drugs, such as vincristine, etoposide, daunorubicin and doxorubicin or unconjugated aflatoxin B1 was enhanced in the presence of GSH (Loe et al., 1998; Evers et al., 2000; Mao et al., 2000). These compounds did not form conjugate with

GSH, implicating that GSH is co-transported with the drug by MRP1 as a cofactor for the substrate transport. Similar to MRP1 and MRP2 that co-transport compounds out of cells with GSH, fibroblasts with transfection of MRP4 acted as co-transporters of bile salts with GSH or S-methyl-GSH (Rius et al., 2003). MDCKII cells with overexpression of MRP4 were found to transport cAMP in the presence of GSH (Lai and Tan, 2002). However, GSH is not able to affect those transporters which do not need the presence of GSH for substrate efflux. For instance, depletion of intracellular GSH by BSO had no effect on the resistance of MRP3-overexpressing cells to etoposide, suggesting that GSH is not involved in the MRP3-mediated transport of etoposide (Zelcer et al., 2001).

In view of these observations, the present study was carried out to examine whether CPT analogs have any effect on MRP4-mediated GSH efflux by the changes of bimane-GS conjugate efflux level in V/HepG2 and MRP4/HepG2 cells. CPT analogs tested at low concentration showed no significant effect on bimane-GS efflux in time-dependent assays. CPT-11 at a high concentration of 10 μM, however, displayed a significant inhibitory effect on bimane-GS efflux in MRP4/HepG2 cells, whereas lower concentrations of all CPT analogs tested had no significant effect. These data may suggest that CPT-11 is able to inhibit MRP4-mediated GSH efflux. MRP4 has been proposed to have multiple allosteric substrate binding sites (Van Aubel et al., 2005), which may explain the complex interaction patterns found with a number of MRP4 substrates. For example, urate was able to activate its own transport, inhibit MTX transport, and stimulate cGMP transport by MRP4. MRP4 was able to transport urate simultaneously with cAMP or cGMP, even under saturated conditions (Van Aubel et al., 2005). Moreover, cAMP showed inhibitory effect on MRP4-mediated cGMP transport (van Aubel et al., 2002). As MRP4 substrates, both GSH and CPT-11

are able to bind with the protein. It is possible that CPT-11 at a high concentration occupies most binding domains on MRP4. At low drug concentration, the GSH-binding site remains occupied by GSH and drug-binding site becomes occupied by CPT-11, resulting in co-transport of both compounds. However, CPT-11 at a high concentration appears to be able to occupy both the GSH- and drug-binding sites, leading to efflux reduction of GSH by MRP4. Moreover, the size and orientation of a substrate in the binding site may greatly affect whether GSH could also bind with the protein and be transported (Ballatori et al., 2005). The steric-hindrance produced by the chemical structure of CPT-11 may make it more difficult for bimane-GS to bind with MRP4 and be effluxed by MRP4. Another MRP4 substrate, MTX, also showed inhibitory effect on MRP4-mediated bimane-GS efflux at a high concentration of 100 μM (Lai and Tan, 2002).

Furthermore, efflux of bimane-GS in MRP4/HepG2 was susceptible to inhibition by 200 μM BSO. The nearly unchanged GSH efflux in V/HepG2 with the presence 200 μM BSO implicates a specific influence of BSO on MRP4, which is supported by decreased resistance to CPTs by MRP4/HepG2 cells in our cytotoxicity assay. BSO has long been reported to be responsible for the increased cytotoxicity of anticancer drugs (Mans et al., 1992; Schneider et al., 1995; Benderra et al., 2000; Zhang et al., 2001). The reduction of intracellular GSH levels by BSO led to decreased transport of MRP1 substrates in cells, such as daunorubicin, doxorubicin, etoposide and vincristine (Gekeler et al., 1995; Schneider et al., 1995; Versantvoort et al., 1995; Zaman et al., 1995; Vanhoefer et al., 1996; Rappa et al., 1997; Salerno and Garnier-Suillerot, 2001). Exposure of V79 cells to 4 h of either 2.5 mM or 5.0 mM BSO led to less than 15% of control GSH depletion (Bonner et al., 1992; Sawyer and Bonner, 1996). Short exposure to BSO for 7 h did not increase the chemosensitization of V79

to CPT-11. When V79 cells were exposed to 2.5 mM BSO for 28 h, the IC_{50} of CPT-11 was decreased by 3-fold. In contrast, BSO pretreatment increased chemosensitization of V79 cells to SN-38 in both long and short exposure (Sawyer and Bonner, 1996). However, BSO pretreatment showed no effect on the intracellular CPT-11 accumulation, conversion of CPT-11 to SN-38, and the efflux of both CPT-11 and SN-38 in V79 cells. Incubation with BSO for 28 h could cause a G_1 arrest and fewer cells were in S-phase. Since CPT-11 and SN-38 are S-phase specific, BSO might increase chemosensitization by redistributing cell cycle (Sawyer and Bonner, 1996). BSO also led to the decrease in the number of stabilized DNA-Top 1 complexes induced by CPT-11, implicating that BSO sensitized V79 cells to CPT-11 in a manner independent of the stabilization of DNA-Top 1 complexes (Sawyer and Bonner, 1996). The mechanism of BSO-induced chemosensitization varies greatly depending on the drugs involved. Pretreatment with 10 mM BSO increased doxorubicin cytotoxicity to V79 cells, while BSO pretreatment had no effect on the cellular accumulation of doxorubicin, the rate of stabilization and dissociation of cleavable doxorubicin complexes, suggesting that BSO-induced doxorubicin sensitization might occur by an independent mechanism (Bonner et al., 1992).

The involvement of GSH depletion caused by BSO in the increased cytotoxicity of CPTs indicates the possible role of GSH in modulating cytotoxicity of CPTs (Niimi et al., 1992; Matsumoto et al., 1995; Sawyer and Bonner, 1996; Gamcsik et al., 2001). Moreover, GSH was reported to affect the activity of CPTs to influence their cellular target, such as Top 1, while the mechanism by which GSH acted was not known (Gamcsik et al., 2001). It has been found that GSH showed no effect on the opening rate of lactone form of CPTs or changed the equilibrium ratio of CPTs in lactone and carboxylate forms, and GSH was not responsible for maintaining the *S*-transferase in a

reduced form. Moreover, SN-38 and topotecan did not form covalent conjugate with GSH in the absence or presence of GST (Gamcsik et al., 2001). Further work should be done to determine whether other CPT analogs, CPT-11, rubitecan and 10-OH-CPT form conjugates with GSH. It would also be interesting to explore whether GSH depletion might involve an enhancement of the cytotoxicity following the stabilization of DNA-Top 1 complexes by CPTs.

In conclusion, CPT analogs showed no significant effect on MRP4-mediated GSH efflux. It is suggested that CPT analogs may not affect their own transport by MRP4 and the transport of some MRP4 substrates that is GSH-dependent. As expected, BSO displayed specific inhibition effect on MRP4-mediated GSH efflux, whereas only CPT-11 at a high concentration showed inhibitory effect on GSH efflux in MRP4/HepG2 cells.

5.2. CONCLUSION & FUTURE WORK

In this thesis, we explored the interactions between CPTs and MRP4. Our results indicate that CPTs are part of the MRP4 resistance profile. HepG2 cells with the overexpression of MRP4 conferred significant resistance to the established MRP4 substrates, bis-POM-PMEA and MTX. The resistance capability of MRP4 to bis-POM-PMEA and MTX was significantly inhibited by the preincubation with BSO, indicating that there is proper functionality of MRP4 in MRP4/HepG2 cells used in this study. We explored the cytotoxicity profiles of a series of CPTs including CPT, CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT in HepG2 cells with stably overexpressed human MRP4. The cytotoxicity of CPTs in V/HepG2 was much higher than in MRP4/HepG2 cells, and overexpression of MRP4 increased the IC_{50} values 1.8-14.2 folds for various CPTs in lactone or carboxylate forms, suggesting that

MRP4 overexpression conferred significant resistance to CPTs. MRP4 showed higher resistance to CPT and CPT-11 in lactone form than their corresponding carboxylate form, while displayed similar resistance to lactone and carboxylate form of SN-38. The resistance of MRP4 to various CPTs tested was significantly reversed in the presence of GSH synthesis inhibitor BSO, and MRP4 inhibitors including, MK571, celecoxib, and diclofenac. A panel of other anticancer agents were also screened in cytotoxicity assays, including vinblastine, vincristine, etoposide, carboplatin, cyclophosphamide, norcantharidin, 5-fluorouracil, and paclitaxel. However, MRP4 displayed no resistance to most of these anticancer drugs. Notably, MRP4 conferred resistance to cyclophosphamide, an alkylating agent, and the resistance was partially reversed by BSO. The accumulation of CPTs, including CPT-11, SN-38, topotecan, rubitecan and 10-OH-CPT, in V/HepG2 and MRP4/HepG2 cells was further examined to investigate the relationship with resistance to CPTs in cytotoxicity assays. With the exception of 10-OH-CPT, the intracellular concentrations of CPT analogs tested in MRP4/HepG2 cells were significantly lower than V/HepG2 cells in both time-dependent and concentration-dependent accumulation assays, and the concentrations of CPT analogs in MRP4/HepG2 cells were increased by known inhibitors of MRP4 that reversed drug resistance in cytotoxicity assay. The higher intracellular accumulation of 10-OH-CPT in MRP4/HepG2 could partly be attributed to the specific binding between the carboxylate form of 10-OH-CPT and MRP4, the particular membrane protein in MRP4/HepG2 cells. These findings strongly suggested that MRP4 overexpression significantly decreases the cellular accumulation of CPTs and is involved in the resistance to CPTs. The intracellular accumulation of CPT-11 and SN-38 were also examined in 7 different MRP4 mutant cell lines, and mutant cell lines displayed different effects on drugs cellular accumulation depending

on the chemical structure of drugs, the drug dose, and the incubation time. For cytotoxicity assay, mutant cell lines showed a different influence on resistance to CPT-11 at 48 h exposure and MTX at 4 h exposure, suggesting changes in amino acids may affect substrate selectivity of MRP4. GSH plays an important role in the function of MRP4, and MRP4 transports a number of its substrates in a GSH-dependent manner. The efflux of bimane-GS from MRP4/HepG2 cells was significantly higher than that from V/HepG2 cells in 15 min. The effects of CPT analogs on GSH efflux were investigated by detecting the changes of bimane-GS conjugate efflux level in V/HepG2 and MRP4/HepG2. The CPT analogs tested showed no significant effect on bimane-GS efflux in both time-dependent and concentration-dependent assays, except that CPT-11 at a high concentration of 10 μM displayed a significant inhibitory effect on the bimane-GS efflux in MRP4/HepG2 cells. Moreover, BSO showed inhibitory effect on the efflux of bimane-GS in MRP4/HepG2, while no effect was observed in V/HepG2 cells, implicating a specific influence of BSO on MRP4. Western blot assays showed that MRP4 was overexpressed in MRP4/HepG2, and was hard to be detected in V/HepG2. The undetectable expression of some related ABC transporters, including PgP, MRP1, MRP2, MRP3, MRP5 and BCRP, excluded their involvement in bimane-GS efflux, and their interaction with CPTs. The resistance to CPTs in cytotoxicity assays, the difference uptake of CPT analogs, and the GSH efflux difference in V/HepG2 and MRP4/HepG2 cells can be mainly attributable to high expression level of MRP4 in MRP4/HepG2 cells.

Further studies are needed to explore the CPTs effects on MRP4 expression level. Recently, celecoxib was reported to be a MRP4 inducer in lung cancer cells. MRP4 expression was greatly increased in terms of mRNA and protein levels by celecoxib

and was localized in the inner plasma membrane in human NSCLC cell line Colo 699N (Gradilone et al., 2007). Thus, celecoxib could decrease the efflux of CPT-11 from cells by inhibiting MRP4-mediated transport and increase the drug efflux by inducing MRP4 expression. The understanding of the effects of drug-induced MRP4 expression level would be helpful in evaluating the combined drug schedules in chemotherapies. In addition, the transport of CPTs in both lactone and carboxylate forms and the effects of inhibitors could be studied in membrane vesicles from cells with overexpression of MRP4. This would help to clarify the influence of CPTs structure and inhibitors on MRP4-related efflux. While we have provided initial results of the effect of GSH in MRP4-mediated resistance to CPTs, several questions have also been raised. In particular, more experiments need to be performed to identifiy whether GSH is a necessary component for CPTs, or GSH has different influence on CPT analogs transport. Finally, studies on lactone-carboxylate equilibrium of CPTs and protein binding in cell culture medium would help to identify the effect of pH and proteins in cell culture medium on CPTs cytotoxicity and resistance.

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