University of Alberta

Transport of Arsenic by the Human Multidrug Resistance Proteins

by

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1 Abstract

Arsenic is a potent environmental contaminant and human carcinogen, occurring naturally in the earth's crust and entering the food chain through leeching into the water supply. Upon entering the body, inorganic arsenic is methylated to mono- and di-methylated forms, the trivalent versions of which have been shown to have increased toxicity over their inorganic counterparts. The majority of arsenic is methylated in the liver and is eliminated from the body in urine. Multidrug resistance protein 2 (MRP2) can transport the seleno-bis(*S*-glutathionyl) arsinium ion, $[(GS)_2AsSe]^-$, and coupled with the apical expression of MRP2 in the liver, provides a mechanistic explanation for the cooperative detoxification observed between arsenic and selenium. Multidrug resistance protein 1 (MRP1) was capable of transporting monomethylarsenic diglutathione ($MMA(GS)_2$) and due to the broad tissue distribution of MRP1, is likely to be important for protecting cells from arsenic. Multidrug resistance protein 4 (MRP4) can transport $MMA(GS)_2$ and dimethylarsinic acid (DMA^V) and due to its basolateral localization in the hepatocyte and apical localization in the renal proximal tubule, is likely crucial in the disposition of arsenic from the liver into the blood stream and then into urine. The results of this thesis demonstrate the role and the importance of MRP1, MRP2, and MRP4 in the protection against arsenic and its metabolites.

1.1 Table List

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1.2 Figure List

Chapter 2

Chapter 3

1.3 Abbreviations

ABC – ATP-binding cassette

Ala - alanine

APL – acute promyelocytic leukemia

AMP – adenosine monophosphate

ATP – adenosine triphosphate

As – arsenic

As^{III} - arsenite

As^{V} – arsenate

As_2O_3 – arsenic trioxide

As3MT - As^{III} methyltransferase

ArsA – arsenic resistance anion translocating ATPase

$As(GS)_3$ – arsenic triglutathione

ATRA – all *trans* retinoic acid

BSO – buthionine sulfoximine

CFTR – cystic fibrosis transmembrane conductance regulator

$CH_3As(GS)_2$ – monomethylarsenic diglutathione

Cys - Cysteine

DMA^{III} – dimethylarsinous acid

DMA^{V} – dimethylarsinic acid

DMA(GS) - darinaparsin

DTT – dithiothreitol

$E_2$17βG - 17β-estradiol 17-(β-$_D$-glucuronide)

E_1SO_4 – estrone sulphate

$[(GS)_2AsSe]^-$ - seleno-bis(S-glutathionyl) arsinium ion

GSH – glutathione

GSSG – oxidized glutathione

GSTP1 – glutathione transferase P1

HEK293 - human embryonic kidney

HEK293T – SV40 transformed human embryonic kidney

HNE – 4-hydroxynonenal

HPLC – high performance liquid chromatography

ICP-MS – inductively coupled plasma mass spectrometry

$KAsO_2$ – potassium arsenite

LTC_4 – leukotriene C_4

m/z – mass-to-charge ratio

mAb – monoclonal antibody

MMA^{III} – monomethylarsonous acid

MMA^{V} – monomethylarsonic acid

$MMA(GS)_2$ – monomethylarsenic diglutathione

MRP – multidrug resistance protein

MSD – membrane spanning domain

MTS - [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NADPH - nicotinamide adenine dinucleotide phosphate

NBD – nucleotide binding domain

NEM - *N*-ethylmaleimide

OA – ophthalmic acid

PEEK – polyetheretherketone

PGE_2 – prostaglandin E2

PML – promyelocytic leukemia

RARα – retinoic acid receptor

Rf - relative retention value

SAM - *S*-adenosylmethionine

Se - selenium

Se^{IV} – selenite

Ser - Serine

SUR – sulfonylurea receptor

TLC – thin layer chromatography

VCR – vincristine

Chapter 2

General Introduction

2 General Introduction

2.1 The ATP-binding cassette (*ABC*) transporter super-family

2.1.1 *ABC transporter superfamily members*

In humans, the ABC transporter superfamily includes 49 genes that have been assigned to 7 different subfamilies, designated A through G [1]. Eukaryotic ABC transporters can transport a wide variety of physiological and drug substrates and were originally identified based on the ability to confer resistance to anti-neoplastic agents [2]. While the majority of ABC transporter superfamily members are ATP-dependant transporters, the cystic fibrosis transmembrane conductance regulator (CFTR/*ABCC7*) [3] is a channel gated by ATP. Other exceptions to the transporter function include the ATP-dependant sulfonylurea receptors SUR1/*ABCC8* and SUR2/*ABCC9*, that serve as ion channel regulators [4]. ABC proteins in subfamilies E and F do not have transmembrane domains, but contain ATP-binding domains that closely resemble those of other ABC transporters [5, 6]. ABCE contains one subfamily member, ABCE-1 (RNase L inhibitor), which binds specifically to RNase L and has roles in cell growth, development and some pathological processes [6]. The human genome contains 3 ABCF genes of unknown function, however in yeast the ABCF1 protein is required for the activation of a kinase that phosphorylates the translation initiation factor eIF2 [7]. In humans it has been shown that the ABCF1 protein copurifies with eIF2, suggesting it performs a similar function [8].

The first eukaryotic ABC transporter to be identified and characterized was P-glycoprotein (*ABCB1*) [2]. The discovery of P-glycoprotein and the idea of multidrug resistance led to the discovery of other ABC transporters notably, multidrug resistance protein 1 (MRP1/*ABCC1*) and the breast cancer resistance protein (BCRP/*ABCG2*) [9-12]. MRP1 was identified in the H69AR small cell lung cancer cell line after step-wise selection of the parental H69 cell line with doxorubicin [9]. MRP1 and P-glycoprotein share only 20% sequence identity, but confer resistance to a similar spectrum of drugs [1]. BCRP/*ABCG2* was independently identified in placenta in addition to the drug resistant breast tumor cell lines, MCF-7/AdrVp and MCF-7/AdVp3000 [10-12]. BCRP confers resistance to anticancer drugs and is expressed in many tissues, including the blood-brain, blood-placental, and blood-testis barriers, where it is thought to play a protective role [13, 14].

2.1.2 *ABC protein structure*

The core functional unit of ABC transporters consists of two membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs) [15]. Some ABC transporters, such as BCRP/*ABCG2*, contain only one MSD and one NBD and require dimerization for functional activity.

The nucleotide binding domains of all ABC transporters contain three common motifs; the Walker A, Walker B, and the "C" signature motif (LSGGQ) [16]. The Walker A, Walker B, and "C" signature motif are essential for ATP binding and hydrolysis [1]. The two NBDs of a functional ABC transporter form

a head-to-tail ‘sandwich’ dimer [17] in which each C-signature motif is opposed to the Walker A motif of the opposite NBD, thus forming two ATP-binding sites.

2.1.3 *ABCC protein structure*

Several ABC proteins in subfamily “C” (MRP1, MRP2, MRP3, MRP6, MRP7, SUR1, SUR2), have an additional NH_2-terminal MSD, known as MSD0 (while CFTR, MRP4, MRP5, MRP8, and MRP9 do not) as shown in Figure 2.1. The function of MSD0 and the associated cytoplasmic loop (CL3) has been investigated for MRP1, MRP2, and SUR1. Unlike SUR1, in which MSD0 has a functional role in trafficking of the protein [18], no functional role of MSD0 has been found for MRP1. MSD0 of SUR1 contains the ER retention signal ‘RXR’ and when expressed alone, is retained in the ER. When expressed with Kir6.2 (the major component of the ATP sensitive K^+ channel), which also contains an ER retention signal, both signals are ‘masked’, preventing recognition of the ER retention signal, resulting in both proteins properly trafficking to the membrane at a 1:1 ratio [19]. This is important because Kir6.2 and SUR1 form an octameric functional channel, consisting of 4 Kir6.2 subunits, and 4 SUR1 subunits [20]. The CL3 of MRP2 contains a lysine-rich sequence responsible for apical expression of the protein [21]. Original studies of MRP1, without MSD0 and CL3, expressed in insect cell lines suggested that MSD0 was involved in function [22, 23], but it was later determined that removal of MSD0 of MRP1 expressed in mammalian cell lines had no effect on protein function or trafficking [24, 25]. Removal of CL3 was responsible for causing the effect on protein trafficking [24].

In the majority of ABC transporter NBDs, the amino acid following the Walker B motif is Glu, which contains the carboxylate side chain involved in the cleavage of the β-phosphodiester bond of ATP. This Glu residue in NBD1 is not conserved in the ABCC family [26]. In addition, while the COOH-proximal NBDs of the ABCC family closely resemble typical ABC structure, the NH_2-proximal NBDs of ABCC proteins are quite divergent [9]. A 13 amino acid deletion exists between the Walker A and C-signature motif (10 in MRP7/*ABCC10*) [27]. As expected from the structural differences between the NBDs of the ABCC family, they play distinct roles in the transport cycle and several studies have shown that NBD1 of MRP1, CFTR, and SUR is incapable of ATP hydrolysis [28-34].

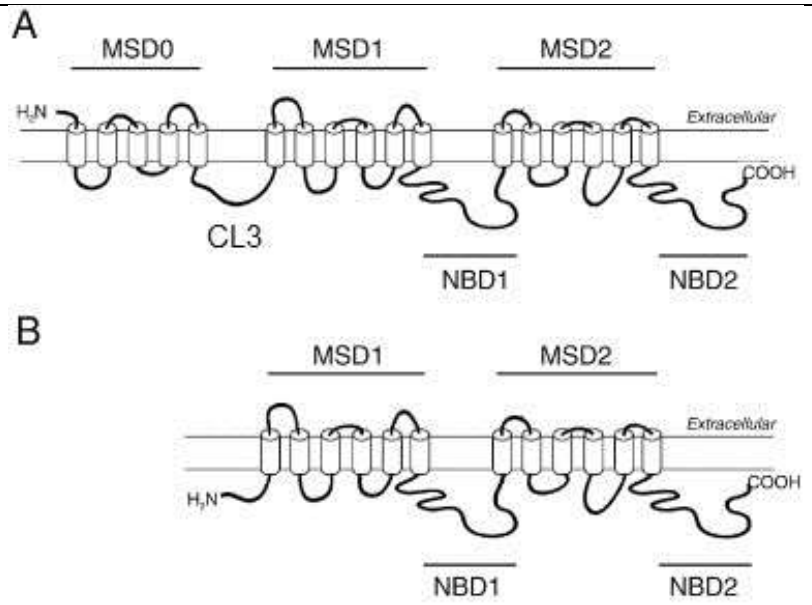

Figure 2.1. Predicted secondary structure of ABCC/MRP family members. (A) Contains the NH_2-terminal membrane spanning domain (MSD0) (MRP1, MRP2, MRP3, MRP6, MRP7, SUR1, SUR2). (B) The 'short' structure resembling typical ABC structure (MRP4, MRP5, MRP8, and MRP9). This figure was modified from [35] with permission.

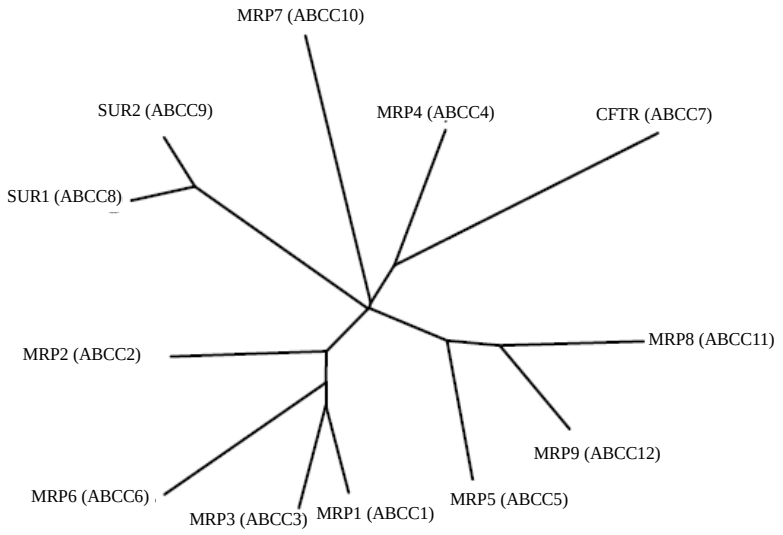

Figure 2.2. Phylogenetic dendrogram of the sequence similarities of the full-length human ABCC proteins. Dendrogram is based on ClustalW2 alignments and generated using TreeView. Accession and version of sequences used for alignment: ABCC1- P33527.3 GI:296439301, ABCC2- Q92887.3 GI:308153583, ABCC3- O15438.3 GI:6920069, ABCC4- O15439.3 GI:206729914, ABCC5- O15440.2 GI:8928547 , ABCC6- O95255.2 GI:269849624, ABCC7- P13569.3 GI:147744553, ABCC8- Q09428.6 GI:311033501, ABCC9- O60706.2 GI:215273925, ABCC10- Q5T3U5.1 GI:74756298, ABCC11- Q96J66.1 GI:74762666, ABCC12- Q96J65.2 GI:161788999

2.1.4 *Mechanism of transport*

The mechanism by which ABC transporters hydrolyze ATP in order to translocate their respective substrates is currently incompletely understood. Two proposed models for the function of ABC transporters have evolved from biochemical data and the solved crystal structures of nine prokaryotic and two eukaryotic ABC transporter proteins [36-48]. One model referred to as "The Switch" mechanism arose from the alternating access model [49] and the first solved structures of the ABC family members, MsbA and Sav1866 [37, 48]. Sav1866 is a bacterial multidrug efflux "half-transporter" that forms a homodimer [37], currently with an unknown physiological role [50]. MsbA is also a bacterical efflux "half-transporter" that effluxes lipid A, preventing toxic accumulation within the inner leaflet of the bacterial membrane [51]. The Switch transport mechanism involves the dimerization of the NBDs upon binding of two ATP molecules. Both NBDs are thought to completely dissociate after the sequential hydrolysis of the two ATP molecules. The Switch mechanism involves the transporter starting from the inward facing conformation with a low affinity for ATP. Substrate binds, inducing a conformational change which increases the affinity for ATP and two ATP molecules bind the NBDs to cause their dimerization. ATP-binding and NBD dimerization results in the transporter shifting to the outward-facing conformation and the release of substrate. Hydrolysis of ATP is thought to return the transporter to the inward-facing conformation, resetting the cycle. Several structures providing evidence in support of the 'Switch' mechanism, in which NBDs are dissociated, are based on crystal structures of proteins in a nucleotide-free state [36, 45, 52], whereas Sav1866 was resolved in the presence of ADP and

showed a close association between NBDs [37]. These nucleotide-free states are unlikely to occur physiologically. Solved crystal structures such as P-gp [52], in the absence of nucleotide, leave significant room for question regarding their mechanistic relevance as the presence of nucleotide would cause significant conformational changes and also better represent an *in situ* snap shot of the protein.

The second proposed mechanism is known as the "Constant Contact" model. The primary difference is that the ATP molecules are proposed to be sequentially hydrolyzed but nucleotide binding domains remain in contact throughout the cycle [53-57]. The constant contact model involves alternating ATP hydrolysis at each site, with one site opening at the point of hydrolysis and the second NBD remaining closed with ATP bound and occluded. While one site opens to allow nucleotide release, the NBDs do not separate fully, ATP binds to the newly opened site, the second site undergoes ATP hydrolysis and this process repeats in alternating cycles. Structural studies of Sav1866, CFTR and MalK support the constant contact model through demonstration of non-equivalent NBDs [58-60]. These studies indicate that only one of the two NBDs is capable of ATP hydrolysis, leaving one pocket NBD permanently occupied by ATP and thus in constant contact and never completely dissociating as proposed in the switch mechanism. Recently, the heterodimeric bacterial multidrug transporter TM287/288, with non-equivalent NBDs was solved in the inward facing state and provided strong evidence for the constant contact model [40]. This structure showed, in contrast to other inward facing crystal structures, an open substrate

binding cavity, while NBDs remained in contact [40]. Large bodies of evidence exist for both the constant contact model and the switch model. Determining the correct representation of the transport cycle will require the difficult task of solving high resolution crystal structures of ABC transporters in all possible conformations (outward facing substrate bound, outward facing substrate free, inward facing substrate free, inward facing substrate bound, closed outward facing, closed inward facing, in addition to the various possible nucleotide bound states of each).

2.1.5 *Multidrug resistance protein 1 (MRP1)*

2.1.5.1 *Expression*

MRP1 is expressed in most tissues throughout the body, with relatively high levels found in the lung, testes, kidney, placenta, skeletal muscles, and peripheral mononuclear cells [61]. MRP1 is not detected in healthy human liver, but is present in liver cancer cell lines, such as HepG2 [62]. MRP1 is localized to the basolateral membrane in epithelial cells, and to the luminal surface of brain microvessel endothelial cells [63].

2.1.5.2 *Role in drug resistance and anticancer drugs transported*

MRP1 was originally discovered in the doxorubicin-selected H69AR small cell lung cancer cell line with an associated multidrug resistant phenotype [9]. Since then, MRP1 has been associated with poor outcome in many different haematological and solid tumors and MRP1 expression has important implications in clinical anticancer drug resistance [15]. Upregulation of MRP1 following anticancer drug treatment occurs in many tumor types and cell lines

including prostate, hepatocellular, myeloid leukemia, renal cell, adrenocortical, and colon cancers [64-67]. Genetic polymorphisms in MRP1 have also been associated with poor clinical outcomes of hepatocellular carcinoma [68]. Recent work has identified several single nucleotide polymorphisms (SNPs) of MRP1, which are associated with increased haematological toxicity of the chemotherapeutical regimen of 5-fluorouracil, epirubicin, and cyclophosphamide (FEC) [69], demonstrating the important role of MRP1 in protecting non-malignant tissues during drug treatment.

In addition to doxorubicin, MRP1 has been shown to confer resistance to other anticancer drugs; including, methotrexate, etoposide, mitoxantrone, vincristine, vinblastine, and tyrosine kinase inhibitors [70-72]. MRP1 also transports many non-cancer related drugs, including the antiviral drugs ritonavir and saquinovir, as well as the antibiotics difloxacin and grepafloxacin [73-76]. MRP1 and Pgp confer resistance to a similar spectrum of anti-cancer drugs, but through different transport mechanisms. Unlike Pgp, MRP1 has complex interactions with the cytosolic tripeptide, glutathione (GSH/ γ-Glu-Cys-Gly). Cellular GSH is the most abundant low molecular weight thiol with cytosolic concentrations ranging from 0.5-10 mM [77]. In addition to MRP1 transporting both GSH and its oxidized form GSSG, MRP1 can transport a number of substrates in which conjugation to GSH is required for transport, and transport of several substrates are stimulated by GSH but do not form conjugates for which a GSH-stimulated transport mechanism has been proposed [15]. Necessity of conjugate formation versus GSH stimulation has been partially elucidated using

the GSH analogues, *S*-methyl GSH and ophthalmic acid (OA) (shown in Figure 2.3), which are structurally similar to GSH but have a methyl 'capped' thiol group or methyl substituted thiol group, respectively. *S*-methyl GSH and/or OA have been shown to substitute functionally for GSH for a number of substrates including vincristine (only *S*-methyl GSH) and mitoxantrone [78-82]. *S*-methyl GSH replaces GSH in vincristine transport whereas dipeptides and reducing agents did not, demonstrating that the tripeptide structure of GSH was required for stimulation [83]. More extensive lists of MRP1 substrates are shown in Tables 2.1, 2.2, and 2.3.

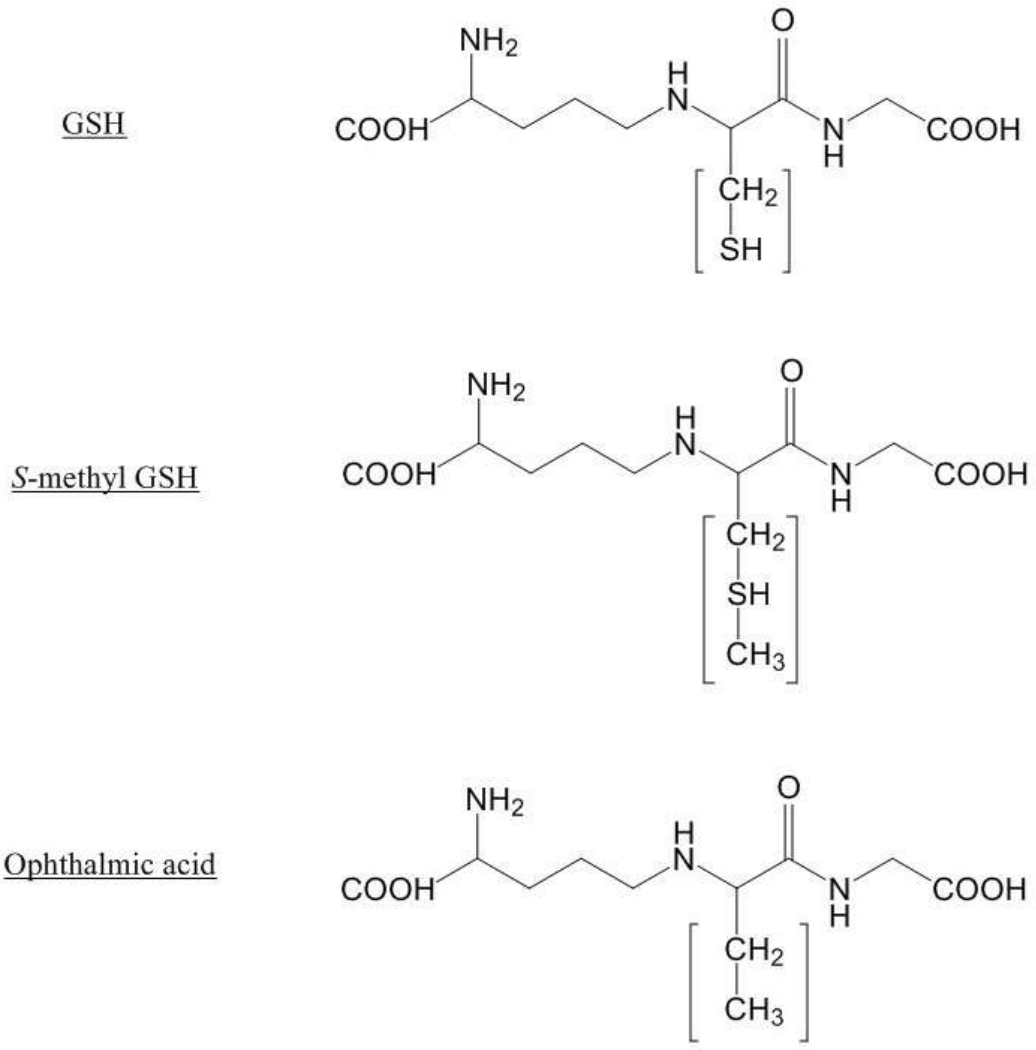

Figure 2.3 Chemical structures of GSH and the GSH analogs ophthalmic acid (OA) and *S*-methyl GSH. Bracketed sections represent the thiol group of GSH, or the substituted group in *S*-methyl GSH and ophthalmic acid.

Table 2.1. Selected Drug Substrates of human MRP/ABCC drug transporters

Substrates	MRP1	MRP2	MRP4
Methotrexate	✓	✓	✓
Sorafenib		✓	
Doxorubicin(+GSH)	✓	✓	
Etoposide (+GSH)	✓	✓	
Mitoxantrone	✓	✓	
Cisplatin [$Pt(GS)_2$]		✓	
Vincristine (+GSH)	✓	✓	
Vinblastine (+GSH)	✓	✓	
Camptothecin		✓	
Leucovorin			✓
Topotecan	✓	✓	✓
Adefovir			✓
Tenovir			✓
Ceftizoxime			✓
Cefotaxime			✓
Cefmetazole			✓
Hydrochlorothiazide			✓
Furosemide			✓
Oimesartan			✓
Edaravone glucuronide			✓
p-aminohippurate			✓
6-mercaptopurine			✓
6-thioguanine			✓
Azathioprine			✓
Morphine 6-glucuronide		✓	
Morphine 3-glucuronide		✓	
Acetominophen-glucuronide		✓	
Tyrosine Kinase Inhibitors[1]	✓		
Paclitaxel	✓	✓	
Saquinovir	✓	✓	✓
Ritonavir	✓		
Difloxacin	✓		
Grepafloxacin	✓		

[1]Small-molecule 4-anilinoquinazoline-based tyrosine-kinase inhibitors

Table 2.2. Selected Physiological Substrates of human MRP/ABCC drug transporters

Substrates	MRP1	MRP2	MRP4
Bilirubin-glucuronide		✓	
Unconjugated bilirubin	✓		
GSSG	✓	✓	
GSH	✓	✓	✓
LTC_4	✓	✓	✓
Folic Acid	✓	✓	✓
DHEAS	✓	✓	✓
$E_2$17βG	✓	✓	✓
Estrone 3-sulphate	✓	✓	
cGMP			✓
cAMP			✓
ADP			✓
urate			✓
PGE_1			✓
PGE_2			✓
$PGE_{2\alpha}$	✓		✓
TXB_2			✓
LTB_4			✓
Taurocholate (+GSH)			✓
Cholate			✓
Cholylglycine			✓
Cholyltaurine	✓		✓
Deoxycholylglycine			✓
Chenodeoxycholylglycine			✓
Ursodeoxycholylglycine			✓
Ursodeoxycholyltaurine			✓

Table 2.3. Selected Toxicological Substrates of human MRP/ABCC drug transporters

Substrates	MRP1	MRP2	MRP4
$As(GS)_3$	✓	✓	
$MMA(GS)_2$	✓	✓	✓
$[(GS)_2AsSe]^-$		✓	
Potassium Antimonite	✓		
Methoxychlor	✓		
Dinitrophenyl-GS	✓	✓	✓
Ethacrynic Acid-GS	✓		
NNAL-O-glucuronide	✓	✓	
Methylmercury-*N*-acetyl-L-cysteine	✓	✓	
N-ethylmaleimide glutathione		✓	
Ochratoxin A		✓	
Metolachlor-GS	✓		

2.1.5.3 *Physiological function and substrates*

In addition to its role in drug resistance, MRP1 is present in non-malignant tissues and transports a diverse array of physiologically relevant substrates. MRP1 is important for the efflux of the leukotriene, LTC_4 from the cell [81]. Leukotrienes elicit a number of biological effects including contraction of bronchial smooth muscles, stimulation of vascular permeability, and the attraction and activation of leukocytes. LTC_4 is produced in mast cells, basophils, neutrophils, eosinophils, dendritic cells, platelets, kidney, and brain [84]. LTC_4 is synthesized within the cell starting from arachidonic acid which is converted to LTA_4 by 5-lipoxygenase. LTA_4 is converted to LTB_4 or LTC_4 by LTA_4 hydrolase and LTC_4 synthase respectively [15]. *Mrp1*$^{-/-}$ mice are healthy and fertile and show no physical or histological abnormalities [85]. Consistent with the role of MRP1 as an LTC_4 transporter, studies in *Mrp1*$^{-/-}$ mice showed a reduced IgE-mediated inflammatory response [85]. The transport of LTC_4 by MRP1 is involved in dendritic cell migration from the skin to the lymph nodes [86]. Regulation of dendritic cell migration to the chemokine CCL19 was shown to be regulated by LTC_4, and Mrp1-/- mice had greatly reduced dendritic cell migration, which could be restored by the addition of exogenous LTC_4 [86].

Human MRP1 transports the GSH conjugate of 4-hydroxy-2-nonenal (HNE) [87]. HNE is a toxic and highly reactive product of lipid peroxidation, and being a strong electrophile, readily reacts with nitrogen in nucleic acids or sulphur in proteins to form covalent adducts [88]. Mouse studies demonstrated an increase in Mrp1 expression after treatment with doxorubicin, which induces oxidative stress and thus the formation of HNE [89]. Together, these data suggest

that MRP1 plays an important role in protecting cells against the toxic products of lipid peroxidation.

MRP1 transports GSH and GSSG and it has been suggested that through this function MRP1 is involved in maintaining the redox state of the cell [90]. Levels of MRP1 have been shown to increase significantly after the induction of oxidative stress [91]. *mrp1*$^{-/-}$ mice have shown increased levels of cellular GSH in tissues normally expressing high levels of Mrp1 [92, 93].

MRP1 transports unconjugated bilirubin and could potentially play a role in preventing bilirubin neurotoxicity in infants [94-96]. In addition to its dramatically increased toxicity compared with conjugated bilirubin, unconjugated bilirubin is insoluble in water and cannot be eliminated in urine or feces. Hyperbilirubinemia occurs in neonates due to immature hepatic and intestinal glucuronidation pathways. Neonates also lack the intestinal flora required for the breakdown and excretion of bilirubin in feces [97]. These factors combined with an incompletely developed blood brain barrier make neurotoxicity a concern. MRP1 is thought to serve as a back-up defense pathway, as its expression is upregulated by unconjugated bilirubin in the glia of the brain [94].

MRP1 has been shown to transport folic acid, suggesting a role in folate homeostasis [98]. Since folate cannot be synthesized *de novo*, and is essential for DNA synthesis, repair, and methylation, the cellular regulation of folate levels has implications for the regulation of cell growth and proliferation. Expression of MRP1 in fetal capillaries within the placenta has been proposed to protect the developing fetus from conjugated estrogen hormones such as estrone sulphate

(E_1SO_4) and 17β estradiol 17β-D-glucuronide ($E_2$17βG) [99, 100]. $E_2$17βG is an estradiol metabolite formed in the liver that is transported into bile under normal circumstances by MRP2 [101]. Accumulation of $E_2$17βG is implicated in cholestasis during late stage pregnancy. Estrone sulphate is the predominant circulating estrogen in post-menopausal women and has also been considered as a prognosis factor in prostate cancer [102]. Additionally, the expression of MRP1 in Leydig cells may contribute to protecting the testis from estrogen [103, 104].

2.1.5.4 *Role in tissue protection and toxicological substrates*

With its broad substrate specificity and tissue distribution, MRP1 is thought to play a protective role in cells and tissues by preventing xenobiotic accumulation. In support of this role, MRP1 transports many substrates of toxicological relevance. For example, MRP1 transports the GSH conjugates of the carcinogen aflatoxin B1. Aflatoxin B1 is a fungal toxin that enters the body via inhalation, oral, or dermal exposure and is activated within the cell by cytochrome P450 to form aflatoxin B1-8,9-epoxide, which can be further metabolized by glutathione *S*-transferases, UDP-glucuronyl-transferases, or sulfotransferases [105]. If not conjugated, aflatoxin B1-8,9-epoxide can react with the nucleophilic centers of DNA, RNA, and protein and thus exhibit its carcinogenic effect. Specifically, aflatoxin B1 induces tumors in lung and more commonly the liver, where it induces mutations of p53 in human hepatocytes [106], a cell which under normal circumstances does not express MRP1, thus lacking the protective role MRP1 has in other tissues. MRP1 also transports the glucuronide conjugate of the nicotine derived tobacco-specific lung carcinogen 4-

(methylnitrosamino-1-(3-pyridyl)-1-butanol (NNAL), but only in the presence of GSH [79]. NNAL is formed from the intracellular carbonyl reduction of NNK, another potent lung carcinogen. NNK and NNAL both been induce tumor formation in rodent models [107].

2.1.6 *Multidrug resistance protein 2 (MRP2)*

2.1.6.1 *Expression*

Prior to the identification and cloning of MRP2 in rats and humans [108-110], it was known as the canalicular multispecific organic anion transporter (cMOAT) where it was found to be expressed apically in hepatocytes and transport amphiphilic anions into bile. MRP2 is localized to the apical membrane of epithelial cells and is expressed in the liver, kidney, small intestine, colon, gall bladder, placenta, and lung [15]. Similar to MRP1, MRP2 isimplicated in providing cellular protection in non-malignant tissues such as liver, testis [111], lung, stomach [112], and placenta, where expression has been shown to increase over the course of pregnancy [113].

2.1.6.2 *Role in drug resistance and anti-cancer drugs transported*

Increased expression levels of MRP2 is found in oesophageal squamous cell carcinoma, ovarian cancer, hepatocellular carcinoma, bladder cancer, and colon cancer cell lines [114-118], however expression is not associated with clinical outcome and MRP2 is not thought to be a factor in drug resistance. Similar to MRP1, MRP2 transports a number of antineoplastic agents, such as the *Vinca* alkaloids through GSH stimulated transport [119] and the GSH conjugates of chlorambucil and cyclophosphamide [120, 121]. The chemotherapeutic agents

sorafenib, doxorubicin, etoposide, methotrexate, mitoxantrone, and camptothecin are also substrates for MRP2 [99, 120, 122, 123]. Although MRP2 shares highly overlapping substrate specificity with MRP1, it differs in having the ability to transport platinum containing drugs, such as the controversial MRP2 substrate, cisplatin [123]. More extensive lists of MRP2 substrates are shown in Tables 2.1, 2.2, and 2.3.

2.1.6.3 *Physiological function and substrates*

MRP2 transports a wide variety of physiological substrates including GSH and estradiol conjugates, leukotrienes, conjugated bile salts, bilirubin glucuronides, and some steroid compounds [123]. Dubin-Johnson syndrome is caused by any of a number of mutations in MRP2 that result in the absence of a functionally active MRP2 protein [124]. This lack of MRP2 function causes conjugated hyperbilirubinemia, due to the accumulation of bilirubin glucuronide in liver and blood. Patients with Dubin-Johnson syndrome are generally asymptomatic under normal conditions and treatment is usually unnecessary [125]. *Mrp2*$^{-/-}$ mice and Mrp2 deficient rat strains (TR$^-$/EHBR) are healthy and exhibit mild abnormalities similar to those seen in Dubin-Johnson patients [126-129]. Transport deficient (TR$^-$) and Eisai hyperbilirubinemic rat (EHBR) rats were originally identified from a spontaneous hereditary mutation of the *Mrp2* gene in Wistar and Sprague Dawley rats, respectively [128, 130]. These rodents have increased levels of serum and urine bilirubin glucuronide, increased hepatocyte GSH concentrations, 16-fold lower biliary GSH, and decreased bile flow rates [126, 131]. MRP2 has a complex interaction with GSH, similar to MRP1, in that it is capable of

transporting GSSG, GSH, GSH conjugates [132, 133], and other conjugated substrates that require or are enhanced by GSH [119, 123, 134].

2.1.6.4 *Role in tissue protection and toxicological substrates*

MRP2 is expressed at several pharmacologically and toxicologically important barriers, including the placenta, the apical surfaces of hepatocytes, enterocytes, and proximal tubule of the kidney. Mrp2 transports a number of toxicologically relevant substrates, including the glucuronide conjugate of the tobacco specific carcinogen (NNAL), and acetaminophen glucuronide [79, 99, 135, 136]. MRP2 also transports the toxic and carcinogenic ochratoxin, and due to its apical expression in proximal tubular cells in the kidney, likely contributes to ochratoxin urinary elimination [135, 137, 138]. Rat and human MRP2 also transport the GSH conjugates of the toxic metals/metalloids arsenic, cadmium, and mercury [132, 133, 135, 139-141]. MRP2 transports As in the forms arsenic triglutathione ($As(GS)_3$), monomethylarsenic diglutathione ($MMA(GS)_2$), and the seleno-bis(S-glutathionyl) arsinium ion ($[(GS)_2AsSe]^-$) [132, 142, 143]. Bile duct cannulated Mrp2-deficient rats (TR^-) were found to eliminate more As in urine [142], likely because less biliary elimination occurred compared to wild-type.

2.1.7 *Multidrug resistance protein 4 (MRP4)*

2.1.7.1 *Expression*

The multidrug resistance protein 4 (MRP4/*ABCC4*) is expressed in many tissues, including cardiovascular, kidney, liver, brain, testis, prostate, ovary, adrenal gland and intestine [144-146]. MRP4 distinguishes itself from other members of the multidrug resistance protein family by its ability to localize apically in some

epithelial cell types and basolaterally in others. In hepatocytes and choroid plexus epithelium, MRP4 is localized to the basolateral membrane, whereas in the kidney, it is expressed at the apical membrane of renal proximal tubule cells [147]. In a colonic epithelial cell line, MRP4 was found to be localized to both apical and basolateral membranes [148]. The COOH-terminus of MRP4 contains a consensus class I PDZ-interaction motif that is similar to what is found in MRP2 and CFTR, both of which express at the apical membrane of polarized cells [149]. The adaptor protein, NHERF1 is involved in trafficking of MRP2, MRP4, and CFTR by interacting with the PDZ interaction motif [150, 151]. High levels of NHERF1 in the LLC-PK1 cell line relative to lower levels expressed in MDCKI cells were associated with apical and basolateral expression of MRP4, respectively [149].

2.1.7.2 *Role in drug resistance and anti-cancer drugs transported*

MRP4 confers resistance to nucleoside and nucleotide analogs, irinotecan, and toptecan [152, 153]. While MRP4 confers resistance to anticancer drugs, it has not been shown to be a factor in clinical drug resistance. However, MRP4 expression is a powerful indicator of clinical outcome in neuroblastoma [153] and plays a complicated and incompletely understood role in prostate cancer. While studies have shown upregulation of MRP4 in prostate cancer [154], recent reports have shown a decrease in expression that is associated with tumor progression [155], possibly due to the androgen depletion associated with advanced cancers and the association between androgen levels and MRP4 expression [156]. Because of its tissue distribution, localization, and broad substrate specificity,

MRP4 plays a role in the disposition of various drugs. Using a mouse knockout model, Mrp4 has been shown to be functionally involved in the renal elimination of the antiviral drugs adefovir and tenofovir [157]. Studies of mrp4-/- knockout mice showed that Mrp4 protects the brain from the anticancer drug topotecan [158]. Similar studies have shown that Mrp4 prevents thiopurine-induced hematopoetic toxicity [159]. Mrp4 greatly decreased the accumulation of 6-thioguanine nucleotides in myelopoetic cells. A more extensive list of MRP4 substrates is shown in Tables 2.1, 2.2, and 2.3.

2.1.7.3 *Physiological function and substrates*

MRP4 transports many important physiological substrates, such as bile salts[160], prostaglandins PGE_1 and PGE_2 [161], cyclic nucleotides, ADP, eicosanoids, urate, conjugated steroid hormones, folic acid, and bile acids [144]. The cyclic nucelotides, cAMP and cGMP, were among the first endogenous substrates proposed for MRP4 and function as second messengers that mediate a very broad range of cellular activities through the activation of protein kinases. MRP4 may plays a role in regulating cAMP and cGMP levels through active efflux from the cell. In the proximal tubule of the kidney, where MRP4 is apically expressed, it has been proposed that MRP4 is involved in tubular regulation of water and salt homeostasis through the efflux of cAMP and cGMP into the primary urine [162]. Chen *et al.* reported the K_m of MRP4 for cAMP and cGMP to be 44.5 µM and 9.7 µM respectively [152]. Cellular cAMP and cGMP levels have been reported to range from ~1-10 µM and ~1 µM respectively [163, 164]. The affinity for cAMP and cGMP, within range of the physiological levels, provides evidence for MRP4

being a potential physiological transporter of cAMP and cGMP. A recent study has demonstrated that MRP4 plays a role in regulating intraocular pressure through regulating cellular concentrations of cAMP and cGMP by efflux [165]. Knock-down of MRP4 in the rat pancreatic cell line, AR42J, using siRNA caused a decrease in extracellular cAMP [166]. In addition, efflux of cAMP in a number of cells was shown to be ATP and probenecid sensitive, demonstrating that MRP4 plays a role in the regulation of cellular levels of cAMP [167]. mrp4-/- mice have an increased unvascularized retinal area when challenged with forskolin, a compound used to raise cAMP levels, suggesting a role for Mrp4 in the protection of the developing retina through the reduction of cAMP [168].

Urate is a possible physiological substrate for MRP4 [169]. Urate is the end product of purine metabolism in humans and higher primates, whereas in other mammals, it is further metabolized to allantoin. The relatively high blood levels of urate (0.3-0.5 mM) in higher primates compared with other mammals has been associated with extended lifespan through its free radical scavenging ability [170]. The basolateral expression of MRP4 in hepatocytes where the majority of urate is produced demonstrates the importance of MRP4 in urate regulation. In addition, the expression of MRP4 at the apical surface of the proximal tubular cells in the kidney, where urate can be transported into urine and eliminated from the body provides evidence for the involvement of MRP4. The reported K_m of MRP4 for urate is 1.5 mM [169], which is within range of serum levels, in addition to the increased concentrations that would likely be found in the liver and kidney. Demonstration of a clear physiological role of MRP4 in the

transport of urate is yet to be shown. Bataille et al. proposed that MRP4 is the dominant apical transport pathway for urate in birds, however this conclusion is grounded on their experiments using the LTD_4 receptor antagonist, MK571, as a "MRP4-specific inhibitor", which is misleading as MK571 also inhibits many of the MRPs and other transport proteins [171, 172].

Similar to MRP1 and P-gp [86, 173], MRP4 also plays an important role in dendritic cell migration, although the MRP4 related mechanism has not been identified [174]. Dendritic cells are key initiators of the immune system, recognizing foreign antigens and presenting them to other cells in the immune system, acting as a bridge between the innate and adaptive immune systems [175].

2.1.7.4 *Role in tissue protection and toxicological substrates*

In the brain, MRP4 is found in the luminal membrane of the capillary endothelium, the basolateral membrane of the choroid plexus, and in astrocytes [158, 176]. MRP4 contributes to blood brain barrier protection as demonstrated by the increased brain levels of adefovir in Mrp4-knockout mice compared to wild-type [158]. It has been proposed that MRP4 also contributes to protection of the liver during cholestasis by preventing the accumulation of toxic bile acids through efflux for their ultimate urinary elimination [160, 177] and is supported by the observation that Mrp4-knockout mice are sensitive to cholestatic induced liver injury [178]. With the expression of Mrp4 in the lumen of brain capillaries and in the basolateral membrane of choroid plexus, it is a candidate for protection of the brain against harmful compounds. Mrp4 is also upregulated in the liver after acetaminophen-induced cholestasis and was proposed to provide a relief to

the chemical burden during critical stages of regeneration and repair following liver injury [179]. In the human bronchoalveolar cell line, H358, MRP4 reduces DNA-adduct formation caused by benzo[a]pyrene [180]. Benzo[a]pyrene is a ubiquitous polycyclic aromatic hydrocarbon, which can be metabolized in cells to form (+)-*anti*-*trans*-B[*a*]PDE-dGuo which can enter the nucleus and react with DNA to form adducts.

2.2 Arsenic

2.2.1 *Arsenic occurrence and exposure*

The metalloid arsenic (As) is a component of the earth's crust and through natural as well as human influences can be released into the environment [181]. Arsenic is water soluble and is therefore susceptible to leeching into surrounding water. One infamous occurrence of As leaching was caused by the digging of shallow tube wells in Bangladesh in order to evade microbial contamination of drinking water [182]. This resulted in the contamination of drinking water with As-containing strata and Bangladesh becoming a site of massive numbers of humans chronically exposed to As. It is estimated that 60% of all ground water in Bangladesh is contaminated with unacceptable levels of arsenic resulting in the exposure of 35-77 million people [183]. In addition to environmental exposure, several industrial sources of As are responsible for human As exposure. For example, treatment of wood with arsenical preservatives, arsenic containing pesticides, and the mining/smelting of copper, gold, lead, and zinc are possible exposure sources of As [184].

The main As-exposure route for populations world-wide is drinking water, and through crops irrigated with contaminated water [185]. The majority of arsenic consumed in a North American diet is in relatively non-toxic organic forms in seafood, such as arsenobetaine, arsenosugars, and arsenolipids [184]. Seaweed and mollusks are high in arsenosugars and arsenobetaine [186], and fatty fish are rich in arsenolipids [187]. The significantly more toxic inorganic arsenic species arsenite (As^{III}) and arsenate (As^{V}) are of global concern as pollutants of drinking water and groundwater. Unacceptable levels (WHO limit of 10 ppb) have been reported in over 70 countries, including regions of USA, China, Chile, Bangladesh, Nepal, Vietnam, Taiwan, Mexico, Argentina, Poland, Italy, Finland, Spain, Canada, Hungary, New Zealand, Japan and India [184, 185, 188].

2.2.2 *Carcinogenicity of Arsenic*

Arsenic was first identified as a human carcinogen in 1977 [189], and is associated with bladder, lung, and skin cancers [190]. Multiple pathways have been reported for the mechanism for As-induced carcinogenesis, and it is likely a result of a complex combination of several factors. Extensive studies have been performed using animal models to investigate arsenic carcinogenicity [191-195]. However, the results of these studies are complicated by species differences in As metabolism. For example, arsenic in rodent drinking water at levels 100-200 times greater than levels in human drinking water result in similar levels of As in blood [196]. Because of these species differences, early studies failed to demonstrate any carcinogenic effect of As [191]. Despite early studies being unable to demonstrate the carcinogenicity of As in rodents, As interacts with

many other carcinogenic substances and increase oncogenesis in many tissues and tumor types, including hepatic, ovarian, pulmonary, adrenal, skin, colorectal, and bladder tumors [197-200]. However, the metabolic differences between rodents and humans result in varied handling and transport pathways. In addition, the As exposure required to cause cancer in rodent models requires very high levels of As (20+ ppm) [192, 196, 198, 201], 2000-fold higher than the WHO limit set at 10 ppb, demonstrating the importance of developing human models for understanding the handling and carcinogenicity of As. In addition to the difference in doses required to cause cancer in humans versus rodents, rodent models are unable to demonstrate dermal cancer [201], which is a prominent pathology in humans exposed to As.

A number of mechanisms have been proposed to be responsible for the carcinogenicity of As. Oxidative stress, chromosomal abnormalities, genotoxicity, inhibition of DNA repair, altered DNA methylation patterns, altered growth factors, enhanced proliferation, promotion/progression, gene amplification, and suppression of p53 have all been proposed to contribute to the carcinogenic mode of action of arsenic [192, 202-208]. Interestingly, inorganic arsenic is metabolized through methylation and uses the same methyl donor (S-adenosyl-L-methionine) as required for DNA methylation [209-211]. This is likely to cause reduced capacity for DNA methylation under arsenic-induced stress and is likely to contribute to the carcinogenic effect of arsenic by reducing normal DNA methylation, resulting in abberant gene expression [209].

2.2.3 *Health effects of environmental arsenic*

Acute arsenic poisoning is rare and historically has occurred due to accidental ingestion or intentional administration to commit suicide or murder [212]. Acute arsenic toxicity presents with severe abdominal pain, nausea, vomiting, and diarrhea and can lead to hypotension, shock, arrhythmia, congestive heart failure, encephalopathy, and coma [184]. In acutely toxic doses, arsenic disrupts the tricarboxylic acid (TCA) cycle of the cell by causing allosteric inhibition of pyruvate dehydrogenase and thus prevents the oxidation of pyruvate to acetyl-CoA eventually causing cell apoptosis [203]. In addition to causing apoptosis due to the disruption of the TCA cycle, free radical formation caused by trivalent arsenic species may result in cell necrosis [213].

Chronic exposure to inorganic arsenic is associated with a multitude of disease states with effects on many organs and systems. One trademark sign of arsenic exposure is skin hyper-pigmentation and palmoplantar hyperkeratosis. These arsenic "skin lesions" are commonly used as a diagnostic tool to identify individuals chronically exposed to dangerous levels of arsenic [214]. Chronic arsenic exposure is linked to diseases of the cardiovascular, neurological, gastrointestinal, haematological, respiratory, hepatic, and dermal systems of the body [184, 215]. Arsenic causes the unique peripheral vascular disease known as blackfoot disease, which is characterized by high arsenic levels in arterial walls, thromboangitis obliterans, and arteriosclerosis [216, 217]. Blackfoot disease results in black colouration of the feet, numbness, and in some cases can lead to

gangrene [216, 217]. In mice, arsenic levels equivalent to the WHO limit of 10 ppb can exhibit an effect on blood vessel remodeling [218].

There is an association between high levels of As exposure in drinking water and risk for type 2 diabetes mellitus [219-222], although this is somewhat controversial. Several mechanisms have been proposed for the association. The first proposed mechanism is through the chemical similarity of arsenate and phosphate, resulting in the uptake of arsenate through phosphate carriers and the formation of ADP-arsenate which could interfere with phosphate uptake and thus ATP synthesis. However, with the low levels of arsenate exposure to the cell, this is an unlikely scenario [223-225]. A second possible mechanism is the affinity of As for sulfhydryl groups and the inhibition of pyruvate dehydrogenase or α-ketoglutarate dehydrogenase. Arsenite, as well as trivalent methylated arsenic species, inhibit these enzymes, but at concentrations much higher (>100-fold) than those encountered during environmental exposure [226]. Oxidative stress and alteration of gene expression have also been proposed for the mechanism behind the association [225], but current evidence remains inconclusive.

2.2.4 *Medicinal use of arsenic*

The medicinal use of arsenic dates back over 5000 years in Traditional Chinese medicine, and the first record of arsenic trioxide is mentioned in the Shennong Materia Medica (200 BC) [227]. In early use, arsenic trioxide was prescribed for a wide variety of ailments including toxin accumulation, obstructive symptoms, gangrene, chronic ulcers, and cervical lymphadenopathy and was recognised as a ‘drug with violent side effects’ [227]. Arsenic made its first appearance in

western medicine as ‘Fowler’s solution’, which contained 1% potassium arsenite ($KAsO_2$). Fowler’s solution was used as a tonic for anaemia and rheumatism, and was later used as a treatment for psoriasis, asthma, cholera, and syphilis [228]. In 1865, Fowler’s solution became the first chemotherapeutic agent used in the treatment of leukemia [229].

The toxicity of inorganic arsenicals as medicinal treatments led to research into the less toxic organic arsenicals and the search for a more ‘specific’ arsenic species [228]. In 1907, Paul Ehrlrich developed an arsenobenzene compound (Salvarsan), for the treatment of the recently discovered syphilis spirochaete [228].

Currently, arsenic-containing drugs are only used for one non-cancer related condition, African trypanosomiasis (African sleeping sickness) [227]. African trypanosomiasis is a fatal parasitic disease caused by *Trypanosoma brucei* and is transmitted by the Tse tse fly [230]. Currently, the most common treatment for this disease is Melarsoprol, an arsenic containing compound. Melarsoprol administration causes very similar side effects to arsenic poisoning and fatality rates from treatment have been reported to be as high as 8% [231].

2.2.5 *Arsenic and cancer treatment*

Acute promyelocytic leukemia (APL) was first characterized in the mid 1900’s and until 1970 had a 100% mortality rate [232]. The majority of cases of APL involve a translocation of retinoic acid receptor alpha (RARα) on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15. This translocation results in a fusion of the PML and RARα proteins and creates a

hybrid protein with altered function. The PML-RARα fusion protein acts by binding DNA with an enhanced affinity and blocks transcription and differentiation of granulocytes [233]. Currently two drugs are used for the treatment of APL, all-*trans* retinoic acid (ATRA) and arsenic trioxide (As_2O_3/Trisenox). Both ATRA and As_2O_3 act by promoting the degradation of PML-RARα. As_2O_3 exerts its effect by binding to cysteine residues in the zinc-finger proteins of PML. This binding causes a conformation change in the PML protein and promotes oligomerization, leading to SUMOylation. Sumolyated PML recruits ring finger protein 4, also known as SUMO-dependent ubiquitin ligase, which leads to ubiquitination and degradation of the PML-RARα oncoprotein, allowing for PML stem cell differentiation [234].

The GSH conjugate of the trivalent dimethylated As metabolite dimethylarsinous acid (DMA^{III}), DMA(GS), is currently in clinical trials as a single agent for the treatment of lymphohematopoietic malignancies and solid tumors, under the drug name Darinaparsin [235, 236]. Interestingly, darinaparsin does not cause PML-RARα degradation in APL, as other arsenicals have been shown to [235], demonstrating a different mechanism for this GSH conjugate of DMA^{III}. It is known that trivalent methylated arsenicals are much more potent toxicants than inorganic arsenic [237], and being conjugated to GSH likely provides DMA^{III} with protection against oxidation during drug delivery to the tumor.

2.3 Arsenic metabolism and transport

2.3.1 *Cellular arsenic uptake*

Contamination of drinking water occurs primarily with the inorganic As species arsenate (As^{V}) and arsenite (As^{III}) and are the forms encountered initially at the intestinal epithelium. As^{III} enters cells at a much faster rate than As^{V}, which accounts for its higher level of toxicity [238, 239]. In solution at physiological pH, As^{V} (pKa values 2.19, 6.94, and 11.5), predominantly $HAsO_4^{-2}$ with some $H_2AsO_4^{-}$) is chemically similar to inorganic phosphate, and enters cells through the sodium/phosphate cotransporter type IIb (SLC34A2) [240]. In solution, As^{III} (pKa 9.2) exists in the neutral form $As(OH)_3$, and has been shown to passively enter cells through aquaglyceroporins (AQPs) [241, 242]. AQP9 is expressed at high levels in hepatocytes, and has been proposed to contribute to As^{III} accumulation in the liver [238]. Cells transfected with liver-specific solute carrier protein, organic anion transporting polypeptide 1B1 (OATP1B1/ *SLCO1B1*) were more sensitive to As^{V} and As^{III} than untransfected cells, suggesting that OATP1B1 could allow the uptake of these inorganic As species [243].

2.3.2 *Overview of arsenic metabolism*

The majority of arsenic ingested from drinking water occurs in the inorganic forms As^{III} and As^{V}, while the majority of arsenic eliminated from the body occurs as the methylated forms of arsenic, monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^{V}), dimethylarsinous acid (DMA^{III}), and

dimethylarsinic acid (DMA^V). The majority of As is eliminated in urine (60-80%). Of the As eliminated in urine, 10-30% is As^V and As^{III}, 10-20% MMA^V and MMA^{III}, and 60-80% DMA^V and DMA^{III} [244]. Several pathways for the methylation of arsenic have been proposed. The older and more commonly accepted pathway, proposed by Challenger in 1951 [245] involves arsenic undergoing a series of oxidative methylation steps as shown in Figure 2.4A.

An alternative pathway for the metabolism of arsenic has been proposed in which arsenic undergoes reductive methylation while complexed to glutathione or another cellular thiol (Figure 2.4B) [246, 247]. Both methylation pathways require the enzyme As (+3 oxidation state) methyltransferase (As3MT), which catalyzes the methylation of As using S-adenosyl-L-methionine as the methyl donor.

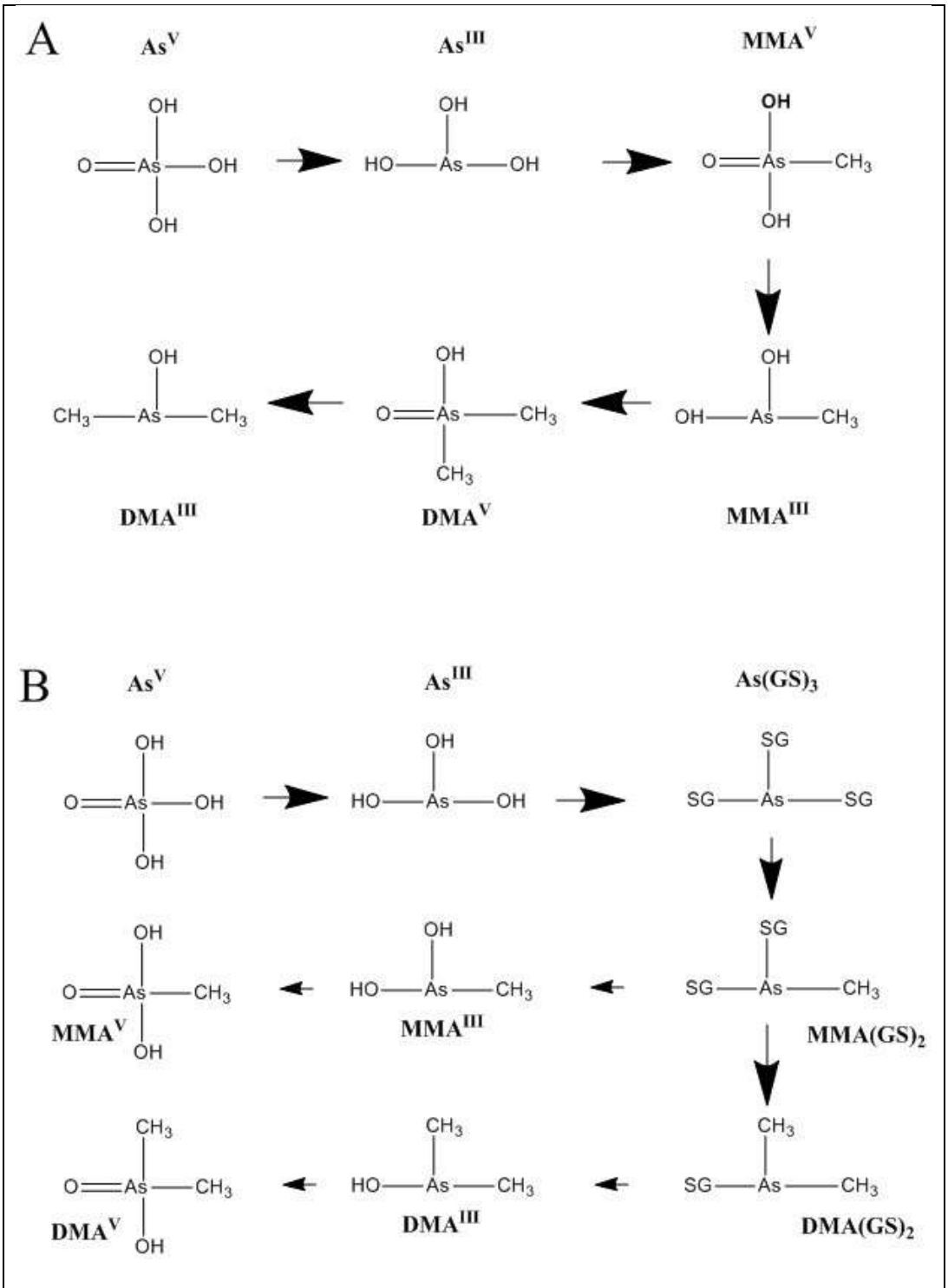

Figure 2.4. Proposed Arsenic methylation pathways. (A) Challenger pathway (B) Alternative 'thiol-bound' pathway. [245, 247]

In cell line studies the trivalent methylated species are at least 250-fold more toxic than their pentavalent forms and ~2-fold more toxic than As^{III} [226, 237, 248, 249]. This contradicts the concept that methylation is a detoxification process, and suggests methylation is an activation step. In contrast, studies with as3mt-/- mice treated with high doses of As^{V}, showed increased As toxicity, lower urinary elimination of As, and higher whole body accumulation [250, 251]. Thus, despite increased toxicity of the trivalent methylated As species, methylation is still protective at least during acute exposures [250, 251]. During chronic human exposure, low urinary excretion rates of DMA compared to MMA are associated with higher tissue retention of arsenic and increased risk of carcinogenesis [252, 253], suggesting that even during chronic exposure, complete methylation to DMA is a crucial process to prevent As-induced carcinogenesis.

2.3.3 *Importance of GSH conjugates in arsenic detoxification*

The cysteinyl thiol group within GSH is responsible for its ability to react with and inactivate many harmful compounds, such as peroxides, free radicals, and many other toxicants. Studies have shown a protective role for GSH in As exposure through its role in As efflux [132, 133, 142, 143, 248, 254]. Depletion of GSH from cells significantly increases sensitivity to As^{III} and MMA^{III} [248, 255-257].

MRP1 transports inorganic arsenic conjugated to GSH, as the triglutathione conjugate, $As(GS)_3$ with a high affinity [143]. Studies with *Mrp1-/-* mice revealed no increased survival advantage of Mrp1 expression, or increased

urinary elimination after treatment with relatively high doses of As [93, 258]. However, the high affinity low capacity transport capabilities of MRP1 for $As(GS)_3$ could play an important protective role against chronic doses of arsenic in drinking water.

GSH forms conjugates with inorganic as well as methylated arsenic species. In addition to the triglutathione conjugate formed by inorganic As, MMA^{III} forms a complex with two GSH molecules to form $MMA(GS)_2$, and DMA^{III} forms a complex with one GSH molecule to form DMA(GS) [259]. The biliary excretion of $MMA(GS)_2$ and $As(GS)_3$ was dependent on the expression of Mrp2 [133]. Mrp2 deficient, TR^- Wistar rats had less than 1% of As in bile compared to control Wistar rats. In addition, Wistar rats treated with buthionine sulfoximine (BSO), which inhibits γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis, caused a marked decrease of As transport into bile, providing support for the importance of GSH in As elimination [133].

Identification of As-GSH conjugates in biological samples has been challenging due to their short half-lives at physiological pH, ranging from 5-40 minutes [133, 260]. $As(GS)_3$ and $MMA(GS)_2$ are stable at acidic pH with half-lives of 35 and 37 minutes, respectively (at pH 2), but have half-lives less than 6 minutes at physiological pH [260]. While the GSH conjugate of DMA^{III} was shown to be the most stable As-GSH conjugate, with a half-life between 23-37 minutes at physiological pH [260], it has never been identified in a biological sample. Although Mrp2 is important for the biliary excretion of $As(GS)_3$ and $MMA(GS)_2$, urinary excretion accounts for the majority of elimination [142].

The instability of $As(GS)_3$ and $MMA(GS)_2$ can result in the dissociation of these compounds in bile and reabsorption of As across the intestine into the portal circulation and therefore undergo enterohepatic circulation. Thus a hepatic transporter(s) at the basolateral surface of the hepatocyte is/are likely responsible for the efflux of As into sinusoidal blood, ultimately for urinary elimination. Mice deficient in γ-glutamyl transpeptidase, a brush border enzyme responsible for the degradation of GSH, had $As(GS)_3$ and $MMA(GS)_2$ in their urine. These conjugates accounted for 60-70% of urinary arsenic with the remainder of urinary arsenic being DMA^V [142]. In addition, similar to studies with rats as mentioned previously, treatment of mice with BSO caused a marked decrease in arsenic elimination [142]. Together, these data suggest that As-GSH conjugates account for a major portion of eliminated As. MRPs are the predominant transporters of GSH conjugates, and thus are also likely to be important for As elimination.

2.4 Selenium

2.4.1 *Selenium overview*

Selenium is an essential trace element for human nutrition, involved in a wide variety of biological processes and has been demonstrated as a requirement for the growth of mammalian cells in culture [261]. In mammals, selenium functions primarily as a part of selenoproteins [262]. Selenium is a unique trace element that is contained in the 21st amino acid, selenocysteine, coded for by UGA [262]. Selenium has been implicated to have a role in the preventation of cancer and the progression of many pathophysiologies including cardiovascular disease. Selenium is also involved in the inhibition of viral expression, delaying the onset

of AIDS in HIV positive patients, and in mammalian development, male reproductive function, and immune function [263, 264].

Within the cell, selenium is reduced and forms diglutathionyl selenide (GS-Se-GS), which is converted to gluathionyl selenol (GS-SeH), and finally is converted to hydrogen selenide (H_2Se) [265]. Hydrogren selenide is serially methylated ultimately to dimethyl selenide and trimethylselonium, which are eliminated through exhalation and in urine, respectively [266]. Methylation of selenium, similar to arsenic requires *S*-adenosylmethionine (SAM) as a methyl-donor [265].

2.4.2 *Arsenic and selenium*

In addition to having similar metabolic and elimination pathways, arsenic and selenium have a reciprocal effect on their biliary elimination [267]. An interaction between arsenic and selenium has been known since the early 1900s when toxicity of inorganic selenium in the form of selenite (Se^{IV}) was alleviated by supplementation of cattle with As^{III} [268].

A seleno-bis(S-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$, has been identified in rabbit bile and erythrocytes [269, 270] and demonstrates a higher stability at the alkaline pH of bile than $As(GS)_3$ and $MMA(GS)_2$ [133, 269]. $As(GS)_3$ and $MMA(GS)_2$ are unstable at neutral and basic pH and this most likely results in the conjugate dissociating after transport into bile and free As^{III} and MMA^{III} being reabsorbed into the enterohepatic circulation [142]. The formation and biliary

excretion of $[(GS)_2AsSe]^-$ could provide a physiological explanation for the cooperative detoxification between arsenic and selenium.

2.4.3 *Objectives, hypothesis, and rationale*

The metabolic and transport pathways of arsenic are complex and incompletely understood. With a high incidence of human arsenic exposure and the clinical use of arsenic compounds as anticancer drugs it is critical to understand the cellular handling of As. Understanding how arsenic is handled and eliminated, will allow the development of strategies to protect against its toxic effects and the optimization of therapeutic use. For example, certain methylated species of arsenic may be crucial for the therapeutic effect of As_2O_3 during treatment of acute promyelocytic leukemia [271]. Tissue disposition and elimination of arsenic can be modulated by various micronutrients, such as selenium [272]. This introduces a further complexity to arsenic handling, and also illustrates the possibility of preventing arsenic toxicity through nutritional supplementation. The overall goal of my research has been to identify and characterize multidrug resistant proteins that are capable of transporting inorganic and methylated arsenic species, with an emphasis on proteins expressed in the liver and kidney. At the time this research was initiated, MRP1 was the only human MRP to be well characterized as a transporter of inorganic arsenic, in the form $As(GS)_3$ [143].

The first objective of my research was to characterize the transport of arsenic by MRP2 and this gave me the opportunity to explore the possibility that MRP2 was involved in the transport of $[(GS)_2AsSe]^-$ in addition to $As(GS)_3$. At

the time, it had been well established that arsenic and selenium decreased the toxicity of each other, but mechanisms for this cooperative detoxification were incomplete without the identification of a canalicular transporter involved in the elimination of $[(GS)_2AsSe]^-$. $[(GS)_2AsSe]^-$ had been recently identified and shown to form *in vivo* and earlier studies had shown an unknown arsenic selenium conjugate in fecal samples [270, 273]. With the apical localization of MRP2 in hepatocytes, it seemed logical that MRP2 might be transporting $[(GS)_2AsSe]^-$ into bile, resulting in its elimination in feces. Characterization of the transport of $[(GS)_2AsSe]^-$ and $As(GS)_3$ by MRP2 is presented in Chapter 3.

The second objective of my research was to determine if MRP1 was capable of transporting any of the methylated arsenic species. This study, presented in Chapter 4, provides evidence for the ability of MRP1 to transport MMA^{III} in the form of the GSH conjugate $MMA(GS)_2$. MRP1 was shown to decrease the cytotoxicity and the cellular accumulation of MMA^{III} in a GSH dependant manner. Direct transport studies showed that GSH was required for the transport of MMA^{III} and could not be substituted by OA, providing evidence that conjugation to GSH was required for transport.

The third and final objective of my research was to characterize the ability of MRP4 to transport inorganic and methylated As species. The expression of MRP4 at the basolateral surface of the hepatocyte and apical surface of proximal tubular cells of the kidney made MRP4 an ideal candidate as an As transporter. The majority of arsenic methylation occurs in the liver and must be transported

across the basolateral surface into the blood stream to ultimately reach the kidney for urinary elimination. Investigation into arsenic metabolites as substrates for MRP4, outlined in Chapter 5, revealed that $MMA(GS)_2$ and DMA^V were both substrates for MRP4. These findings demonstrated the potential importance of MRP4 in the elimination of arsenic.

2.5 Bibliography

1. Dean M, Rzhetsky A, Allikmets R: **The human ATP-binding cassette (ABC) transporter superfamily**. *Genome Res* 2001, **11**(7):1156-1166.
2. Juliano RL, Ling V: **A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants**. *Biochim Biophys Acta* 1976, **455**(1):152-162.
3. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL: **Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA**. *Science* 1989, **245**(4922):1066-1073.
4. Inagaki N, Gonoi T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J, Seino S: **A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K+ channels**. *Neuron* 1996, **16**(5):1011-1017.
5. Tian Y, Han X, Tian DL: **The biological regulation of ABCE1**. *IUBMB Life* 2012, **64**(10):795-800.
6. Ren Y, Li Y, Tian D: **Role of the ABCE1 gene in human lung adenocarcinoma**. *Oncol Rep* 2012, **27**(4):965-970.
7. Marton MJ, Vazquez de Aldana CR, Qiu H, Chakraburtty K, Hinnebusch AG: **Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2alpha kinase GCN2**. *Mol Cell Biol* 1997, **17**(8):4474-4489.

8. Tyzack JK, Wang X, Belsham GJ, Proud CG: **ABC50 interacts with eukaryotic initiation factor 2 and associates with the ribosome in an ATP-dependent manner**. *J Biol Chem* 2000, **275**(44):34131-34139.

9. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG: **Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line**. *Science* 1992, **258**(5088):1650-1654.

10. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD: **A multidrug resistance transporter from human MCF-7 breast cancer cells**. *Proc Natl Acad Sci U S A* 1998, **95**(26):15665-15670.

11. Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M: **A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance**. *Cancer Res* 1998, **58**(23):5337-5339.

12. Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, Brangi M, Greenberger L, Dean M, Fojo T, Bates SE: **Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes**. *Cancer Res* 1999, **59**(1):8-13.

13. Doyle L, Ross DD: **Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)**. *Oncogene* 2003, **22**(47):7340-7358.

14. Vlaming ML, Lagas JS, Schinkel AH: **Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice**. *Adv Drug Deliv Rev* 2009, **61**(1):14-25.

15. Deeley RG, Westlake C, Cole SP: **Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins**. *Physiol Rev* 2006, **86**(3):849-899.

16. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF: **Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport**. *Nature* 1990, **346**(6282):362-365.

17. Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, Hunt JF: **ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer**. *Mol Cell* 2002, **10**(1):139-149.

18. Babenko AP, Bryan J: **Sur domains that associate with and gate KATP pores define a novel gatekeeper**. *J Biol Chem* 2003, **278**(43):41577-41580.

19. Hough E, Beech DJ, Sivaprasadarao A: **Identification of molecular regions responsible for the membrane trafficking of Kir6.2**. *Pflugers Arch* 2000, **440**(3):481-487.

20. Aguilar-Bryan L, Clement JPt, Gonzalez G, Kunjilwar K, Babenko A, Bryan J: **Toward understanding the assembly and structure of KATP channels**. *Physiol Rev* 1998, **78**(1):227-245.

21. Bandler PE, Westlake CJ, Grant CE, Cole SP, Deeley RG: **Identification of regions required for apical membrane localization of human multidrug resistance protein 2**. *Mol Pharmacol* 2008, **74**(1):9-19.

22. Bakos E, Hegedus T, Hollo Z, Welker E, Tusnady GE, Zaman GJ, Flens MJ, Varadi A, Sarkadi B: **Membrane topology and glycosylation of the human multidrug resistance-associated protein**. *J Biol Chem* 1996, **271**(21):12322-12326.

23. Gao M, Loe DW, Grant CE, Cole SP, Deeley RG: **Reconstitution of ATP-dependent leukotriene C4 transport by Co-expression of both half-molecules of human multidrug resistance protein in insect cells**. *J Biol Chem* 1996, **271**(44):27782-27787.

24. Bakos E, Evers R, Szakacs G, Tusnady GE, Welker E, Szabo K, de Haas M, van Deemter L, Borst P, Varadi A, Sarkadi B: **Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain**. *J Biol Chem* 1998, **273**(48):32167-32175.

25. Westlake CJ, Qian YM, Gao M, Vasa M, Cole SP, Deeley RG: **Identification of the structural and functional boundaries of the multidrug resistance protein 1 cytoplasmic loop 3**. *Biochemistry* 2003, **42**(48):14099-14113.

26. Payen LF, Gao M, Westlake CJ, Cole SP, Deeley RG: **Role of carboxylate residues adjacent to the conserved core Walker B motifs in the catalytic cycle of multidrug resistance protein 1 (ABCC1)**. *J Biol Chem* 2003, **278**(40):38537-38547.

27. Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD: **Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily**. *Cancer Lett* 2001, **162**(2):181-191.

28. Gao M, Cui HR, Loe DW, Grant CE, Almquist KC, Cole SP, Deeley RG: **Comparison of the functional characteristics of the nucleotide binding domains of multidrug resistance protein 1**. *J Biol Chem* 2000, **275**(17):13098-13108.

29. Hou Y, Cui L, Riordan JR, Chang X: **Allosteric interactions between the two non-equivalent nucleotide binding domains of multidrug resistance protein MRP1**. *J Biol Chem* 2000, **275**(27):20280-20287.

30. Nagata K, Nishitani M, Matsuo M, Kioka N, Amachi T, Ueda K: **Nonequivalent nucleotide trapping in the two nucleotide binding folds of the human multidrug resistance protein MRP1**. *J Biol Chem* 2000, **275**(23):17626-17630.

31. Zhao Q, Chang XB: **Mutation of the aromatic amino acid interacting with adenine moiety of ATP to a polar residue alters the properties of multidrug resistance protein 1**. *J Biol Chem* 2004, **279**(47):48505-48512.

32. Aleksandrov L, Aleksandrov AA, Chang XB, Riordan JR: **The First Nucleotide Binding Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is a Site of Stable Nucleotide Interaction,**

whereas the Second Is a Site of Rapid Turnover. *J Biol Chem* 2002, **277**(18):15419-15425.

33. Basso C, Vergani P, Nairn AC, Gadsby DC: **Prolonged nonhydrolytic interaction of nucleotide with CFTR's NH2-terminal nucleotide binding domain and its role in channel gating**. *J Gen Physiol* 2003, **122**(3):333-348.

34. Matsuo M, Tanabe K, Kioka N, Amachi T, Ueda K: **Different binding properties and affinities for ATP and ADP among sulfonylurea receptor subtypes, SUR1, SUR2A, and SUR2B**. *J Biol Chem* 2000, **275**(37):28757-28763.

35. Leslie EM: **Arsenic-glutathione conjugate transport by the human multidrug resistance proteins (MRPs/ABCCs)**. *J Inorg Biochem* 2012, **108**:141-149.

36. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G: **Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding**. *Science* 2009, **323**(5922):1718-1722.

37. Dawson RJ, Locher KP: **Structure of a bacterial multidrug ABC transporter**. *Nature* 2006, **443**(7108):180-185.

38. Dawson RJ, Hollenstein K, Locher KP: **Uptake or extrusion: crystal structures of full ABC transporters suggest a common mechanism**. *Mol Microbiol* 2007, **65**(2):250-257.

39. Gerber S, Comellas-Bigler M, Goetz BA, Locher KP: **Structural basis of trans-inhibition in a molybdate/tungstate ABC transporter**. *Science* 2008, **321**(5886):246-250.

40. Hohl M, Briand C, Grutter MG, Seeger MA: **Crystal structure of a heterodimeric ABC transporter in its inward-facing conformation**. *Nat Struct Mol Biol* 2012, **19**(4):395-402.

41. Hollenstein K, Frei DC, Locher KP: **Structure of an ABC transporter in complex with its binding protein**. *Nature* 2007, **446**(7132):213-216.

42. Hvorup RN, Goetz BA, Niederer M, Hollenstein K, Perozo E, Locher KP: **Asymmetry in the structure of the ABC transporter-binding protein complex BtuCD-BtuF**. *Science* 2007, **317**(5843):1387-1390.

43. Kadaba NS, Kaiser JT, Johnson E, Lee A, Rees DC: **The high-affinity E. coli methionine ABC transporter: structure and allosteric regulation**. *Science* 2008, **321**(5886):250-253.

44. Khare D, Oldham ML, Orelle C, Davidson AL, Chen J: **Alternating access in maltose transporter mediated by rigid-body rotations**. *Mol Cell* 2009, **33**(4):528-536.

45. Locher KP, Lee AT, Rees DC: **The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism**. *Science* 2002, **296**(5570):1091-1098.

46. Oldham ML, Chen J: **Crystal structure of the maltose transporter in a pretranslocation intermediate state**. *Science* 2011, **332**(6034):1202-1205.

47. Pinkett HW, Lee AT, Lum P, Locher KP, Rees DC: **An inward-facing conformation of a putative metal-chelate-type ABC transporter**. *Science* 2007, **315**(5810):373-377.

48. Ward A, Reyes CL, Yu J, Roth CB, Chang G: **Flexibility in the ABC transporter MsbA: Alternating access with a twist**. *Proc Natl Acad Sci U S A* 2007, **104**(48):19005-19010.

49. Jardetzky O: **Simple allosteric model for membrane pumps**. *Nature* 1966, **211**(5052):969-970.

50. Beck A, Aanismaa P, Li-Blatter X, Dawson R, Locher K, Seelig A: **Sav1866 from Staphylococcus aureus and P-Glycoprotein: Similarities and Differences in ATPase Activity Assessed with Detergents as Allocrites**. *Biochemistry* 2013.

51. Zhou Z, White KA, Polissi A, Georgopoulos C, Raetz CR: **Function of Escherichia coli MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis**. *J Biol Chem* 1998, **273**(20):12466-12475.

52. Gottesman MM, Ambudkar SV, Xia D: **Structure of a multidrug transporter**. *Nat Biotechnol* 2009, **27**(6):546-547.

53. Jones PM, George AM: **Opening of the ADP-bound active site in the ABC transporter ATPase dimer: evidence for a constant contact, alternating sites model for the catalytic cycle**. *Proteins* 2009, **75**(2):387-396.

54. George AM, Jones PM: **Perspectives on the structure-function of ABC transporters: the Switch and Constant Contact models**. *Prog Biophys Mol Biol* 2012, **109**(3):95-107.

55. Loo TW, Bartlett MC, Clarke DM: **Human P-glycoprotein is active when the two halves are clamped together in the closed conformation**. *Biochem Biophys Res Commun* 2010, **395**(3):436-440.

56. Siarheyeva A, Liu R, Sharom FJ: **Characterization of an asymmetric occluded state of P-glycoprotein with two bound nucleotides: implications for catalysis**. *J Biol Chem* 2010, **285**(10):7575-7586.

57. Verhalen B, Wilkens S: **P-glycoprotein retains drug-stimulated ATPase activity upon covalent linkage of the two nucleotide binding domains at their C-terminal ends**. *J Biol Chem* 2011, **286**(12):10476-10482.

58. Aittoniemi J, de Wet H, Ashcroft FM, Sansom MS: **Asymmetric switching in a homodimeric ABC transporter: a simulation study**. *PLoS Comput Biol* 2010, **6**(4):e1000762.

59. Tsai MF, Li M, Hwang TC: **Stable ATP binding mediated by a partial NBD dimer of the CFTR chloride channel**. *J Gen Physiol* 2010, **135**(5):399-414.

60. Samanta S, Ayvaz T, Reyes M, Shuman HA, Chen J, Davidson AL: **Disulfide cross-linking reveals a site of stable interaction between C-terminal regulatory domains of the two MalK subunits in the maltose transport complex**. *J Biol Chem* 2003, **278**(37):35265-35271.

61. Haimeur A, Conseil G, Deeley RG, Cole SP: **The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation**. *Curr Drug Metab* 2004, **5**(1):21-53.

62. Roelofsen H, Vos TA, Schippers IJ, Kuipers F, Koning H, Moshage H, Jansen PL, Muller M: **Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells**. *Gastroenterology* 1997, **112**(2):511-521.

63. Zhang Y, Schuetz JD, Elmquist WF, Miller DW: **Plasma membrane localization of multidrug resistance-associated protein homologs in brain capillary endothelial cells**. *J Pharmacol Exp Ther* 2004, **311**(2):449-455.

64. Wang J, Zu X, Li Y: **[TGF-alpha increases neuroendocrine differentiation and strengthens chemoresistance in prostate cancer DU145 cells]**. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2013, **38**(2):142-147.

65. Lin S, Hoffmann K, Xiao Z, Jin N, Galli U, Mohr E, Buchler MW, Schemmer P: **MEK inhibition induced downregulation of MRP1 and MRP3 expression in experimental hepatocellular carcinoma**. *Cancer Cell Int* 2013, **13**(1):3.

66. Camgoz A, Gencer EB, Ural AU, Baran Y: **Mechanisms responsible for nilotinib resistance in human chronic myeloid leukemia cells and reversal of resistance**. *Leuk Lymphoma* 2012.

67. Leonard GD, Fojo T, Bates SE: **The role of ABC transporters in clinical practice**. *Oncologist* 2003, **8**(5):411-424.

68. Zhao J, Yu BY, Wang DY, Yang JE: **Promoter polymorphism of MRP1 associated with reduced survival in hepatocellular carcinoma**. *World J Gastroenterol* 2010, **16**(48):6104-6110.

69. Vulsteke C, Lambrechts D, Dieudonne A, Hatse S, Brouwers B, van Brussel T, Neven P, Belmans A, Schoffski P, Paridaens R, Wildiers H: **Genetic variability in the multidrug resistance associated protein-1 (ABCC1/MRP1) predicts hematological toxicity in breast cancer patients receiving (neo-)adjuvant chemotherapy with 5-fluorouracil, epirubicin and cyclophosphamide (FEC)**. *Ann Oncol* 2013.

70. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG: **Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells**. *Cancer Res* 1994, **54**(22):5902-5910.

71. Breuninger LM, Paul S, Gaughan K, Miki T, Chan A, Aaronson SA, Kruh GD: **Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution**. *Cancer Res* 1995, **55**(22):5342-5347.

72. Sodani K, Patel A, Kathawala RJ, Chen ZS: **Multidrug resistance associated proteins in multidrug resistance**. *Chin J Cancer* 2012, **31**(2):58-72.

73. Perloff MD, Von Moltke LL, Marchand JE, Greenblatt DJ: **Ritonavir induces P-glycoprotein expression, multidrug resistance-associated protein (MRP1) expression, and drug transporter-mediated activity in a human intestinal cell line**. *J Pharm Sci* 2001, **90**(11):1829-1837.

74. Konig SK, Herzog M, Theile D, Zembruski N, Haefeli WE, Weiss J: **Impact of drug transporters on cellular resistance towards saquinavir and darunavir**. *J Antimicrob Chemother* 2010, **65**(11):2319-2328.

75. Watts RN, Hawkins C, Ponka P, Richardson DR: **Nitrogen monoxide (NO)-mediated iron release from cells is linked to NO-induced glutathione efflux via multidrug resistance-associated protein 1**. *Proc Natl Acad Sci U S A* 2006, **103**(20):7670-7675.

76. Li Q, Kato Y, Sai Y, Imai T, Tsuji A: **Multidrug resistance-associated protein 1 functions as an efflux pump of xenobiotics in the skin**. *Pharm Res* 2005, **22**(6):842-846.

77. Kosower NS, Kosower EM: **The glutathione status of cells**. *Int Rev Cytol* 1978, **54**:109-160.

78. Leslie EM, Bowers RJ, Deeley RG, Cole SP: **Structural requirements for functional interaction of glutathione tripeptide analogs with the human multidrug resistance protein 1 (MRP1)**. *J Pharmacol Exp Ther* 2003, **304**(2):643-653.

79. Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, Cole SP: **Transport of the beta -O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol**

(NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog. *J Biol Chem* 2001, **276**(30):27846-27854.

80. Rothnie A, Conseil G, Lau AY, Deeley RG, Cole SP: **Mechanistic differences between GSH transport by multidrug resistance protein 1 (MRP1/ABCC1) and GSH modulation of MRP1-mediated transport**. *Mol Pharmacol* 2008, **74**(6):1630-1640.

81. Loe DW, Almquist KC, Deeley RG, Cole SP: **Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport**. *J Biol Chem* 1996, **271**(16):9675-9682.

82. Morrow CS, Peklak-Scott C, Bishwokarma B, Kute TE, Smitherman PK, Townsend AJ: **Multidrug resistance protein 1 (MRP1, ABCC1) mediates resistance to mitoxantrone via glutathione-dependent drug efflux**. *Mol Pharmacol* 2006, **69**(4):1499-1505.

83. Loe DW, Deeley RG, Cole SP: **Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione**. *Cancer Res* 1998, **58**(22):5130-5136.

84. Mayatepek E: **Leukotriene C4 synthesis deficiency: a member of a probably underdiagnosed new group of neurometabolic diseases**. *Eur J Pediatr* 2000, **159**(11):811-818.

85. Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P: **Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein**. *Nat Med* 1997, **3**(11):1275-1279.

86. Robbiani DF, Finch RA, Jager D, Muller WA, Sartorelli AC, Randolph GJ: **The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes**. *Cell* 2000, **103**(5):757-768.

87. Renes J, de Vries EE, Hooiveld GJ, Krikken I, Jansen PL, Muller M: **Multidrug resistance protein MRP1 protects against the toxicity of the major lipid peroxidation product 4-hydroxynonenal**. *Biochem J* 2000, **350 Pt 2**:555-561.

88. Esterbauer H, Schaur RJ, Zollner H: **Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes**. *Free Radic Biol Med* 1991, **11**(1):81-128.

89. Jungsuwadee P, Cole MP, Sultana R, Joshi G, Tangpong J, Butterfield DA, St Clair DK, Vore M: **Increase in Mrp1 expression and 4-hydroxy-2-nonenal adduction in heart tissue of Adriamycin-treated C57BL/6 mice**. *Mol Cancer Ther* 2006, **5**(11):2851-2860.

90. Cole SP, Deeley RG: **Transport of glutathione and glutathione conjugates by MRP1**. *Trends Pharmacol Sci* 2006, **27**(8):438-446.

91. Hayashi A, Suzuki H, Itoh K, Yamamoto M, Sugiyama Y: **Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts**. *Biochem Biophys Res Commun* 2003, **310**(3):824-829.

92. Cole SP, Downes HF, Mirski SE, Clements DJ: **Alterations in glutathione and glutathione-related enzymes in a multidrug-resistant small cell lung cancer cell line**. *Mol Pharmacol* 1990, **37**(2):192-197.

93. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC: **Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione**. *Cancer Res* 1997, **57**(23):5238-5242.

94. Rigato I, Pascolo L, Fernetti C, Ostrow JD, Tiribelli C: **The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin**. *Biochem J* 2004, **383**(Pt 2):335-341.

95. Calligaris S, Cekic D, Roca-Burgos L, Gerin F, Mazzone G, Ostrow JD, Tiribelli C: **Multidrug resistance associated protein 1 protects against bilirubin-induced cytotoxicity**. *FEBS Lett* 2006, **580**(5):1355-1359.

96. Falcao AS, Bellarosa C, Fernandes A, Brito MA, Silva RF, Tiribelli C, Brites D: **Role of multidrug resistance-associated protein 1 expression in the in vitro susceptibility of rat nerve cell to unconjugated bilirubin**. *Neuroscience* 2007, **144**(3):878-888.

97. Gamaleldin R, Iskander I, Seoud I, Aboraya H, Aravkin A, Sampson PD, Wennberg RP: **Risk factors for neurotoxicity in newborns with severe neonatal hyperbilirubinemia**. *Pediatrics* 2011, **128**(4):e925-931.

98. Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G: **Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2**. *Cancer Res* 1999, **59**(11):2532-2535.

99. Leslie EM, Deeley RG, Cole SP: **Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense**. *Toxicol Appl Pharmacol* 2005, **204**(3):216-237.

100. St-Pierre MV, Serrano MA, Macias RI, Dubs U, Hoechli M, Lauper U, Meier PJ, Marin JJ: **Expression of members of the multidrug resistance protein family in human term placenta**. *Am J Physiol Regul Integr Comp Physiol* 2000, **279**(4):R1495-1503.

101. Vore M: **Canalicular transport: discovery of ATP-dependent mechanisms**. *Toxicol Appl Pharmacol* 1993, **118**(1):2-7.

102. Giton F, de la Taille A, Allory Y, Galons H, Vacherot F, Soyeux P, Abbou CC, Loric S, Cussenot O, Raynaud JP, Fiet J: **Estrone sulfate (E1S), a prognosis marker for tumor aggressiveness in prostate cancer (PCa)**. *J Steroid Biochem Mol Biol* 2008, **109**(1-2):158-167.

103. Wijnholds J, Scheffer GL, van der Valk M, van der Valk P, Beijnen JH, Scheper RJ, Borst P: **Multidrug resistance protein 1 protects the**

oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. *J Exp Med* 1998, **188**(5):797-808.

104. Qian YM, Song WC, Cui H, Cole SP, Deeley RG: **Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1**. *J Biol Chem* 2001, **276**(9):6404-6411.

105. Eaton DL, Gallagher EP: **Mechanisms of aflatoxin carcinogenesis**. *Annu Rev Pharmacol Toxicol* 1994, **34**:135-172.

106. Aguilar F, Hussain SP, Cerutti P: **Aflatoxin B1 induces the transversion of G-->T in codon 249 of the p53 tumor suppressor gene in human hepatocytes**. *Proc Natl Acad Sci U S A* 1993, **90**(18):8586-8590.

107. Hecht SS: **Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines**. *Chem Res Toxicol* 1998, **11**(6):559-603.

108. Mayer R, Kartenbeck J, Buchler M, Jedlitschky G, Leier I, Keppler D: **Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes**. *J Cell Biol* 1995, **131**(1):137-150.

109. Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D: **cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats**. *J Biol Chem* 1996, **271**(25):15091-15098.

110. Paulusma CC, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RP: **Congenital jaundice in rats with**

a mutation in a multidrug resistance-associated protein gene. *Science* 1996, **271**(5252):1126-1128.

111. Bart J, Hollema H, Groen HJ, de Vries EG, Hendrikse NH, Sleijfer DT, Wegman TD, Vaalburg W, van der Graaf WT: **The distribution of drug-efflux pumps, P-gp, BCRP, MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours**. *Eur J Cancer* 2004, **40**(14):2064-2070.

112. Cherrington NJ, Hartley DP, Li N, Johnson DR, Klaassen CD: **Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mrp1, 2, and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats**. *J Pharmacol Exp Ther* 2002, **300**(1):97-104.

113. Meyer zu Schwabedissen HE, Jedlitschky G, Gratz M, Haenisch S, Linnemann K, Fusch C, Cascorbi I, Kroemer HK: **Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation**. *Drug Metab Dispos* 2005, **33**(7):896-904.

114. Taniguchi K, Wada M, Kohno K, Nakamura T, Kawabe T, Kawakami M, Kagotani K, Okumura K, Akiyama S, Kuwano M: **A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation**. *Cancer Res* 1996, **56**(18):4124-4129.

115. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P: **Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated**

protein gene (MRP1), in human cancer cell lines. *Cancer Res* 1997, **57**(16):3537-3547.

116. Liedert B, Materna V, Schadendorf D, Thomale J, Lage H: **Overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA adducts and decreased G2-arrest in melanoma cells resistant to cisplatin**. *J Invest Dermatol* 2003, **121**(1):172-176.

117. Materna V, Liedert B, Thomale J, Lage H: **Protection of platinum-DNA adduct formation and reversal of cisplatin resistance by anti-MRP2 hammerhead ribozymes in human cancer cells**. *Int J Cancer* 2005, **115**(3):393-402.

118. Ma JJ, Chen BL, Xin XY: **Inhibition of multi-drug resistance of ovarian carcinoma by small interfering RNA targeting to MRP2 gene**. *Arch Gynecol Obstet* 2009, **279**(2):149-157.

119. Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D: **Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells**. *Mol Pharmacol* 1999, **55**(5):929-937.

120. Smitherman PK, Townsend AJ, Kute TE, Morrow CS: **Role of multidrug resistance protein 2 (MRP2, ABCC2) in alkylating agent detoxification: MRP2 potentiates glutathione S-transferase A1-1-mediated resistance to chlorambucil cytotoxicity**. *J Pharmacol Exp Ther* 2004, **308**(1):260-267.

121. Qiu R, Kalhorn TF, Slattery JT: **ABCC2-mediated biliary transport of 4-glutathionylcyclophosphamide and its contribution to elimination of 4-hydroxycyclophosphamide in rat**. *J Pharmacol Exp Ther* 2004, **308**(3):1204-1212.

122. Shibayama Y, Nakano K, Maeda H, Taguchi M, Ikeda R, Sugawara M, Iseki K, Takeda Y, Yamada K: **Multidrug resistance protein 2 implicates anticancer drug-resistance to sorafenib**. *Biol Pharm Bull* 2011, **34**(3):433-435.

123. Jedlitschky G, Hoffmann U, Kroemer HK: **Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition**. *Expert Opin Drug Metab Toxicol* 2006, **2**(3):351-366.

124. Wada M, Toh S, Taniguchi K, Nakamura T, Uchiumi T, Kohno K, Yoshida I, Kimura A, Sakisaka S, Adachi Y, Kuwano M: **Mutations in the canilicular multispecific organic anion transporter (cMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin-Johnson syndrome**. *Hum Mol Genet* 1998, **7**(2):203-207.

125. Jemnitz K, Heredi-Szabo K, Janossy J, Ioja E, Vereczkey L, Krajcsi P: **ABCC2/Abcc2: a multispecific transporter with dominant excretory functions**. *Drug Metab Rev* 2010, **42**(3):402-436.

126. Chu XY, Strauss JR, Mariano MA, Li J, Newton DJ, Cai X, Wang RW, Yabut J, Hartley DP, Evans DC, Evers R: **Characterization of mice lacking the multidrug resistance protein MRP2 (ABCC2)**. *J Pharmacol Exp Ther* 2006, **317**(2):579-589.

127. Chu XY, Kato Y, Niinuma K, Sudo KI, Hakusui H, Sugiyama Y: **Multispecific organic anion transporter is responsible for the biliary excretion of the camptothecin derivative irinotecan and its metabolites in rats**. *J Pharmacol Exp Ther* 1997, **281**(1):304-314.

128. Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, Sugiyama Y: **Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR**. *Am J Physiol* 1997, **272**(1 Pt 1):G16-22.

129. Huber M, Guhlmann A, Jansen PL, Keppler D: **Hereditary defect of hepatobiliary cysteinyl leukotriene elimination in mutant rats with defective hepatic anion excretion**. *Hepatology* 1987, **7**(2):224-228.

130. Jansen PL, van Klinken JW, van Gelder M, Ottenhoff R, Elferink RP: **Preserved organic anion transport in mutant TR- rats with a hepatobiliary secretion defect**. *Am J Physiol* 1993, **265**(3 Pt 1):G445-452.

131. Paulusma CC, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, Oude Elferink RP: **Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione**. *Biochem J* 1999, **338 (Pt 2)**:393-401.

132. Carew MW, Leslie EM: **Selenium-dependent and -independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/ABCC2): implications for the mutual detoxification of arsenic and selenium**. *Carcinogenesis* 2010, **31**(8):1450-1455.

133. Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW: **The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic**. *J Biol Chem* 2000, **275**(43):33404-33408.

134. Ito K, Koresawa T, Nakano K, Horie T: **Mrp2 is involved in benzylpenicillin-induced choleresis**. *Am J Physiol Gastrointest Liver Physiol* 2004, **287**(1):G42-49.

135. Leslie EM, Ghibellini G, Nezasa K, Brouwer KL: **Biotransformation and transport of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in bile duct-cannulated wild-type and Mrp2/Abcc2-deficient (TR) Wistar rats**. *Carcinogenesis* 2007, **28**(12):2650-2656.

136. Xiong H, Turner KC, Ward ES, Jansen PL, Brouwer KL: **Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR(-) rats**. *J Pharmacol Exp Ther* 2000, **295**(2):512-518.

137. Anzai N, Jutabha P, Endou H: **Molecular mechanism of ochratoxin a transport in the kidney**. *Toxins (Basel)* 2010, **2**(6):1381-1398.

138. Leier I, Hummel-Eisenbeiss J, Cui Y, Keppler D: **ATP-dependent para-aminohippurate transport by apical multidrug resistance protein MRP2**. *Kidney Int* 2000, **57**(4):1636-1642.

139. Terlouw SA, Graeff C, Smeets PH, Fricker G, Russel FG, Masereeuw R, Miller DS: **Short- and long-term influences of heavy metals on anionic**

drug efflux from renal proximal tubule. *J Pharmacol Exp Ther* 2002, **301**(2):578-585.

140. Aleo MF, Morandini F, Bettoni F, Giuliani R, Rovetta F, Steimberg N, Apostoli P, Parrinello G, Mazzoleni G: **Endogenous thiols and MRP transporters contribute to Hg2+ efflux in HgCl2-treated tubular MDCK cells**. *Toxicology* 2005, **206**(1):137-151.

141. Dietrich CG, Ottenhoff R, de Waart DR, Oude Elferink RP: **Role of MRP2 and GSH in intrahepatic cycling of toxins**. *Toxicology* 2001, **167**(1):73-81.

142. Kala SV, Kala G, Prater CI, Sartorelli AC, Lieberman MW: **Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione**. *Chem Res Toxicol* 2004, **17**(2):243-249.

143. Leslie EM, Haimeur A, Waalkes MP: **Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required**. *J Biol Chem* 2004, **279**(31):32700-32708.

144. Russel FG, Koenderink JB, Masereeuw R: **Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules**. *Trends Pharmacol Sci* 2008, **29**(4):200-207.

145. Borst P, de Wolf C, van de Wetering K: **Multidrug resistance-associated proteins 3, 4, and 5**. *Pflugers Arch* 2007, **453**(5):661-673.

146. Ritter CA, Jedlitschky G, Meyer zu Schwabedissen H, Grube M, Kock K, Kroemer HK: **Cellular export of drugs and signaling molecules by the**

ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). *Drug Metab Rev* 2005, **37**(1):253-278.

147. van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG: **The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP**. *J Am Soc Nephrol* 2002, **13**(3):595-603.

148. Li C, Krishnamurthy PC, Penmatsa H, Marrs KL, Wang XQ, Zaccolo M, Jalink K, Li M, Nelson DJ, Schuetz JD, Naren AP: **Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia**. *Cell* 2007, **131**(5):940-951.

149. Hoque MT, Conseil G, Cole SP: **Involvement of NHERF1 in apical membrane localization of MRP4 in polarized kidney cells**. *Biochem Biophys Res Commun* 2009, **379**(1):60-64.

150. Swiatecka-Urban A, Duhaime M, Coutermarsh B, Karlson KH, Collawn J, Milewski M, Cutting GR, Guggino WB, Langford G, Stanton BA: **PDZ domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator**. *J Biol Chem* 2002, **277**(42):40099-40105.

151. Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Stutts MJ, Milgram SL: **An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton**. *J Biol Chem* 1998, **273**(31):19797-19801.

152. Chen ZS, Lee K, Kruh GD: **Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine**. *J Biol Chem* 2001, **276**(36):33747-33754.

153. Norris MD, Smith J, Tanabe K, Tobin P, Flemming C, Scheffer GL, Wielinga P, Cohn SL, London WB, Marshall GM, Allen JD, Haber M: **Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan in vitro**. *Mol Cancer Ther* 2005, **4**(4):547-553.

154. Ho LL, Kench JG, Handelsman DJ, Scheffer GL, Stricker PD, Grygiel JG, Sutherland RL, Henshall SM, Allen JD, Horvath LG: **Androgen regulation of multidrug resistance-associated protein 4 (MRP4/ABCC4) in prostate cancer**. *Prostate* 2008, **68**(13):1421-1429.

155. Montani M, Herrmanns T, Muntener M, Wild P, Sulser T, Kristiansen G: **Multidrug resistance protein 4 (MRP4) expression in prostate cancer is associated with androgen signaling and decreases with tumor progression**. *Virchows Arch* 2013, **462**(4):437-443.

156. Cai C, Omwancha J, Hsieh CL, Shemshedini L: **Androgen induces expression of the multidrug resistance protein gene MRP4 in prostate cancer cells**. *Prostate Cancer Prostatic Dis* 2007, **10**(1):39-45.

157. Imaoka T, Kusuhara H, Adachi M, Schuetz JD, Takeuchi K, Sugiyama Y: **Functional involvement of multidrug resistance-associated protein 4**

(MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. *Mol Pharmacol* 2007, **71**(2):619-627.

158. Leggas M, Adachi M, Scheffer GL, Sun D, Wielinga P, Du G, Mercer KE, Zhuang Y, Panetta JC, Johnston B, Scheper RJ, Stewart CF, Schuetz JD: **Mrp4 confers resistance to topotecan and protects the brain from chemotherapy**. *Mol Cell Biol* 2004, **24**(17):7612-7621.

159. Krishnamurthy P, Schwab M, Takenaka K, Nachagari D, Morgan J, Leslie M, Du W, Boyd K, Cheok M, Nakauchi H, Marzolini C, Kim RB, Poonkuzhali B, Schuetz E, Evans W, Relling M, Schuetz JD: **Transporter-mediated protection against thiopurine-induced hematopoietic toxicity**. *Cancer Res* 2008, **68**(13):4983-4989.

160. Zelcer N, Reid G, Wielinga P, Kuil A, van der Heijden I, Schuetz JD, Borst P: **Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4)**. *Biochem J* 2003, **371**(Pt 2):361-367.

161. Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P: **The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs**. *Proc Natl Acad Sci U S A* 2003, **100**(16):9244-9249.

162. Hofer AM, Lefkimmiatis K: **Extracellular calcium and cAMP: second messengers as "third messengers"?** *Physiology (Bethesda)* 2007, **22**:320-327.

163. Tan Y, Tang Q, Hu BR, Xiang JZ: **[Effects of berberine on cyclic GMP and cyclic AMP levels in rabbit corpus cavernosum in vitro]**. *Zhonghua Nan Ke Xue* 2005, **11**(6):406-408.

164. Iancu RV, Ramamurthy G, Warrier S, Nikolaev VO, Lohse MJ, Jones SW, Harvey RD: **Cytoplasmic cAMP concentrations in intact cardiac myocytes**. *Am J Physiol Cell Physiol* 2008, **295**(2):C414-422.

165. Pattabiraman PP, Pecen PE, Rao PV: **MRP4-mediated regulation of intracellular cAMP and cGMP levels in trabecular meshwork cells and homeostasis of intraocular pressure**. *Invest Ophthalmol Vis Sci* 2013, **54**(3):1636-1649.

166. Rodriguez MR, Diez F, Ventimiglia MS, Morales V, Copsel S, Vatta MS, Davio CA, Bianciotti LG: **Atrial natriuretic factor stimulates efflux of cAMP in rat exocrine pancreas via multidrug resistance-associated proteins**. *Gastroenterology* 2011, **140**(4):1292-1302.

167. Copsel S, Garcia C, Diez F, Vermeulem M, Baldi A, Bianciotti LG, Russel FG, Shayo C, Davio C: **Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation**. *J Biol Chem* 2011, **286**(9):6979-6988.

168. Matsumiya W, Kusuhara S, Hayashibe K, Maruyama K, Kusuhara H, Tagami M, Schuetz JD, Negi A: **Forskolin modifies retinal vascular development in Mrp4-knockout mice**. *Invest Ophthalmol Vis Sci* 2012, **53**(13):8029-8035.

169. Van Aubel RA, Smeets PH, van den Heuvel JJ, Russel FG: **Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites**. *Am J Physiol Renal Physiol* 2005, **288**(2):F327-333.

170. Ames BN, Cathcart R, Schwiers E, Hochstein P: **Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis**. *Proc Natl Acad Sci U S A* 1981, **78**(11):6858-6862.

171. Letschert K, Faulstich H, Keller D, Keppler D: **Molecular characterization and inhibition of amanitin uptake into human hepatocytes**. *Toxicol Sci* 2006, **91**(1):140-149.

172. Renes J, de Vries EG, Nienhuis EF, Jansen PL, Muller M: **ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1**. *Br J Pharmacol* 1999, **126**(3):681-688.

173. Randolph GJ, Beaulieu S, Pope M, Sugawara I, Hoffman L, Steinman RM, Muller WA: **A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels**. *Proc Natl Acad Sci U S A* 1998, **95**(12):6924-6929.

174. van de Ven R, Scheffer GL, Reurs AW, Lindenberg JJ, Oerlemans R, Jansen G, Gillet JP, Glasgow JN, Pereboev A, Curiel DT, Scheper RJ, de Gruijl TD: **A role for multidrug resistance protein 4 (MRP4; ABCC4) in human dendritic cell migration**. *Blood* 2008, **112**(6):2353-2359.

175. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**(6673):245-252.

176. Nies AT, Jedlitschky G, Konig J, Herold-Mende C, Steiner HH, Schmitt HP, Keppler D: **Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain**. *Neuroscience* 2004, **129**(2):349-360.

177. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D: **Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane**. *Hepatology* 2003, **38**(2):374-384.

178. Mennone A, Soroka CJ, Cai SY, Harry K, Adachi M, Hagey L, Schuetz JD, Boyer JL: **Mrp4-/- mice have an impaired cytoprotective response in obstructive cholestasis**. *Hepatology* 2006, **43**(5):1013-1021.

179. Aleksunes LM, Campion SN, Goedken MJ, Manautou JE: **Acquired resistance to acetaminophen hepatotoxicity is associated with induction of multidrug resistance-associated protein 4 (Mrp4) in proliferating hepatocytes**. *Toxicol Sci* 2008, **104**(2):261-273.

180. Gelhaus SL, Gilad O, Hwang WT, Penning TM, Blair IA: **Multidrug resistance protein (MRP) 4 attenuates benzo[a]pyrene-mediated DNA-adduct formation in human bronchoalveolar H358 cells**. *Toxicol Lett* 2012, **209**(1):58-66.

181. Rahman MM, Ng JC, Naidu R: **Chronic exposure of arsenic via drinking water and its adverse health impacts on humans**. *Environ Geochem Health* 2009, **31 Suppl 1**:189-200.

182. Bergstedt E: **[Bangladesh has to choose between arsenic or cholera. A campaign for good drinking water may have caused a national disaster]**. *Lakartidningen* 1999, **96**(47):5266-5267.

183. Argos M, Kalra T, Rathouz PJ, Chen Y, Pierce B, Parvez F, Islam T, Ahmed A, Rakibuz-Zaman M, Hasan R, Sarwar G, Slavkovich V, van Geen A, Graziano J, Ahsan H: **Arsenic exposure from drinking water, and all-cause and chronic-disease mortalities in Bangladesh (HEALS): a prospective cohort study**. *Lancet* 2010, **376**(9737):252-258.

184. Mandal BK, Suzuki KT: **Arsenic round the world: a review**. *Talanta* 2002, **58**(1):201-235.

185. Jiang JQ, Ashekuzzaman SM, Jiang A, Sharifuzzaman SM, Chowdhury SR: **Arsenic contaminated groundwater and its treatment options in Bangladesh**. *Int J Environ Res Public Health* 2013, **10**(1):18-46.

186. Moll AE, Heimburger R, Lagarde F, Leroy MJ, Maier E: **Arsenic speciation in marine organisms: from the analytical methodology to the constitution of reference materials**. *Anal Bioanal Chem* 1996, **354**(5-6):550-556.

187. Raber G, Khoomrung S, Taleshi MS, Edmonds JS, Francesconi KA: **Identification of arsenolipids with GC/MS**. *Talanta* 2009, **78**(3):1215-1218.

188. Balakumar P, Kaur J: **Arsenic exposure and cardiovascular disorders: an overview**. *Cardiovasc Toxicol* 2009, **9**(4):169-176.

189. Tseng WP: **Effects and dose--response relationships of skin cancer and blackfoot disease with arsenic**. *Environ Health Perspect* 1977, **19**:109-119.

190. Thomas DJ, Styblo M, Lin S: **The cellular metabolism and systemic toxicity of arsenic**. *Toxicol Appl Pharmacol* 2001, **176**(2):127-144.

191. Jager JW, Ostrosky-Wegman P: **Arsenic: a paradoxical human carcinogen**. *Mutat Res* 1997, **386**(3):181-184.

192. Waalkes MP, Liu J, Diwan BA: **Transplacental arsenic carcinogenesis in mice**. *Toxicol Appl Pharmacol* 2007, **222**(3):271-280.

193. Stepnik M, Stetkiewicz J, Krajnow A, Domeradzka K, Gradecka-Meesters D, Arkusz J, Stanczyk M, Palus J, Dziubaltowska E, Sobala W, Gromadzinska J, Wasowicz W, Rydzynski K: **Carcinogenic effect of arsenate in C57BL/6J/Han mice and its modulation by different dietary selenium status**. *Ecotoxicol Environ Saf* 2009, **72**(8):2143-2152.

194. Suzuki S, Arnold LL, Ohnishi T, Cohen SM: **Effects of inorganic arsenic on the rat and mouse urinary bladder**. *Toxicol Sci* 2008, **106**(2):350-363.

195. Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L: **Results of a long-term carcinogenicity bioassay on Sprague-Dawley rats exposed to sodium arsenite administered in drinking water**. *Ann N Y Acad Sci* 2006, **1076**:578-591.

196. Tokar EJ, Benbrahim-Tallaa L, Ward JM, Lunn R, Sams RL, 2nd, Waalkes MP: **Cancer in experimental animals exposed to arsenic and arsenic compounds**. *Crit Rev Toxicol* 2010, **40**(10):912-927.

197. Wei M, Wanibuchi H, Morimura K, Iwai S, Yoshida K, Endo G, Nakae D, Fukushima S: **Carcinogenicity of dimethylarsinic acid in male F344 rats and genetic alterations in induced urinary bladder tumors**. *Carcinogenesis* 2002, **23**(8):1387-1397.

198. Waalkes MP, Ward JM, Liu J, Diwan BA: **Transplacental carcinogenicity of inorganic arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice**. *Toxicol Appl Pharmacol* 2003, **186**(1):7-17.

199. Yamanaka K, Mizol M, Kato K, Hasegawa A, Nakano M, Okada S: **Oral administration of dimethylarsinic acid, a main metabolite of inorganic arsenic, in mice promotes skin tumorigenesis initiated by dimethylbenz(a)anthracene with or without ultraviolet B as a promoter**. *Biol Pharm Bull* 2001, **24**(5):510-514.

200. Wang X, Mandal AK, Saito H, Pulliam JF, Lee EY, Ke ZJ, Lu J, Ding S, Li L, Shelton BJ, Tucker T, Evers BM, Zhang Z, Shi X: **Arsenic and chromium in drinking water promote tumorigenesis in a mouse colitis-associated colorectal cancer model and the potential mechanism is ROS-mediated Wnt/beta-catenin signaling pathway**. *Toxicol Appl Pharmacol* 2012, **262**(1):11-21.

201. Waalkes MP, Ward JM, Diwan BA: **Induction of tumors of the liver, lung, ovary and adrenal in adult mice after brief maternal gestational exposure to inorganic arsenic: promotional effects of postnatal phorbol ester exposure on hepatic and pulmonary, but not dermal cancers**. *Carcinogenesis* 2004, **25**(1):133-141.

202. Yu HS, Liao WT, Chai CY: **Arsenic carcinogenesis in the skin**. *J Biomed Sci* 2006, **13**(5):657-666.

203. Hughes MF: **Arsenic toxicity and potential mechanisms of action**. *Toxicol Lett* 2002, **133**(1):1-16.

204. Kessel M, Liu SX, Xu A, Santella R, Hei TK: **Arsenic induces oxidative DNA damage in mammalian cells**. *Mol Cell Biochem* 2002, **234-235**(1-2):301-308.

205. Hei TK, Liu SX, Waldren C: **Mutagenicity of arsenic in mammalian cells: role of reactive oxygen species**. *Proc Natl Acad Sci U S A* 1998, **95**(14):8103-8107.

206. Kitchin KT: **Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites**. *Toxicol Appl Pharmacol* 2001, **172**(3):249-261.

207. Cui X, Kobayashi Y, Akashi M, Okayasu R: **Metabolism and the paradoxical effects of arsenic: carcinogenesis and anticancer**. *Curr Med Chem* 2008, **15**(22):2293-2304.

208. Huang S, Guo S, Guo F, Yang Q, Xiao X, Murata M, Ohnishi S, Kawanishi S, Ma N: **CD44v6 expression in human skin keratinocytes**

as a possible mechanism for carcinogenesis associated with chronic arsenic exposure. *Eur J Histochem* 2013, **57**(1):e1.

209. Thomas DJ, Li J, Waters SB, Xing W, Adair BM, Drobna Z, Devesa V, Styblo M: **Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals**. *Exp Biol Med (Maywood)* 2007, **232**(1):3-13.

210. Lin S, Shi Q, Nix FB, Styblo M, Beck MA, Herbin-Davis KM, Hall LL, Simeonsson JB, Thomas DJ: **A novel S-adenosyl-L-methionine:arsenic(III) methyltransferase from rat liver cytosol**. *J Biol Chem* 2002, **277**(13):10795-10803.

211. Walton FS, Waters SB, Jolley SL, LeCluyse EL, Thomas DJ, Styblo M: **Selenium compounds modulate the activity of recombinant rat AsIII-methyltransferase and the methylation of arsenite by rat and human hepatocytes**. *Chem Res Toxicol* 2003, **16**(3):261-265.

212. Poklis A, Saady JJ: **Arsenic poisoning: acute or chronic? Suicide or murder?** *Am J Forensic Med Pathol* 1990, **11**(3):226-232.

213. Chattopadhyay S, Bhaumik S, Purkayastha M, Basu S, Nag Chaudhuri A, Das Gupta S: **Apoptosis and necrosis in developing brain cells due to arsenic toxicity and protection with antioxidants**. *Toxicol Lett* 2002, **136**(1):65-76.

214. Chen CJ, Hsu LI, Wang CH, Shih WL, Hsu YH, Tseng MP, Lin YC, Chou WL, Chen CY, Lee CY, Wang LH, Cheng YC, Chen CL, Chen SY, Wang YH, Hsueh YM, Chiou HY, Wu MM: **Biomarkers of exposure,**

effect, and susceptibility of arsenic-induced health hazards in Taiwan. *Toxicol Appl Pharmacol* 2005, **206**(2):198-206.

215. Roy P, Saha A: **Metabolism and toxicity of arsenic: A human carcinogen**. *Current Science* 2002, **82**(1):38-45.

216. Navas-Acien A, Silbergeld EK, Sharrett R, Calderon-Aranda E, Selvin E, Guallar E: **Metals in urine and peripheral arterial disease**. *Environ Health Perspect* 2005, **113**(2):164-169.

217. Navas-Acien A, Sharrett AR, Silbergeld EK, Schwartz BS, Nachman KE, Burke TA, Guallar E: **Arsenic exposure and cardiovascular disease: a systematic review of the epidemiologic evidence**. *Am J Epidemiol* 2005, **162**(11):1037-1049.

218. Straub AC, Clark KA, Ross MA, Chandra AG, Li S, Gao X, Pagano PJ, Stolz DB, Barchowsky A: **Arsenic-stimulated liver sinusoidal capillarization in mice requires NADPH oxidase-generated superoxide**. *J Clin Invest* 2008, **118**(12):3980-3989.

219. Lai MS, Hsueh YM, Chen CJ, Shyu MP, Chen SY, Kuo TL, Wu MM, Tai TY: **Ingested inorganic arsenic and prevalence of diabetes mellitus**. *Am J Epidemiol* 1994, **139**(5):484-492.

220. Tseng CH, Chong CK, Heng LT, Tseng CP, Tai TY: **The incidence of type 2 diabetes mellitus in Taiwan**. *Diabetes Res Clin Pract* 2000, **50 Suppl 2**:S61-64.

221. Rahman M, Griffin SJ, Rathmann W, Wareham NJ: **How should peripheral neuropathy be assessed in people with diabetes in primary**

care? A population-based comparison of four measures. *Diabet Med* 2003, **20**(5):368-374.

222. Wang SL, Chiou JM, Chen CJ, Tseng CH, Chou WL, Wang CC, Wu TN, Chang LW: **Prevalence of non-insulin-dependent diabetes mellitus and related vascular diseases in southwestern arseniasis-endemic and nonendemic areas in Taiwan**. *Environ Health Perspect* 2003, **111**(2):155-159.

223. Gresser MJ: **ADP-arsenate. Formation by submitochondrial particles under phosphorylating conditions**. *J Biol Chem* 1981, **256**(12):5981-5983.

224. Moore SA, Moennich DM, Gresser MJ: **Synthesis and hydrolysis of ADP-arsenate by beef heart submitochondrial particles**. *J Biol Chem* 1983, **258**(10):6266-6271.

225. Tseng CH: **The potential biological mechanisms of arsenic-induced diabetes mellitus**. *Toxicol Appl Pharmacol* 2004, **197**(2):67-83.

226. Petrick JS, Jagadish B, Mash EA, Aposhian HV: **Monomethylarsonous acid (MMA(III)) and arsenite: LD(50) in hamsters and in vitro inhibition of pyruvate dehydrogenase**. *Chem Res Toxicol* 2001, **14**(6):651-656.

227. Au WY: **A biography of arsenic and medicine in Hong Kong and China**. *Hong Kong Med J* 2011, **17**(6):507-513.

228. Jolliffe DM: **A history of the use of arsenicals in man**. *J R Soc Med* 1993, **86**(5):287-289.

229. Chen SJ, Zhou GB, Zhang XW, Mao JH, de The H, Chen Z: **From an old remedy to a magic bullet: molecular mechanisms underlying the therapeutic effects of arsenic in fighting leukemia**. *Blood* 2011, **117**(24):6425-6437.

230. Baker N, de Koning HP, Maser P, Horn D: **Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story**. *Trends Parasitol* 2013, **29**(3):110-118.

231. Balasegaram M, Harris S, Checchi F, Ghorashian S, Hamel C, Karunakara U: **Melarsoprol versus eflornithine for treating late-stage Gambian trypanosomiasis in the Republic of the Congo**. *Bull World Health Organ* 2006, **84**(10):783-791.

232. Hillestad LK: **Acute promyelocytic leukemia**. *Acta Med Scand* 1957, **159**(3):189-194.

233. Chen Z, Brand NJ, Chen A, Chen SJ, Tong JH, Wang ZY, Waxman S, Zelent A: **Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia**. *EMBO J* 1993, **12**(3):1161-1167.

234. Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M, Zhang QY, Yang HY, Huang QH, Zhou GB, Tong JH, Zhang Y, Wu JH, Hu HY, de The H, Chen SJ, Chen Z: **Arsenic trioxide controls the fate of the PML-**

RARalpha oncoprotein by directly binding PML. *Science* 2010, **328**(5975):240-243.

235. Mann KK, Wallner B, Lossos IS, Miller WH, Jr.: **Darinaparsin: a novel organic arsenical with promising anticancer activity**. *Expert Opin Investig Drugs* 2009, **18**(11):1727-1734.

236. Tian J, Zhao H, Nolley R, Reese SW, Young SR, Li X, Peehl DM, Knox SJ: **Darinaparsin: solid tumor hypoxic cytotoxin and radiosensitizer**. *Clin Cancer Res* 2012, **18**(12):3366-3376.

237. Petrick JS, Ayala-Fierro F, Cullen WR, Carter DE, Vasken Aposhian H: **Monomethylarsonous acid (MMA(III)) is more toxic than arsenite in Chang human hepatocytes**. *Toxicol Appl Pharmacol* 2000, **163**(2):203-207.

238. McDermott JR, Jiang X, Beene LC, Rosen BP, Liu Z: **Pentavalent methylated arsenicals are substrates of human AQP9**. *Biometals* 2010, **23**(1):119-127.

239. Hirano S, Cui X, Li S, Kanno S, Kobayashi Y, Hayakawa T, Shraim A: **Difference in uptake and toxicity of trivalent and pentavalent inorganic arsenic in rat heart microvessel endothelial cells**. *Arch Toxicol* 2003, **77**(6):305-312.

240. Villa-Bellosta R, Sorribas V: **Arsenate transport by sodium/phosphate cotransporter type IIb**. *Toxicol Appl Pharmacol* 2010, **247**(1):36-40.

241. Rosen BP, Liu Z: **Transport pathways for arsenic and selenium: a minireview**. *Environ Int* 2009, **35**(3):512-515.

242. Liu Z, Carbrey JM, Agre P, Rosen BP: **Arsenic trioxide uptake by human and rat aquaglyceroporins**. *Biochem Biophys Res Commun* 2004, **316**(4):1178-1185.

243. Lu WJ, Tamai I, Nezu J, Lai ML, Huang JD: **Organic anion transporting polypeptide-C mediates arsenic uptake in HEK-293 cells**. *J Biomed Sci* 2006, **13**(4):525-533.

244. Navas-Acien A, Umans JG, Howard BV, Goessler W, Francesconi KA, Crainiceanu CM, Silbergeld EK, Guallar E: **Urine arsenic concentrations and species excretion patterns in American Indian communities over a 10-year period: the Strong Heart Study**. *Environ Health Perspect* 2009, **117**(9):1428-1433.

245. Challenger F: **Biological methylation**. *Adv Enzymol Relat Subj Biochem* 1951, **12**:429-491.

246. Naranmandura H, Suzuki N, Suzuki KT: **Trivalent arsenicals are bound to proteins during reductive methylation**. *Chem Res Toxicol* 2006, **19**(8):1010-1018.

247. Hayakawa T, Kobayashi Y, Cui X, Hirano S: **A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19**. *Arch Toxicol* 2005, **79**(4):183-191.

248. Carew MW, Naranmandura H, Shukalek CB, Le XC, Leslie EM: **Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1)**. *Drug Metab Dispos* 2011, **39**(12):2298-2304.

249. Kumagai Y, Sumi D: **Arsenic: signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity**. *Annu Rev Pharmacol Toxicol* 2007, **47**:243-262.

250. Drobna Z, Naranmandura H, Kubachka KM, Edwards BC, Herbin-Davis K, Styblo M, Le XC, Creed JT, Maeda N, Hughes MF, Thomas DJ: **Disruption of the arsenic (+3 oxidation state) methyltransferase gene in the mouse alters the phenotype for methylation of arsenic and affects distribution and retention of orally administered arsenate**. *Chem Res Toxicol* 2009, **22**(10):1713-1720.

251. Yokohira M, Arnold LL, Pennington KL, Suzuki S, Kakiuchi-Kiyota S, Herbin-Davis K, Thomas DJ, Cohen SM: **Effect of sodium arsenite dose administered in the drinking water on the urinary bladder epithelium of female arsenic (+3 oxidation state) methyltransferase knockout mice**. *Toxicol Sci* 2011, **121**(2):257-266.

252. Steinmaus C, Bates MN, Yuan Y, Kalman D, Atallah R, Rey OA, Biggs ML, Hopenhayn C, Moore LE, Hoang BK, Smith AH: **Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States**. *J Occup Environ Med* 2006, **48**(5):478-488.

253. Pu YS, Yang SM, Huang YK, Chung CJ, Huang SK, Chiu AW, Yang MH, Chen CJ, Hsueh YM: **Urinary arsenic profile affects the risk of urothelial carcinoma even at low arsenic exposure**. *Toxicol Appl Pharmacol* 2007, **218**(2):99-106.

254. Cui X, Kobayashi Y, Hayakawa T, Hirano S: **Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats**. *Toxicol Sci* 2004, **82**(2):478-487.

255. Chen ZS, Mutoh M, Sumizawa T, Furukawa T, Haraguchi M, Tani A, Akiyama S: **Reversal of heavy metal resistance in multidrug-resistant human KB carcinoma cells**. *Biochem Biophys Res Commun* 1997, **236**(3):586-590.

256. Liu J, Chen H, Miller DS, Saavedra JE, Keefer LK, Johnson DR, Klaassen CD, Waalkes MP: **Overexpression of glutathione S-transferase II and multidrug resistance transport proteins is associated with acquired tolerance to inorganic arsenic**. *Mol Pharmacol* 2001, **60**(2):302-309.

257. Ong PS, Chan SY, Ho PC: **Microarray analysis revealed dysregulation of multiple genes associated with chemoresistance to As(2)O(3) and increased tumor aggressiveness in a newly established arsenic-resistant ovarian cancer cell line, OVCAR-3/AsR**. *Eur J Pharm Sci* 2012, **45**(3):367-378.

258. Lorico A, Bertola A, Baum C, Fodstad O, Rappa G: **Role of the Multidrug Resistance Protein 1 in protection from heavy metal oxyanions: investigations in vitro and in MRP1-deficient mice**. *Biochem Biophys Res Commun* 2002, **291**(3):617-622.

259. Delnomdedieu M, Basti MM, Otvos JD, Thomas DJ: **Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study**. *Chem Biol Interact* 1994, **90**(2):139-155.

260. Yehiayan L, Pattabiraman M, Kavallieratos K, Wang X, Boise LH, Cai Y: **Speciation, formation, stability and analytical challenges of human arsenic metabolites**. *J Anal At Spectrom* 2009, **24**(10):1397-1405.

261. Zeng H: **Selenite and selenomethionine promote HL-60 cell cycle progression**. *J Nutr* 2002, **132**(4):674-679.

262. Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R, Gladyshev VN: **Characterization of mammalian selenoproteomes**. *Science* 2003, **300**(5624):1439-1443.

263. Rayman MP: **Selenium in cancer prevention: a review of the evidence and mechanism of action**. *Proc Nutr Soc* 2005, **64**(4):527-542.

264. Brigelius-Flohe R: **Selenium compounds and selenoproteins in cancer**. *Chem Biodivers* 2008, **5**(3):389-395.

265. Jackson MI, Cao J, Zeng H, Uthus E, Combs GF, Jr.: **S-adenosylmethionine-dependent protein methylation is required for expression of selenoprotein P and gluconeogenic enzymes in HepG2 human hepatocytes**. *J Biol Chem* 2012, **287**(43):36455-36464.

266. Csanaky I, Gregus Z: **Effect of selenite on the disposition of arsenate and arsenite in rats**. *Toxicology* 2003, **186**(1-2):33-50.

267. Levander OA, Baumann CA: **Selenium metabolism. VI. Effect of arsenic on the excretion of selenium in the bile**. *Toxicol Appl Pharmacol* 1966, **9**(1):106-115.

268. Moxon AL: **The Effect of Arsenic on the Toxicity of Seleniferous Grains**. *Science* 1938, **88**(2273):81.

269. Gailer J, Madden S, Burke MF, Denton MB, Aposhian HV: **Simultaneous multielement-specific detection of a novel glutathione-arsenic-selenium ion [(GS)(2)AsSe](-) by ICP AES after micellar size-exclusion chromatography**. *Appl Organomet Chem* 2000, **14**(7):355-363.

270. Manley SA, George GN, Pickering IJ, Glass RS, Prenner EJ, Yamdagni R, Wu Q, Gailer J: **The seleno bis(S-glutathionyl) arsinium ion is assembled in erythrocyte lysate**. *Chem Res Toxicol* 2006, **19**(4):601-607.

271. Yoshino Y, Yuan B, Miyashita SI, Iriyama N, Horikoshi A, Shikino O, Toyoda H, Kaise T: **Speciation of arsenic trioxide metabolites in blood cells and plasma of a patient with acute promyelocytic leukemia**. *Anal Bioanal Chem* 2008.

272. Molin Y, Frisk P, Ilback NG: **Arsenic trioxide affects the trace element balance in tissues in infected and healthy mice differently**. *Anticancer Res* 2009, **29**(1):83-90.

273. Zeng HW, Uthus EO, Combs GF: **Mechanistic aspects of the interaction between selenium and arsenic**. *Journal of Inorganic Biochemistry* 2005, **99**(6):1269-1274.

Chapter 3

Selenium-dependent and -independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/*ABCC2*): Implications for the mutual detoxification of arsenic and selenium

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3 Selenium-dependent and –independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/*ABCC2*): Implications for the mutual detoxification of arsenic and selenium

3.1 Abstract

Simultaneous exposure of lab animals to toxic doses of the human carcinogen arsenic (As) and the essential trace element selenium (Se), results in a remarkable mutual detoxification. A likely basis for this is the *in vivo* formation and biliary excretion of seleno-bis(S-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$; however, the transport protein responsible for the biliary efflux of $[(GS)_2AsSe]^-$ has not been identified. The multidrug resistance protein 2 (MRP2/*ABCC2*) is an ATP-binding cassette transporter expressed at the canalicular membrane of hepatocytes. Rat Mrp2 is known to excrete the As-GSH conjugates $As(GS)_3$ and $MMA(GS)_2$ into bile, and *in vitro* studies have established $As(GS)_3$ as a substrate for human MRP2. In the present study, membrane vesicles prepared from HEK293T cells transfected with human MRP2 were used to demonstrate that MRP2 transports $[(GS)_2AsSe]^-$. In addition, the characteristics of MRP2 transport of $As(GS)_3$ and $[(GS)_2AsSe]^-$ were investigated. $As(GS)_3$ and $[(GS)_2AsSe]^-$ are chemically labile and have the potential to dissociate. However, As^{III} ($\pm$ Se^{IV}) transport was not detected in the absence of GSH or in the presence of the non-reducing GSH analog, ophthalmic acid, suggesting that the conjugates are the transported forms. The apparent K_m values for $[(GS)_2AsSe]^-$ and $As(GS)_3$ were 1.7 μM and 4.2 μM, respectively, signifying high relative affinities. Membrane vesicles prepared from

human erythrocytes, which express the MRP2-related MRP1/*ABCC1*, MRP4/*ABCC4* and MRP5/*ABCC5*, transported $As(GS)_3$ in an MRP1- and ATP-dependent manner, but did not transport $[(GS)_2AsSe]^-$. These results have important implications for the selenium dependent and independent disposition of arsenic.

3.2 Introduction

The metalloid arsenic is a type I human carcinogen and a high priority environmental contaminant capable of causing both chronic and acute toxicity [1]. Arsenic occurs naturally in the earth's crust and is a serious health concern in many countries including Bangladesh and India, where wells were dug deep into arsenic containing bedrock in order to evade microbial contamination of drinking water [2]. This resulted in mg/L levels of inorganic arsenic, including arsenate (As^V) and arsenite (As^{III}), being released into the water in some areas. These levels were 100-fold in excess of the World Health Organization's standard of 10 μg/L. In addition to developing nations, natural and industrial contamination of well water by arsenic is also an issue in some areas of North America [3].

Selenium is an essential trace element for human nutrition, involved in a wide variety of biological processes and has been demonstrated as a requirement for the growth of mammalian cells in culture [4]. Selenium is found in soil, accumulates in plants, and is therefore naturally present in the human diet [5]. A mutual protective relationship exists between arsenic and selenium that was discovered when arsenic containing drinking water protected rats against selenium induced liver damage [5]. It was later shown that selenium and arsenic

enhanced the biliary excretion of each other, resulting in a decreased retention of both metalloids in the liver, proportional to the levels excreted into bile [6]. More recently, a seleno-bis(S-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$, has been identified in the bile of rabbits co-injected with selenite (Se^{IV}) and As^{III} providing a molecular basis for the antagonistic interaction between these two metalloids [7-9]. $[(GS)_2AsSe]^-$ can also be formed in rabbit erythrocyte lysate and it has been hypothesized that it is actively exported from the red blood cells into plasma for excretion by the liver [7, 8, 10].

The multidrug resistance protein 1 (MRP1, encoded by *ABCC1*) and the related MRP2 (*ABCC2*) are ATP-binding cassette transporter proteins that can work synergistically with phase II conjugation pathways to reduce the accumulation of a broad range of glutathione- (GSH, γ-Glu-Cys-Gly), glucuronide-, and sulfate-conjugated organic anions [11, 12]. In addition, MRP1 and MRP2 require GSH for the transport of certain unconjugated and conjugated compounds [13-15]. For example, the transport of several chemotherapeutics that do not form conjugates is dependent on GSH [12, 14, 16]. In addition, GSH is required for MRP1 mediated transport of the *O*-glucuronide conjugate of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, and enhances the transport of several other conjugated compounds including estrone-3-sulfate [17-22]. The thiol group of GSH is not required for GSH-dependent transport as non-reducing GSH analogs such as S-methyl GSH and ophthalmic acid (γ-Glu-aminobutyrate (Abu)-Gly) have been shown to substitute functionally for GSH in GSH-dependent or GSH-enhanced transport [14, 17, 21, 23].

MRP2 is localized at the apical surface of polarized epithelia including hepatocytes and the proximal tubular cells of the kidney [24]. Studies comparing wild-type and Mrp2-deficient Wistar rats revealed that Mrp2 is responsible for the biliary excretion of two arsenic glutathione conjugates [$As(GS)_3$ and $MMA(GS)_2$] [15]. *In vitro* transport assays have shown that $As(GS)_3$ is also a substrate for human MRP2 [25], but the properties of this transport have never been characterized. In addition, the ability of MRP2 to transport $[(GS)_2AsSe]^-$ is unknown.

In the current study, membrane vesicles prepared from human MRP2-transfected HEK293T cells were used to identify $[(GS)_2AsSe]^-$ as a MRP2 substrate. The characteristics and mechanism of MRP2 transport of $As(GS)_3$ and $[(GS)_2AsSe]^-$ were also investigated. Human erythrocyte membrane vesicles were used to determine if erythrocytes transport $As(GS)_3$ and/or $[(GS)_2AsSe]^-$ in an ATP-dependent manner.

3.3 Methods

3.3.1 *Chemicals and Reagents*

$^{73}As^V$ was purchased from Los Alamos Meson Production Facility (Los Alamos, NM). GSH, ATP, AMP, sucrose, Tris base, sodium selenite, sodium arsenite and $MgCl_2$ were purchased from Sigma-Aldrich (Oakville, Ontario). Creatine kinase, glutathione reductase, creatine phosphate, NADPH, and protease inhibitor tablets (Complete™, mini EDTA free) were purchased from Roche Applied Science. Ophthalmic acid was purchased from Bachem (Torrance, CA).

3.3.2 *MRP2 expression in HEK293T cells*

The HEK293T cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. MRP2 complementary DNA was a gift from Dr Susan P.C.Cole (Queen's University) and its construction in the pcDNA3.1(−) vector has been described previously [26].

HEK293T cells were transfected in 150 mm culture dishes using the calcium phosphate method as described previously, with minor modifications [27]. Briefly, 3×10^6 cells were seeded onto 150 mm plates and 24 h later, 18 μg of DNA was prepared with calcium chloride (250 mM, final volume 1.3 ml) and added dropwise to 1.3 ml *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (HEPES) (275 mM NaCl, 1.5 mM Na_2HPO_4 and 55 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0). The DNA solution was added dropwise to cells, incubated for 24 h and then the media was changed. Seventy-two hours post-transfection, cells were washed twice with Tris-sucrose buffer (50 mM Tris, pH 7.4, 250 mM sucrose), scraped into Tris-sucrose buffer and collected by centrifuging at 800*g* for 10 min. Cell pellets were stored at −80°C until membrane vesicles were prepared.

3.3.3 *Membrane vesicle preparation*

Plasma membrane vesicles were prepared from MRP2-transfected HEK293T cells according to methods described previously [25]. Briefly, cells were thawed and resuspended in buffer containing 250 mM sucrose, 50 mM Tris, pH 7.4, 0.25 mM $CaCl_2$ and protease inhibitor tablets. Cells were then disrupted by nitrogen cavitation (pressurized to 200 p.s.i. and released to atmospheric pressure) and

ethylenediaminetetraacetic acid (1 mM) was added. The disrupted cells were centrifuged at 800*g* at 4°C for 10 min to remove unbroken cells and nuclei. The supernatant was layered onto 12 ml of 35% (wt/wt) sucrose, 50 mM Tris, pH 7.4 and centrifuged at 100,000*g* for 1 h at 4°C. The interphase containing the plasma membrane-enriched fraction was removed and diluted with 25 mM sucrose, 50 mM Tris, pH 7.4 and centrifuged at 100,000*g* for 30 min at 4°C. The membranes were washed with Tris-sucrose buffer and centrifuged at 100,000*g* for 30 min at 4°C. The membranes were then resuspended in Tris-sucrose buffer by passing through a 27 gauge needle, then aliquoted and frozen at −80°C. Expression of MRP2 in membrane vesicles was confirmed by immunoblot analysis, using the mouse monoclonal antibody (mAb), M_2 I-4 (1:2000) (Chemicon, Rosemont, IL).

3.3.4 *Erythrocyte membrane vesicle preparation*

Plasma membrane vesicles were prepared from human erythrocytes, according to a method described previously [28] with minor modifications. Human blood was obtained with the approval of the University of Alberta Human Ethics Review Board and with donor consent. Briefly, 12 ml of blood was drawn from human volunteers into K3 EDTA Vacutainer® tubes (BD, Franklin Lakes, NJ). Blood was centrifuged at 2000*g* for 10 min and washed three times with isotonic saline (150 mM NaCl and 5 mM sodium phosphate; pH 8.0). Packed cells were lysed by the addition of 40 vol of concentrated phosphate buffer (5 mM sodium phosphate, pH 8.0) and then centrifuged at 20,000*g* for 10 min, washed with phosphate buffer (0.5 mM sodium phosphate, pH 8.0), resuspended and incubated for 1 h on ice. Membranes were centrifuged at 100,000*g* for 30 min and resuspended in Tris

sucrose buffer by passage through a 27 gauge needle, then aliquoted and frozen at −80°C.

3.3.5 *Chemical synthesis of $^{73}As^{III}$, $^{73}As(GS)_3$ and $[(GS)_2{}^{73}AsSe]^-$*

$^{73}As^{III}$ was prepared from $^{73}As^{V}$ with a metabisulfite-thiosulfate treatment as described previously [29]. $^{73}As^{III}$ + As^{III} was prepared to a concentration of 50 μM (50 nCi/μl). The formation of $^{73}As^{III}$ was monitored using a previously established thin-layer chromatography method [30]. $^{73}As(GS)_3$ was prepared as described previously [31], with minor modifications. Briefly, $^{73}As^{III}$ (final concentration 25 μM) and GSH (final concentration 75 mM) were mixed under a nitrogen atmosphere in Tris–sucrose buffer and incubated at room temperature for 30 min. $[(GS)_2{}^{73}AsSe]^-$ was prepared as described previously [10] with minor modifications. Briefly, GSH (150 mM) was dissolved in Tris-sucrose buffer and the pH was adjusted to 8.5 with sodium hydroxide (NaOH). $^{73}As^{III}$ (50 μM, 50 nCi/μl) and sodium selenite (50 μM) were added to GSH and the final pH was confirmed to be above 7.5 using a pH meter.

3.3.6 *$^{73}As^{III}$, $^{73}As(GS)_3$ and $[(GS)_2{}^{73}AsSe]^-$ transport assays*

Transport studies were completed using the rapid filtration method [25, 32]. Briefly, membrane vesicles were incubated at 37°C in Tris-sucrose buffer, with adenosine triphosphate (ATP) or adenosine monophosphate (AMP) (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 μg/ml), GSH reductase (5 μg/ml), NADPH (0.35 mM), GSH (3 mM) and $^{73}As(GS)_3$ or $[(GS)_2{}^{73}AsSe]^-$. At 3 min, transport was stopped by diluting the transport reaction with 800 μl of ice-cold Tris-sucrose buffer.

The effects of Se^{IV} (1 μM), GSH (3 mM), the GSH analog OA (3 mM), the reducing agent dithiothreitol (DTT) (3 mM) or the MRP1-specific mAbs QCRL-1 and QCRL-3 (100 μg/ml) on $^{73}As^{III}$ transport were measured at a 3 min time point. Kinetic parameters of $^{73}As(GS)_3$ and $[(GS)_2{}^{73}AsSe]^-$ transport were determined by measuring the initial rate of uptake at eight different substrate concentrations (0.1–10 μM, 20–110 nCi) at a 3 min time point.

Samples were filtered through glass fiber filters and then radioactivity was quantified by liquid scintillation counting. ATP-dependent transport was determined by subtracting transport in the presence of AMP from the transport in the presence of ATP.

3.4 Results

3.4.1 *Transport of $As(GS)_3$ and $[(GS)_2AsSe]^-$ by MRP2-enriched membrane vesicles*

To determine if $[(GS)_2{}^{73}AsSe]^-$ was a substrate for MRP2, the conjugate was synthesized and ATP-dependent transport into MRP2-enriched membrane vesicles was determined (Figure 3.1A). $[(GS)_2{}^{73}AsSe]^-$ (200 nM) was transported at ~5 pmol mg^{-1} 3 min^{-1} compared to $^{73}As(GS)_3$ (200 nM) which was transported at ~3 pmol mg^{-1} 3 min^{-1} (Figure 3.1A). When $^{73}As^{III}$ (200 nM) and GSH (3 mM) were added separately to the transport mix, uptake was not significantly different than that of $^{73}As(GS)_3$. The addition of Se^{IV} (200 nM) to $^{73}As^{III}$ and GSH did not enhance transport activity above the level observed for $^{73}As^{III}$ + GSH or $^{73}As(GS)_3$ (Figure 3.1A). In the presence of Se^{IV} and absence of GSH, $^{73}As^{III}$ transport was not detected, even in the presence of the GSH analog ophthalmic

acid. No ATP-dependent transport was detected under any of the conditions tested using membrane vesicles prepared from untransfected HEK293T cells (data not shown). Thus, MRP2 transports $[(GS)_2{}^{73}AsSe]^-$ and the data suggests that the preformed conjugate is required for transport to occur.

To characterize the mechanism of GSH-dependence involved in MRP2-mediated transport of $^{73}As^{III}$, a series of experimental conditions were tested. In the presence of GSH (3 mM), $^{73}As^{III}$ (200 nM) was transported at ~2 pmol mg^{-1} 3 min^{-1} (Figure 3.1B). To confirm that this was not due strictly to the reducing capacity of GSH, transport assays were undertaken with the reducing agent DTT (3 mM). Consistent with previous studies of MRP1, DTT did not substitute for GSH and $^{73}As^{III}$ transport was not detected (Figure 3.1B) [32]. The non-reducing glutathione analog ophthalmic acid also did not substitute for GSH to enhance $^{73}As^{III}$ transport even in the presence of DTT (Figure 3.1B). Similar results were obtained when the reducing agent β-mercaptoethanol was used in place of DTT (data not shown). Overall, this demonstrated that the free thiol group of GSH, and not its reducing capacity, is required for $^{73}As^{III}$ transport by MRP2. These data provide evidence for transport being dependent upon the formation of $^{73}As(GS)_3$ (Figure 3.1B).

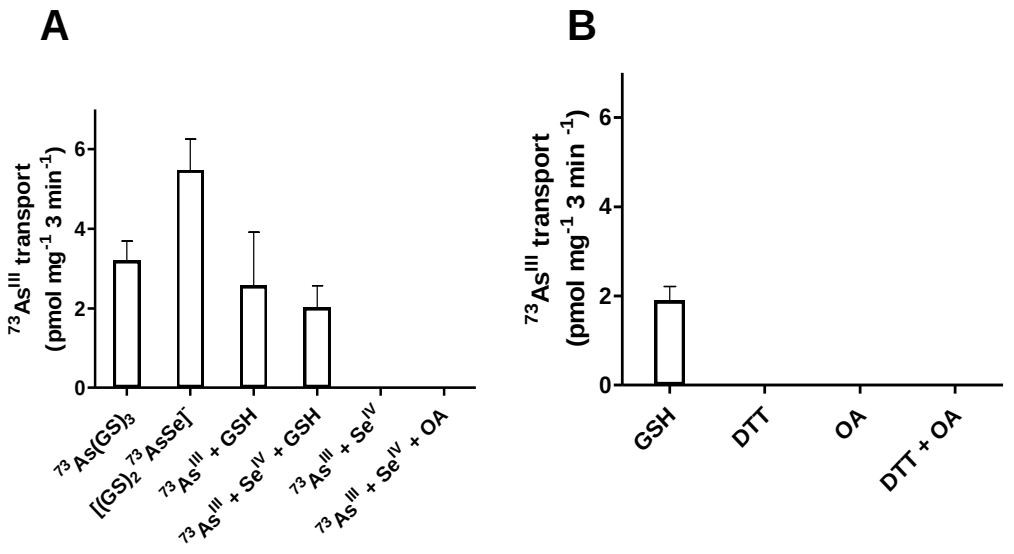

Figure 3.1. MRP2-mediated transport of $^{73}As(GS)_3$, $[(GS)_2{}^{73}AsSe]^-$, or free $^{73}As^{III}$ in the presence and absence of Se^{IV}, GSH or ophthalmic acid. Membrane vesicles (20 μg of protein) prepared from MRP2 transiently transfected HEK293T cells were incubated for 3 min at 37°C in transport buffer with A) $^{73}As(GS)_3$, $[(GS)_2{}^{73}AsSe]^-$, or free $^{73}As^{III}$ (200 nM, 50 nCi) and Se^{IV} (200 nM) in the presence or absence of GSH (3 mM) or ophthalmic acid (3 mM). B) Incubations with $^{73}As^{III}$ (200 nM, 50 nCi) were carried out in the absence, or presence of GSH (3 mM), ophthalmic acid (3 mM), or DTT (3 mM). Columns represent the means of triplicate determinations (± S.D.) in a single experiment; similar results were obtained in at least two additional experiments. No activity was detected in vector control vesicles.

3.4.2 *Kinetic analysis of $As(GS)_3$ transport by MRP2*

Time courses of $^{73}As(GS)_3$ (100 nM) transport by MRP2-enriched membrane vesicles were completed to determine the linear range of uptake. Transport was linear for up to 5 min with a maximum activity of ~8 pmol mg^{-1} at 10 min (Figure 3.2A). MRP2 dependent transport of $^{73}As(GS)_3$ was further characterized by determining the initial rates of transport over several concentrations of $^{73}As(GS)_3$. According to Michaelis-Menten kinetic analysis, the apparent K_m for $^{73}As(GS)_3$ was found to be 4.2 ± 0.9 μM and the V_{max} was 134 ± 12 pmol mg^{-1} min^{-1} (mean ± SD of three independent determinations) (Figure 3.2B and Table 3.1).

3.4.3 *Kinetic Analysis of $[(GS)_2AsSe]^-$ by MRP2.*

Time courses of $[(GS)_2{}^{73}AsSe]^-$ (100 nM) transport by MRP2-enriched membrane vesicles were completed to determine the linear range of uptake. Transport was linear for up to 3 min with a maximum activity of ~5 pmol mg^{-1} at 10 min. MRP2 dependent transport of $[(GS)_2{}^{73}AsSe]^-$ was further characterized by determining the initial rates of transport over several concentrations of $[(GS)_2{}^{73}AsSe]^-$ (Figure 3.3A). According to Michaelis-Menten kinetic analysis, the apparent K_m for $[(GS)_2{}^{73}AsSe]^-$ was found to be 1.7 ± 0.8 μM and the V_{max} was 45 ± 8 pmol mg^{-1} min^{-1} (mean ± SD of three independent determinations) (Figure 3.3B and Table 3.1).

3.4.4 *Transport of $As(GS)_3$ and $[(GS)_2AsSe]^-$ by human erythrocytes*

$[(GS)_2AsSe]^-$ is formed in rabbit erythrocyte lysate and it has been hypothesized that it is actively exported from red blood cells into plasma for excretion at the liver [7, 8, 10]. MRP1, MRP4 and MRP5 are known to be expressed in the

plasma membrane of human erythrocytes, while MRP2 is not [33]. To determine if human erythrocytes have the ability to efflux $^{73}As(GS)_3$ or $[(GS)_2{}^{73}AsSe]^-$ membrane vesicles were prepared and ATP-dependent transport measured. $^{73}As(GS)_3$ (1 μM) was found to be transported at a rate of ~22 pmol mg^{-1} 3 min^{-1} (Figure 3.4A) while no activity was detected for $[(GS)_2AsSe]^-$ (Figure 3.4B). Membrane vesicles prepared from HEK293T cells expressing MRP2 were used as positive controls for each conjugate (Figure 3.4A and B).

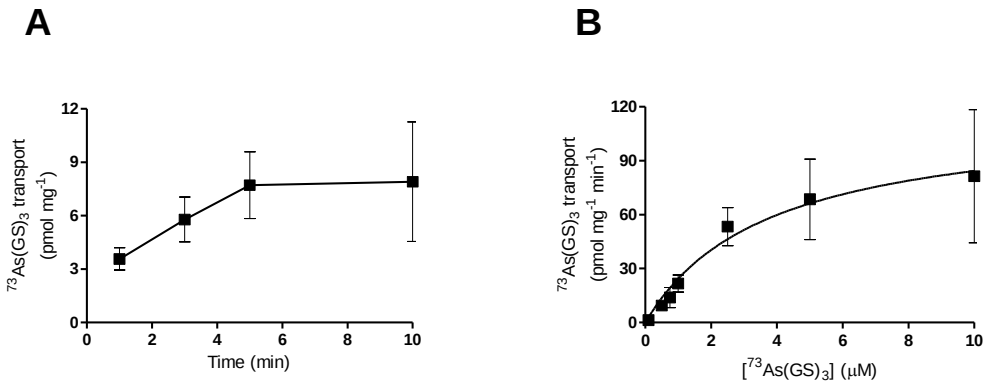

Figure 3.2. Kinetic Analysis of MRP2-mediated $^{73}As(GS)_3$ transport. A) Time course of ATP-dependent transport of $^{73}As(GS)_3$ by membrane vesicles prepared from MRP2 transfected HEK293T cells. Membrane vesicles (20 μg per time point) were incubated at 37 °C in transport buffer with $^{73}As(GS)_3$ (100 nM, 50 nCi) for the indicated time points. B) Membrane vesicles (20 μg of protein) prepared from MRP2 transfected HEK293T cells were incubated for 3 min at 37 °C in transport buffer with various concentrations of $^{73}As(GS)_3$ (0.1-10 μM). Kinetic parameters were determined using the Graphpad Prism 5™ Michaelis-Menten kinetic modeling software. Points shown represent the means (± S.D.) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments.

Table 3.1 Summary of MRP1 and MRP2 transport kinetic parameters for arsenic glutathione conjugates.

	K_m (μM)	V_{max} (pmol/mg/min)	V_{max}/K_m (μl/mg/min)
$As(GS)_3$ (MRP1)[a]	0.32 ± 0.08	17 ± 5	53
$As(GS)_3$ (MRP2)	4.2 ± 0.9	134 ± 12	32
$[(GS)_2AsSe]^-$ (MRP2)	1.7 ± 0.8	45 ± 8	27

[a]Previous Study [25]

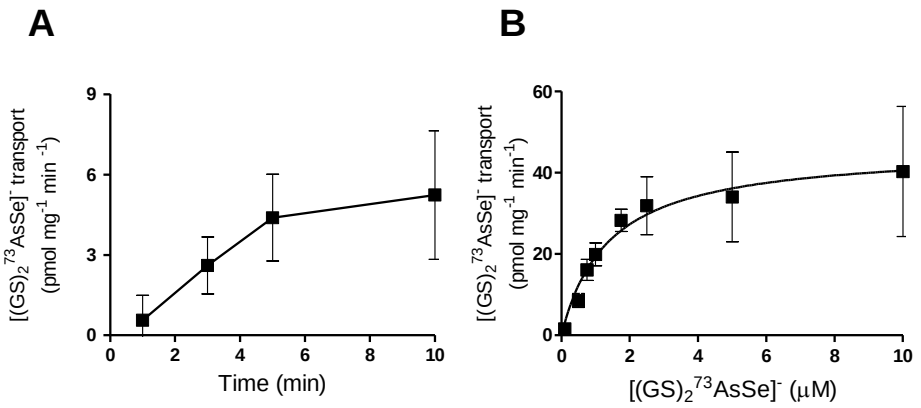

Figure 3.3. Kinetic Analysis of MRP2-mediated $[(GS)_2{}^{73}AsSe]^-$ transport. A) Time course of ATP-dependent transport of $[(GS)_2{}^{73}AsSe]^-$ by membrane vesicles prepared from MRP2 transfected HEK293T cells. Membrane vesicles (20 μg per time point) were incubated at 37 ^{0}C in transport buffer with $[(GS)_2{}^{73}AsSe]^-$ (100 nM, 50 nCi). B) Membrane vesicles (20 μg of protein) prepared from MRP2 transfected HEK293T cells were incubated for 3 min at 37 ^{0}C in transport buffer with various concentrations of $[(GS)_2{}^{73}AsSe]^-$ (0.1-10 μM, 50-100 nCi). Kinetic parameters were determined using the Graphpad Prism 5™ Michaelis-Menten kinetic modeling software. Points shown represent the means (±S.D.) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments.

In order to determine the contribution of MRP1 in erythrocytes to the overall transport of $As(GS)_3$, inhibition studies with MRP1-specific antibodies were completed. When membrane vesicles were incubated with the MRP1-specifc mAb QRCL-3, which recognizes a conformational dependent epitope, and has been shown previously to inhibit the transport of many MRP1 substrates [25, 32, 34], complete inhibition of $As(GS)_3$ transport was observed (Figure 3.4C). The MRP1-specific mAb QCRL-1, which recognizes a linear epitope in the linker region of MRP1 and does not inhibit the transport of other substrates [25, 34, 35], had no effect on the transport of $As(GS)_3$ (Figure 3.4C). Thus, MRP1 is responsible for the ATP-dependent transport of $As(GS)_3$ in erythrocyte membrane vesicles, suggesting it can efflux this conjugate from erythrocytes into plasma.

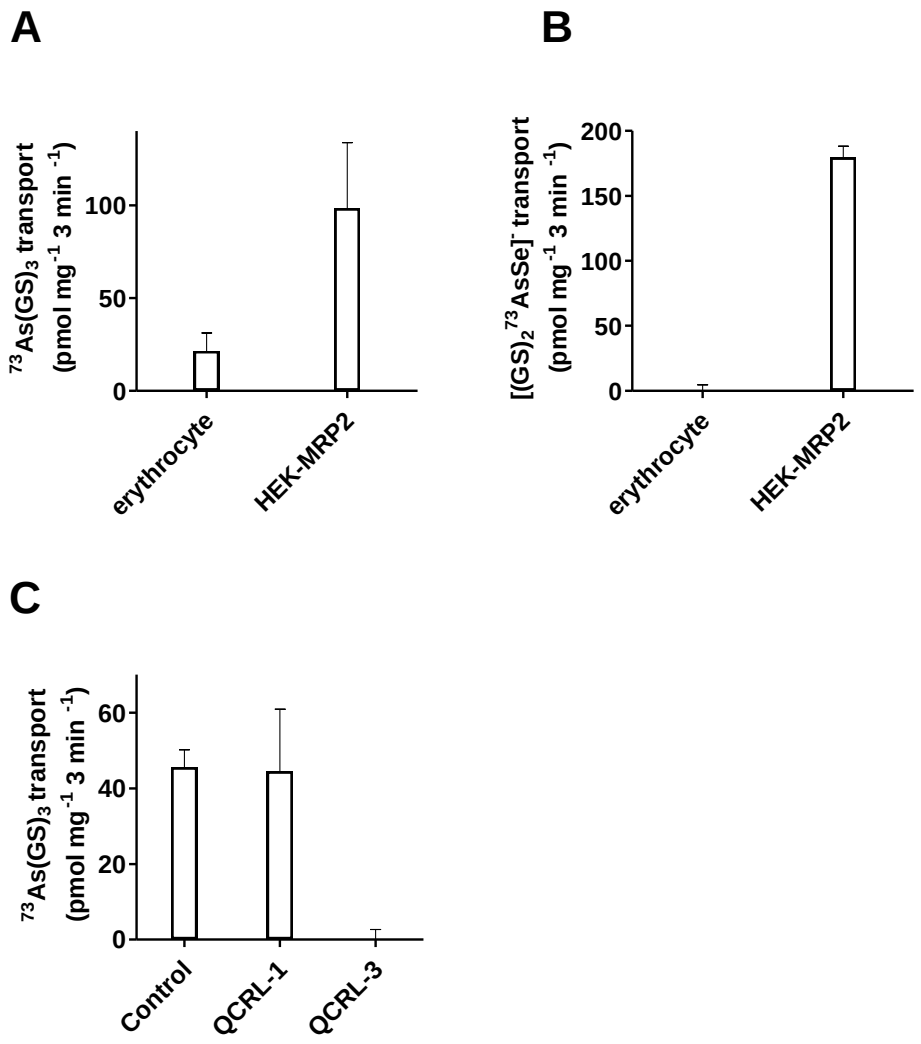

Figure 3.4. Erythrocyte Vesicle Transport of As^{III}. A) $^{73}As^{III}$ uptake was measured after incubation with $^{73}As(GS)_3$ or B) $[(GS)_2{}^{73}AsSe]^-$ (1 µM, 50 nCi). The results shown are the means (±S.D.) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments. C) $^{73}As(GS)_3$ uptake into membrane vesicles was measured after preincubation with the indicated MRP1-specific mAb (100 µg/ml) Vesicles were incubated at 37^0C in transport buffer with $As(GS)_3$ (1 µM, 50 nCi). The results shown are the means (±S.D.) of triplicate determinations in a single experiment; similar results were obtained in one additional experiment.

3.5 Discussion

The formation and biliary excretion of the seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ provides a molecular basis for the mutual detoxification of arsenic and selenium [7, 8, 36]. In the present study, we have identified $[(GS)_2AsSe]^-$ as a substrate for human MRP2, a protein that is required for the biliary excretion of a broad array of endogenous and exogenous chemicals, including GSH conjugates [11, 12]. In addition to identifying $[(GS)_2AsSe]^-$ as an MRP2 substrate, we have characterized the transport characteristics of this conjugate and that of $As(GS)_3$. The apparent K_m values determined for $As(GS)_3$ and $[(GS)_2AsSe]^-$ transport by MRP2 were 4.2 μM and 1.7 μM, respectively, signifying high relative affinities. The chemical instability of $As(GS)_3$ and $[(GS)_2AsSe]^-$ combined with the ability of MRP2 to transport compounds either covalently attached to GSH or through a GSH co-transport mechanism [13, 15, 37], led us to confirm the chemical species transported. Thus, As^{III} (± Se^{IV}) transport was not detected in the absence of GSH or in the presence of the non-reducing GSH analog, ophthalmic acid, suggesting that the conjugates are the transported forms. Transport assays using membrane vesicles prepared from human erythrocytes indicated that $[(GS)_2AsSe]^-$ was not effluxed by erythrocytes, but that $As(GS)_3$ was.

Studies using Mrp2-deficient (TR^-) rats have shown that Mrp2 is required for the biliary excretion of As^{III} as $As(GS)_3$ and the monomethylated diglutathione conjugate $MMA(GS)_2$ [15, 38, 39]. $As(GS)_3$ and $MMA(GS)_2$ are chemically unstable at the alkaline pH of bile (pH 8) with half-lives of 20 and 40 min, respectively [15]. Thus, once biliary excretion of $As(GS)_3$ and $MMA(GS)_2$ has

occurred these conjugates will dissociate to GSH and As^{III} or MMA^{III}, the arsenic will then be reabsorbed across the intestine and cycled back to the liver [13, 38]. Therefore, biliary excretion of $As(GS)_3$ and $MMA(GS)_2$ does not necessarily represent elimination of arsenic from the body. In contrast, $[(GS)_2AsSe]^-$ is relatively stable at biliary pH and is more likely to remain intact and be excreted in feces than $As(GS)_3$ and $MMA(GS)_2$ [10]. $[(GS)_2AsSe]^-$ has never been identified in feces, but As^{III} and Se^{IV} co-treatment of rats resulted in a 4- to 5-fold increase in arsenic and selenium excretion into the intestine and feces [6]. In addition, treatment of hamsters with As^{III} and Se^{IV} significantly increased the total amount of arsenic in feces as well as the rate of fecal excretion [40]. Taken together these data suggest that $[(GS)_2AsSe]^-$ prevents arsenic from undergoing enterohepatic cycling and is therefore a true detoxification product.

In addition to the higher stability of $[(GS)_2AsSe]^-$ in bile compared to $As(GS)_3$, we originally predicted that $[(GS)_2AsSe]^-$ would have a higher affinity for MRP2 compared with $As(GS)_3$, resulting in preferential excretion into bile. While the apparent K_m of $[(GS)_2AsSe]^-$ for MRP2 was indeed 2.5-fold lower than for $As(GS)_3$, the overall transport efficiencies (V_{max}/K_m) for the two compounds were remarkably similar (Figures 3.2 and 3.3, Table 3.1). Thus, it is likely that the higher stability of $[(GS)_2AsSe]^-$ at biliary pH compared to $As(GS)_3$ is the most critical component of the $[(GS)_2AsSe]^-$ contribution to the arsenic/selenium mutual detoxification.

The apparent K_m of $As(GS)_3$ for MRP2 was 4.2 μM which is approximately 10-fold higher than the apparent K_m of 0.32 μM reported

previously for MRP1 [25]. However, assuming comparable expression, MRP2 is a higher capacity (V_{max} of 134 pmol mg^{-1} min^{-1}) transporter of $As(GS)_3$ than MRP1 (V_{max} 17 pmol mg^{-1} min^{-1}) resulting in a similar overall transport efficiency (Table 3.1). The lower K_m value for MRP1 is consistent with its potential role in cellular protection against low levels of arsenic. MRP2 is more likely to be involved in a higher capacity excretory role at the canalicular membrane of hepatocytes or apical surface of proximal tubular cells. The apparent K_m values for MRP1 and MRP2 are of physiological relevance for clinical arsenic exposure. Pharmacological studies of patients undergoing chemotherapy with arsenic trioxide for acute promyelocytic leukemia showed plasma levels of arsenic nearing 0.5 μM [41] and hepatic concentrations would likely exceed this value.

Previously, we reported that the formation of $As(GS)_3$ is necessary for the transport of As^{III} by MRP1 [25]. Thus, MRP1-mediated transport of free As^{III} only occurred in the presence of GSH and glutathione *S*-transferase P1 (GSTP1), implying that catalysis was required for the formation and subsequent transport of $As(GS)_3$ [25]. In the present study, MRP2 transport of free As^{III} (± Se^{IV}) only occurred in the presence of GSH and was not supported by the non-reducing glutathione analog ophthalmic acid. This indicates that the free thiol group of GSH is required and that the conjugates are the transported forms. The MRP2 transport of As^{III} in the presence of GSH was not significantly different from the transport of $As(GS)_3$, likely due to the high level of membrane associated GSTP1 in the HEK293T membrane vesicles [42]. The conditions required

physiologically for the formation of $[(GS)_2AsSe]^-$ are unknown. Transport of As^{III} in the presence of Se^{IV} and GSH was significantly lower than $[(GS)_2AsSe]^-$ and equivalent to As^{III} + GSH or the preformed $As(GS)_3$. The comparable activity suggests that this was the transport of $As(GS)_3$ rather than spontaneously formed $[(GS)_2AsSe]^-$. Further investigation is needed but this could imply that the formation of $[(GS)_2AsSe]^-$ at physiologically relevant concentrations of metalloids and pH (as reported in rabbit erythrocyte lysate [7]) is not efficient and potentially requires catalysis.

In addition to the detoxification of arsenic and selenium through the biliary excretion of $[(GS)_2AsSe]^-$, *in vivo* data have shown that injection of arsenic and selenium delays the transfer of these metalloids to the liver by extending their time in blood [43, 44]. After a two minute incubation of As^{III} and Se^{IV} with rabbit erythrocyte lysate, ~70% of the metalloids were in the form $[(GS)_2AsSe]^-$ [7]. Manley et al., proposed that the delay in transfer from the blood to the liver could be caused by the time taken for $[(GS)_2AsSe]^-$ formation within the erythrocyte followed by active transport into plasma [7]. In the current study, membrane vesicles prepared from erythrocytes did not transport $[(GS)_2AsSe]^-$. Human erythrocytes do not express MRP2 but do express the related MRP1, MRP4 and MRP5 [33]. MRP1 and MRP2 have similar substrate specificity [11] so it was unexpected that $[(GS)_2AsSe]^-$ would not be transported by the erythrocyte membrane vesicles. The lack of ATP-dependent transport of $[(GS)_2AsSe]^-$ across the erythrocyte membrane suggests that formation of this conjugate within human erythrocytes would result in sequestration. This offers

an alternative explanation for the delay in processing of As^{III} and Se^{IV} from the blood to the liver. In contrast with $[(GS)_2AsSe]^-$, $As(GS)_3$ was transported by erythrocyte membrane vesicles. Although multiple MRPs are expressed in the membrane of erythrocytes, $As(GS)_3$ transport was completely inhibited by the MRP1-specific mAb QCRL-3. This suggests that MRP1 is responsible for the ATP-dependent efflux of $As(GS)_3$ from human erythrocytes and potentially contributes to the clearance of arsenic from human blood.

The oral co-administration of Se and As could result in the efficient formation of $[(GS)_2AsSe]^-$ at the liver, followed by MRP2-mediated efflux into bile and the prevention of the distribution of arsenic to peripheral tissues. In support of this, selenium in rodent chow completely blocked the passage of arsenic administered in drinking water to the skin and prevented arsenic enhanced skin carcinogenesis in a hairless mouse model [45]. The cooperative detoxification of arsenic and selenium may result in depletion of selenium, which is an important component of cellular antioxidant defense as well as many other anti-carcinogenic pathways, in addition to the formation of $[(GS)_2AsSe]^-$ [9]. Low selenium status could make individuals more susceptible to arsenic induced carcinogenesis [8]. Investigation of selenium status and arsenic-related premalignant skin lesions in humans have shown that reduced blood selenium levels increased the risk for such lesions [46]. Results from selenium supplementation trials in arsenic endemic regions have shown some promise and benefit to oral supplementation with selenium and prevention of carcinogenesis [46].

Active efflux of conjugated toxicants is the last step in cellular detoxification, preventing not only the reformation of active parent compound within the cell, but also the end-product inhibition of detoxification pathways [12]. The response of different individuals to chronic inorganic arsenic exposure is highly variable and not well understood [47]. Arsenic metabolism and excretion is complex and multiple pathways are likely responsible for the inter-individual differences; however, the importance of transport proteins in arsenic excretion has become increasingly evident [48]. In the current study we have characterized MRP2 as a high affinity, high capacity transporter of $As(GS)_3$ and $[(GS)_2AsSe]^-$. Polymorphic variants of MRP2 exist, and the influence of these variants on selenium-dependent or independent arsenic excretion, and ultimately carcinogenic risk, is worthy of further investigation.

3.6 Bibliography

1. Cui X, Kobayashi Y, Akashi M, Okayasu R: **Metabolism and the paradoxical effects of arsenic: carcinogenesis and anticancer**. *Curr Med Chem* 2008, **15**(22):2293-2304.
2. Bergstedt E: **[Bangladesh has to choose between arsenic or cholera. A campaign for good drinking water may have caused a national disaster]**. *Lakartidningen* 1999, **96**(47):5266-5267.
3. Wang S, Mulligan CN: **Occurrence of arsenic contamination in Canada: sources, behavior and distribution**. *Sci Total Environ* 2006, **366**(2-3):701-721.
4. Zeng H: **Selenite and selenomethionine promote HL-60 cell cycle progression**. *J Nutr* 2002, **132**(4):674-679.
5. Moxon AL: **The Effect of Arsenic on the Toxicity of Seleniferous Grains**. *Science* 1938, **88**(2273):81.
6. Levander OA, Baumann CA: **Selenium metabolism. VI. Effect of arsenic on the excretion of selenium in the bile**. *Toxicol Appl Pharmacol* 1966, **9**(1):106-115.
7. Manley SA, George GN, Pickering IJ, Glass RS, Prenner EJ, Yamdagni R, Wu Q, Gailer J: **The seleno bis(S-glutathionyl) arsinium ion is assembled in erythrocyte lysate**. *Chem Res Toxicol* 2006, **19**(4):601-607.
8. Gailer J: **Chronic toxicity of As(III) in mammals: the role of (GS)(2)AsSe(-)**. *Biochimie* 2009, **91**(10):1268-1272.

9. Zeng H, Uthus EO, Combs GF, Jr.: **Mechanistic aspects of the interaction between selenium and arsenic**. *J Inorg Biochem* 2005, **99**(6):1269-1274.

10. Gailer J, Madden S, Burke MF, Denton MB, Aposhian HV: **Simultaneous multielement-specific detection of a novel glutathione-arsenic-selenium ion [(GS)(2)AsSe](-) by ICP AES after micellar size-exclusion chromatography**. *Appl Organomet Chem* 2000, **14**(7):355-363.

11. Deeley RG, Westlake C, Cole SP: **Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins**. *Physiol Rev* 2006, **86**(3):849-899.

12. Leslie EM, Deeley RG, Cole SP: **Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense**. *Toxicol Appl Pharmacol* 2005, **204**(3):216-237.

13. Dietrich CG, Ottenhoff R, de Waart DR, Oude Elferink RP: **Role of MRP2 and GSH in intrahepatic cycling of toxins**. *Toxicology* 2001, **167**(1):73-81.

14. Loe DW, Deeley RG, Cole SP: **Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione**. *Cancer Res* 1998, **58**(22):5130-5136.

15. Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW: **The MRP2/cMOAT transporter and arsenic-glutathione**

complex formation are required for biliary excretion of arsenic. *J Biol Chem* 2000, **275**(43):33404-33408.

16. Renes J, de Vries EG, Nienhuis EF, Jansen PL, Muller M: **ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1**. *Br J Pharmacol* 1999, **126**(3):681-688.

17. Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, Cole SP: **Transport of the beta -O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog**. *J Biol Chem* 2001, **276**(30):27846-27854.

18. Peklak-Scott C, Townsend AJ, Morrow CS: **Dynamics of glutathione conjugation and conjugate efflux in detoxification of the carcinogen, 4-nitroquinoline 1-oxide: contributions of glutathione, glutathione S-transferase, and MRP1**. *Biochemistry* 2005, **44**(11):4426-4433.

19. Ballatori N, Hammond CL, Cunningham JB, Krance SM, Marchan R: **Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins**. *Toxicol Appl Pharmacol* 2005, **204**(3):238-255.

20. Cole SP, Deeley RG: **Transport of glutathione and glutathione conjugates by MRP1**. *Trends Pharmacol Sci* 2006, **27**(8):438-446.

21. Qian YM, Song WC, Cui H, Cole SP, Deeley RG: **Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1**. *J Biol Chem* 2001, **276**(9):6404-6411.

22. Zelcer N, Reid G, Wielinga P, Kuil A, van der Heijden I, Schuetz JD, Borst P: **Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4)**. *Biochem J* 2003, **371**(Pt 2):361-367.

23. Leslie EM, Bowers RJ, Deeley RG, Cole SP: **Structural requirements for functional interaction of glutathione tripeptide analogs with the human multidrug resistance protein 1 (MRP1)**. *J Pharmacol Exp Ther* 2003, **304**(2):643-653.

24. Nies AT, Keppler D: **The apical conjugate efflux pump ABCC2 (MRP2)**. *Pflugers Arch* 2007, **453**(5):643-659.

25. Leslie EM, Haimeur A, Waalkes MP: **Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required**. *J Biol Chem* 2004, **279**(31):32700-32708.

26. Ito K, Oleschuk CJ, Westlake C, Vasa MZ, Deeley RG, Cole SP: **Mutation of Trp1254 in the multispecific organic anion transporter, multidrug resistance protein 2 (MRP2) (ABCC2), alters substrate specificity and results in loss of methotrexate transport activity**. *J Biol Chem* 2001, **276**(41):38108-38114.

27. Chen CA, Okayama H: **Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA**. *Biotechniques* 1988, **6**(7):632-638.

28. Steck TL, Weinstein RS, Straus JH, Wallach DF: **Inside-out red cell membrane vesicles: preparation and purification**. *Science* 1970, **168**(3928):255-257.

29. Reay PF, Asher CJ: **Preparation and purification of 74As-labeled arsenate and arsenite for use in biological experiments**. *Anal Biochem* 1977, **78**(2):557-560.

30. Styblo M, Thomas DJ: **In vitro inhibition of glutathione reductase by arsenotriglutathione**. *Biochem Pharmacol* 1995, **49**(7):971-977.

31. Delnomdedieu M, Basti MM, Otvos JD, Thomas DJ: **Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study**. *Chem Biol Interact* 1994, **90**(2):139-155.

32. Loe DW, Almquist KC, Deeley RG, Cole SP: **Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport**. *J Biol Chem* 1996, **271**(16):9675-9682.

33. Kock K, Grube M, Jedlitschky G, Oevermann L, Siegmund W, Ritter CA, Kroemer HK: **Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for**

physiology and pharmacotherapy. *Clin Pharmacokinet* 2007, **46**(6):449-470.

34. Hipfner DR, Mao Q, Qiu W, Leslie EM, Gao M, Deeley RG, Cole SP: **Monoclonal antibodies that inhibit the transport function of the 190-kDa multidrug resistance protein, MRP. Localization of their epitopes to the nucleotide-binding domains of the protein**. *J Biol Chem* 1999, **274**(22):15420-15426.

35. Hipfner DR, Almquist KC, Stride BD, Deeley RG, Cole SP: **Location of a protease-hypersensitive region in the multidrug resistance protein (MRP) by mapping of the epitope of MRP-specific monoclonal antibody QCRL-1**. *Cancer Res* 1996, **56**(14):3307-3314.

36. Gailer J, George GN, Pickering IJ, Prince RC, Younis HS, Winzerling JJ: **Biliary excretion of [(GS)(2)AsSe](-) after intravenous injection of rabbits with arsenite and selenate**. *Chem Res Toxicol* 2002, **15**(11):1466-1471.

37. Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, Keppler D: **ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2**. *Biochem J* 1997, **327 (Pt 1)**:305-310.

38. Suzuki KT, Tomita T, Ogra Y, Ohmichi M: **Glutathione-conjugated arsenics in the potential hepato-enteric circulation in rats**. *Chem Res Toxicol* 2001, **14**(12):1604-1611.

39. Kala SV, Kala G, Prater CI, Sartorelli AC, Lieberman MW: **Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione**. *Chem Res Toxicol* 2004, **17**(2):243-249.

40. Ishizaki M, Ueno S, Okazaki T, Suzuki T, Oyamada N: **Interaction of arsenic and selenium on the metabolism of these elements in hamsters**. *Applied Organometallic Chemistry* 1988, **2**:323-331.

41. Yoshino Y, Yuan B, Miyashita SI, Iriyama N, Horikoshi A, Shikino O, Toyoda H, Kaise T: **Speciation of arsenic trioxide metabolites in blood cells and plasma of a patient with acute promyelocytic leukemia**. *Anal Bioanal Chem* 2008.

42. Qazi SS, Osoria Perez A, Sam M, Leslie EM: **Glutathione transferase P1 interacts strongly with the inner leaflet of the plasma membrane**. *Drug Metab Dispos* 2011, **39**(7):1122-1126.

43. Gregus Z, Gyurasics A, Koszorus L: **Interactions between selenium and group Va-metalloids (arsenic, antimony and bismuth) in the biliary excretion**. *Environmental Toxicology and Pharmacology* 1998, **5**:89-99.

44. Palmer IS, Bonhorst CW: **Modification of Selenite Metabolism by Arsenite**. *Agricultural and food chemistry* 1957, **5**(12):928-930.

45. Burns FJ, Rossman T, Vega K, Uddin A, Vogt S, Lai B, Reeder RJ: **Mechanism of selenium-induced inhibition of arsenic-enhanced UVR carcinogenesis in mice**. *Environ Health Perspect* 2008, **116**(6):703-708.

46. Chen Y, Hall M, Graziano JH, Slavkovich V, van Geen A, Parvez F, Ahsan H: **A prospective study of blood selenium levels and the risk of**

arsenic-related premalignant skin lesions. *Cancer Epidemiol Biomarkers Prev* 2007, **16**(2):207-213.

47. Hernandez A, Marcos R: **Genetic variations associated with interindividual sensitivity in the response to arsenic exposure**. *Pharmacogenomics* 2008, **9**(8):1113-1132.

48. Rosen BP, Liu Z: **Transport pathways for arsenic and selenium: a minireview**. *Environ Int* 2009, **35**(3):512-515.

Chapter 4

Monomethylarsenic Diglutathione [$MMA(GS)_2$] transport by the human multidrug resistance protein 1 (MRP1/*ABCC1*)

*A version of this chapter has been published

Carew M.W., Naranmandura H., Shukalek C.B., Le X.C., Leslie E.M. Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/*ABCC1*). Drug Metabolism and Disposition. 2011, 39(12):2298-304.

Contributions: The work presented here utilized an ICP-MS method that was established by Hua Naranmandura. Shukalek C.B. performed the $MMA(GS)_2$ inhibition of $E_2$17βG experiments (Figure 4.5B).

4 Monomethylarsenic Diglutathione [$MMA(GS)_2$] transport by the human multidrug resistance protein 1 (MRP1/*ABCC1*)

4.1 Abstract

The ATP-binding cassette (ABC) transporter protein MRP1 (*ABCC1*) plays an important role in the cellular efflux of the high priority environmental carcinogen arsenic, as a triglutathione conjugate [$As(GS)_3$]. Most mammalian cells can methylate arsenic to monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^{V}), dimethylarsinous acid (DMA^{III}) and dimethylarsinic acid (DMA^{V}). The trivalent forms MMA^{III} and DMA^{III} are more reactive and toxic than their inorganic precursors, arsenite (As^{III}) and arsenate (As^{V}). The ability of MRP1 to transport methylated arsenicals is unknown and was the focus of the current study. HeLa cells expressing MRP1 (HeLa-MRP1) conferred a 2.6-fold higher level of resistance than empty vector control (HeLa-vector) cells to MMA^{III}, and this resistance was dependent on glutathione (GSH). In contrast, MRP1 did not confer resistance to DMA^{III}, MMA^{V} or DMA^{V}. HeLa-MRP1 cells accumulated 4.5-fold less MMA^{III} than HeLa-vector cells. Experiments using MRP1-enriched membrane vesicles showed that transport of MMA^{III} was GSH-dependent, but not supported by the non-reducing GSH analogue, ophthalmic acid, suggesting that $MMA(GS)_2$ was the transported form. $MMA(GS)_2$ was a high affinity high capacity substrate for MRP1 with apparent K_m and V_{max} values of 11 μM and 11 nmol $mg^{-1}min^{-1}$, respectively. $MMA(GS)_2$ transport was osmotically sensitive and inhibited by several MRP1 substrates including 17β-estradiol 17-(β-D-

glucuronide) ($E_2$17βG). MMA$(GS)_2$ competitively inhibited the transport of $E_2$17βG with a K_i value of 16 μM, indicating that these two substrates have overlapping binding sites. These results suggest that MRP1 is an important cellular protective pathway for the highly toxic MMA^{III} and have implications for environmental and clinical exposure to arsenic.

4.2 Introduction

Arsenic (As) is a multi-target human carcinogen: chronic exposure is associated with increased incidences of skin, lung, and bladder tumours [1]. Greater than 100 million people worldwide are chronically exposed to unacceptable levels of nonanthropogenic sources of As [both arsenate (As^V) and arsenite (As^{III})] in their drinking water, the most common exposure route for adverse health effects [2]. Arsenic compounds are also used in chemotherapy. Arsenic trioxide (As_2O_3) has been approved for treating both newly diagnosed and relapsed acute promyelocytic leukemia, with high remission rates [3, 4]. Furthermore, As_2O_3 and another arsenical, dimethylarsenic glutathione [DMA(GS)] are in clinical trials for the treatment of multiple haematological and solid tumours [3].

Arsenic undergoes extensive methylation in humans and most other mammals [5]. Originally it was thought that As methylation was a detoxification process because it increases the rate of whole body clearance of arsenic, and the methylated As^V species monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) are less toxic than As^V and As^{III} [5]. However, *in vitro* studies have revealed that the trivalent methylated species MMA^{III} and DMA^{III} are substantially more potent toxicants than As^{III}, resulting in methylation being

considered an activation pathway [6].

The multidrug resistance protein 1 (MRP1/*ABCC1*) is an ATP-binding cassette transporter protein that was originally identified based on its ability to confer multidrug resistance in tumour cell lines through an ATP-dependent decrease in cellular drug accumulation [7]. MRP1 expression is elevated in a variety of hematological and solid tumours, and it has an important function in clinical anti-cancer drug resistance [8]. In addition, MRP1 is expressed in non-malignant tissues and transports a chemically diverse array of endogenous molecules including reduced and oxidized glutathione (GSH and GSSG, respectively), conjugated organic anions such as the cholestatic steroid 17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG) and the cysteinyl leukotriene LTC_4 [9, 10]. Several substrates of MRP1, including the natural product drugs to which it confers resistance, are not conjugated to any extent *in vivo*, but their transport is stimulated by GSH [11]. Evidence suggests that at least some of these substrates are co-exported with GSH across the plasma membrane.

In addition to anti-cancer agents and physiological substrates, MRP1 transports many other drugs, carcinogens and toxicants and is believed to play a protective role by preventing xenobiotic accumulation and resulting toxicity [12]. MRP1 is well known to play an important role in conferring cellular protection against inorganic arsenicals [13], and transports As^{III} in its triglutathione conjugate form $As(GS)_3$ [14]. MRP1 is expressed in most tissues throughout the body; however, its levels are almost undetectable in the healthy human hepatocytes [8]. MRP1 localizes to the basolateral surface of epithelia and the

apical surface of brain capillaries, generally resulting in the efflux of MRP1 substrates into the blood. MRP1 is therefore unlikely to be directly involved in the elimination of arsenic from the body. However, MRP1 is potentially important to prevent the accumulation of arsenicals in certain cell types and tissues. The expression of MRP1 in tumours could also have negative consequences by conferring resistance to arsenic-based chemotherapeutics.

The purpose of the current study was to identify methylated arsenicals that are substrates for MRP1 and characterize the mechanism by which they are transported. MRP1 was found to confer cellular protection against MMA^{III} in a GSH-dependent manner and also reduced MMA^{III} cellular accumulation. Direct transport studies using MRP1-enriched membrane vesicles revealed that MMA^{III} was a substrate for MRP1, but only in the presence of GSH or as $MMA(GS)_2$. Transport of MMA^{III} was not supported by ophthalmic acid, a GSH analog lacking a free thiol group, suggesting that the GSH conjugate is the transported form. The transport of $MMA(GS)_2$ was then extensively characterized.

4.3 Methods

4.3.1 *Chemicals and Reagents.*

GSH, GSSG, ATP, AMP, sucrose, Tris base, As^{III}, As^{V}, MMA^{V}, DMA^{V}, L-buthionine sulfoximine (BSO) and $MgCl_2$ were purchased from Sigma-Aldrich (Oakville, Ontario). Carrier free [6,7-^{3}H]$E_2$17βG (50.1 Ci/mmol) was purchased from Perkin Elmer (Woodbridge, Ontario). Creatine kinase, glutathione reductase, creatine phosphate, NADPH, and protease inhibitor cocktail tablets (Complete™, mini EDTA free) were purchased from Roche Applied Science

(Torrance, CA). Nitric acid was purchased from Fisher Scientific (Ottawa, Ontario). $E_2$17βG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ophthalmic acid was purchased from Bachem (Torrance, CA). MMA^{III} and DMA^{III} in the form of diiodomethylarsine (CH_3AsI_2) and iododimethylarsine ($[CH_3]_2AsI$) were gifts from Dr. William Cullen (University of British Columbia).

4.3.2 *Cell lines.*

The HeLa-vector and HeLa-MRP1 cell lines were gifts from Dr. Susan P.C. Cole (Queen's University, Kingston, ON, Canada) and generated and maintained as described previously [15]. The HeLa-MRP1 stable cell population was checked routinely for MRP1 expression using immunostaining followed by confocal microscopy or fluorescence-activated cell sorting, as described previously [15]. The HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 7.5% fetal bovine serum.

4.3.3 *Cytotoxicity testing.*

HeLa-MRP1 and HeLa-vector cells were seeded in 96-well plates at 1×10^4cells/well and grown for 24 h. In quadruplicate, cells were then treated with As^{III} (0.1–100 μM), MMA^{III} (0.1–100 μM), DMA^{III} (0.1–100 μM), As^{V} (0.001–10 mM), MMA^{V} (0.3–100 mM), or DMA^{V} (0.01–100 mM) for 72 h. To measure the influence of GSH depletion on As^{III} and MMA^{III} cytotoxicity, cells were treated with the γ-glutamylcysteine synthetase inhibitor BSO (100 μM) at the time of seeding and when the arsenicals were introduced. Reduction in cellular GSH levels by BSO was confirmed using the method described previously [16]. HeLa-

vector and HeLa-MRP1 cells had cellular GSH levels of 1.8 and 1.1 ng/μg protein, respectively, before GSH depletion and 0.1 and 0.03 ng/μg, respectively, after BSO treatment. Cell viability was determined using the CellTiter96 AQueous NonRadioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Data were analyzed using the sigmoidal dose-response equation in GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA), and EC_{50} values were determined. Relative resistance values were calculated as the ratio of the HeLa-MRP1 EC_{50}/HeLa-vector EC_{50}.

4.3.4 *Cellular accumulation of arsenic species.*

HeLa-vector and HeLa-MRP1 cells were seeded in six-well plates at 5×10^5cells/well and grown for 24 h. Cells were treated with 1 μM As^{III}, MMA^{III}, or DMA^{III} in culture media for 24 h. Cells were then washed three times with ice-cold phosphate-buffered saline, trypsinized, pelleted by centrifugation at 1000*g* for 10 min at 4°C, and then digested with 250 μl of concentrated nitric acid for >48 h. Digested cells were diluted 1:1 with deionized distilled water and filtered through 0.45-μm syringe filters (Whatman, Toronto, ON, Canada). The total concentration of arsenic in samples was determined using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500; Yokogawa Analytical Systems, Hachiouji, Japan) using the standard addition method as described previously [17]. Samples were introduced directly into the nebulizer of the ICP-MS.

4.3.5 *Transient expression of MRP1 in HEK293 cells.*

HEK293 cells were transfected with pcDNA3.1(−)MRP1 (a gift from Dr. Susan P.C. Cole, constructed as described previously [15]) using the calcium phosphate method as described previously [18]. In brief, 3×10^6 cells were seeded onto 150-mm plates, and 24 h later, 18 μg of DNA was mixed with calcium chloride (250 mM, final volume 1.3 ml) and added dropwise to 1.3 ml of HEPES buffer (275 mM NaCl, 1.5 mM Na_2HPO_4, 55 mM HEPES, pH 7.0). The DNA solution was then added dropwise to cells and incubated for 24 h followed by a media change. Seventy-two hours after transfection, cells were washed twice with Tris (50 mM, pH 7.4) sucrose (250 mM) buffer, scraped into 10 ml of Tris sucrose buffer per plate and collected by centrifugation at 800*g* for 10 min. Cell pellets were stored at −80°C until membrane vesicles were prepared.

4.3.6 *Membrane vesicle preparation.*

Plasma membrane-enriched vesicles were prepared from MRP1 and empty pcDNA3.1(−)-transfected HEK293 cells, according to previously described methods [18]. In brief, cells were thawed and resuspended in buffer containing 250 mM sucrose, 50 mM Tris, pH 7.4, 0.25 mM $CaCl_2$, and protease inhibitor tablets. Cells were then disrupted by nitrogen cavitation (pressurized to 200 psi and released to atmospheric pressure), and EDTA (1 mM) was added. The disrupted cells were centrifuged at 800*g* at 4°C for 10 min to remove unbroken cells and nuclei. The supernatant was layered onto 12 ml of 35% (w/w) sucrose, 50 mM Tris, pH 7.4, and centrifuged at 100,000 *g* for 1 h at 4°C. The interphase containing the plasma membrane-enriched fraction was removed and diluted with

25 mM sucrose, 50 mM Tris, pH 7.4, and centrifuged at 100,000 *g* for 30 min at 4°C. The membranes were washed with Tris sucrose buffer and centrifuged at 100,000*g* for 30 min at 4°C. The membranes were then resuspended in Tris sucrose buffer by passing through a 27-gauge needle approximately 20-times, then aliquoted and frozen at −80°C. Expression of MRP1 in membrane vesicles was confirmed by immunoblot analysis, using the rat monoclonal antibody, MRPr1 (1:10,000) (Novus Biologicals, Littleton, CO).

4.3.7 *MMA^{III} and $MMA(GS)_2$ transport assays.*

$MMA(GS)_2$ was synthesized from MMA^{III} and GSH according to previously described methods for $As(GS)_3$ [14], with modifications. In brief, MMA^{III} (final concentration of 50 μM) was combined with GSH (final concentration of 75 mM) in degassed Tris sucrose buffer and incubated under a nitrogen atmosphere for >30 min at 4°C. MRP1-enriched and vector control membrane vesicles were incubated at 37°C in Tris sucrose buffer, with MMA^{III} or $MMA(GS)_2$ (1 μM), ATP or AMP (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), GSH reductase (5 μg/ml), and NADPH (0.35 mM). To increase the stability of $MMA(GS)_2$, physiological concentrations of GSH (3 mM) were maintained in all $MMA(GS)_2$-containing transport reactions. At 3 min, transport was stopped by diluting the transport reaction in 800 μl of ice-cold Tris sucrose buffer and vesicles pelleted by centrifugation at 100,000*g* for 20 min. Pelleted membrane vesicles were washed twice with 1 ml of Tris sucrose buffer, digested in 250 μl of concentrated nitric acid for 48 h, diluted 1:1 with deionized distilled water, and filtered through 0.45-μm syringe filters (Whatman). The total concentration of

arsenic in samples was determined using ICP-MS, as described for the cellular accumulation assay.

The influence of GSH (1, 3, or 5 mM) and the GSH analog ophthalmic acid (3 mM) on the transport of MMA^{III} was measured at a 3-min time point. Kinetic parameters of transport were determined by measuring the initial rate of uptake (1-min time point) at eight different concentrations of $MMA(GS)_2$ (0.1–10 μM). The modulation of $MMA(GS)_2$ transport by the MRP1 substrates $E_2$17βG (25 μM), GSSG (500 μM), and VCR (100 μM) was measured at a 1-min time point.

4.3.8 *$E_2$17βG transport inhibition assays.*

Inhibition of $E_2$17βG transport by $MMA(GS)_2$ was performed essentially as described previously [9]. In brief, 5 μg of membrane vesicles were incubated with [^{3}H]$E_2$17βG (0.1–30 μM, 40–80 nCi), AMP or ATP (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 μg/ml), GSH (3 mM), GSH reductase (5 μg/ml), and NADPH (0.35 mM) at 37°C for 60 s in the absence or presence of $MMA(GS)_2$ (25, 40, or 50 μM). The transport reaction was stopped by diluting with 800 μl of ice-cold Tris sucrose buffer and then filtered through glass fiber filters (type GF/B) using a 96-well plate cell harvester (PerkinElmer Life and Analytical Sciences) and washed five times with Tris sucrose buffer, and radioactivity was quantified by liquid scintillation counting using a Microbeta^2 (PerkinElmer Life and Analytical Sciences). Data were plotted using GraphPad Prism 5 Software, and apparent K_m and V_{max} values were determined using Michaelis-Menten analysis.

4.4 Results

4.4.1 *MRP1 decreases the cytotoxicity of MMA^{III}*

To determine if MRP1 is capable of conferring resistance to inorganic and methylated species of arsenic, HeLa-MRP1 and HeLa-vector cell lines were treated with increasing concentrations of As^{III}, MMA^{III}, DMA^{III}, As^{V}, MMA^{V}, and DMA^{V}. The EC_{50} value for each arsenical was determined and the relative resistance was calculated from the ratio of the EC_{50} values for HeLa-MRP1 and HeLa-vector (summarized in Table 4.1). Consistent with previously published work, HeLa-MRP1 cells conferred resistance to As^{III} and As^{V}(Fig. 1A and Table 4.1) [19]. HeLa-MRP1 cells were approximately 3-fold more resistant to MMA^{III} compared to the vector control cell line (Figure 4.1B), however, no difference in the sensitivity of the cell lines was observed for MMA^{V}, DMA^{III}, or DMA^{V} (Table 4.1).

In order to determine if resistance conferred by MRP1 to As^{III} and MMA^{III} was dependent upon GSH, cytotoxicity was tested after depleting the GSH levels of HeLa-vector and HeLa-MRP1 cells using BSO (100 μM). BSO alone had no effect on cell viability. High intracellular GSH levels confer resistance to arsenicals partly through the neutralization of reactive oxygen species [20]. Consistent with this, both arsenicals became significantly more toxic to HeLa-vector and HeLa-MRP1 cell lines after GSH depletion (Tables 4.1 and 4.2, Figure 4.1). Furthermore, GSH depletion resulted in the loss of MRP1-dependent cellular protection to both As^{III} and MMA^{III} (Figure 4.2 and Table 4.2).

4.4.2 *MRP1 reduces the accumulation of As^{III} and MMA^{III}*

In order to assess the effect of MRP1 on the cellular accumulation of As^{III}, MMA^{III}, and DMA^{III}, the accumulation of total arsenic was determined after HeLa-vector and HeLa-MRP1 exposure to As^{III}, MMA^{III} or DMA^{III} (1 μM for 24 h). Consistent with the cytotoxicity data, HeLa-MRP1 cells accumulated 3.5 and 4.5-fold less As^{III} and MMA^{III}, respectively, than HeLa-vector cells (Figure 4.2). No significant difference in DMA^{III} accumulation between the HeLa-MRP1 and HeLa-vector cell lines was observed (Figure 4.2).

Table 4.1 Relative resistance of HeLa-MRP1 compared to vector control

	EC_{50} (μM) ± SEM		
Arsenic Species	HeLa-Vector	HeLa-MRP1	Relative Resistance[a]
As^{III} *(n=11)*	7.0 ± 0.7	22.0 ± 5.0	3.1*
As^{V} *(n=5)*	510 ± 70	1000 ± 220	2*
MMA^{III} *(n=6)*	3.1 ± 0.4	8.2 ± 1.8	2.6*
MMA^{V} *(n=5)*	9800 ± 780	9400 ± 1000	1.0
DMA^{III} *(n=3)*	6.0 ± 0.3	7.0 ± 1.3	1.2
DMA^{V} *(n=5)*	740 ± 50	710 ± 180	1

[a]Ratio of EC_{50}HeLa-MRP1/EC_{50}HeLa-Vector

* $p < 0.05$ (student's t-test)

Table 4.2 Relative resistance of HeLa-MRP1 compared to vector control after GSH depletion

	EC_{50} (μM) ± SEM		
Arsenic Species	HeLa-Vector	HeLa-MRP1	Relative Resistance[a]
As^{III} *(n=5)*	0.37 ± 0.28	0.11 ± 0.01	0.3
MMA^{III} *(n=6)*	0.019 ± 0.004	0.01 ± 0.003	0.5

[a]Ratio of EC_{50}HeLa-MRP1/EC_{50}HeLa-Vector

* $p < 0.05$ (student's t-test)

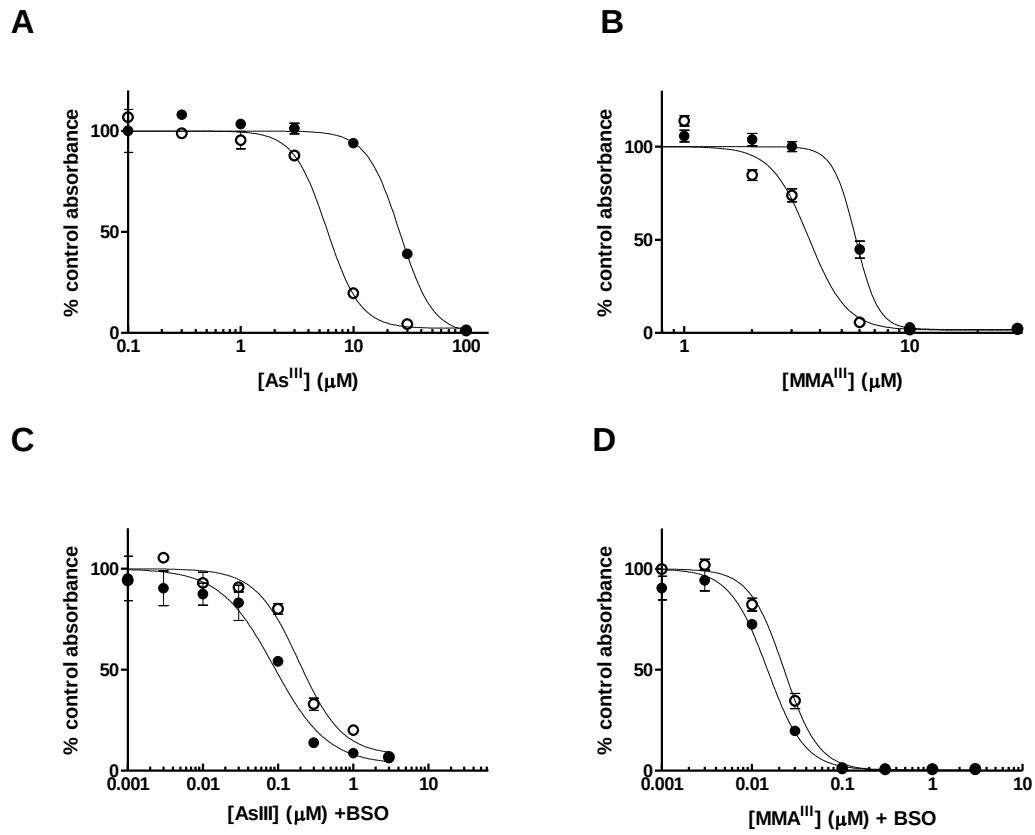

Fig 4.1. Effect of As^{III}, MMA^{III} and DMA^{III} on the viability of transfected HeLa cells. Vector control transfected HeLa cells (○) and MRP1 transfected HeLa cells (●) were incubated in the presence of (A) As^{III} and (B) MMA^{III}. (C) Cells were preincubated for 24 hours in the presence of 100 μM BSO, before the addition of As^{III} and BSO and (D) MMA^{III} + BSO for 72 h then absorbance proportional to cell viability was determined using a tetrazolium-based assay. Data points are means (± S.E.) of quadruplicate determinations in a representative experiment; similar results were obtained in at least three additional experiments.

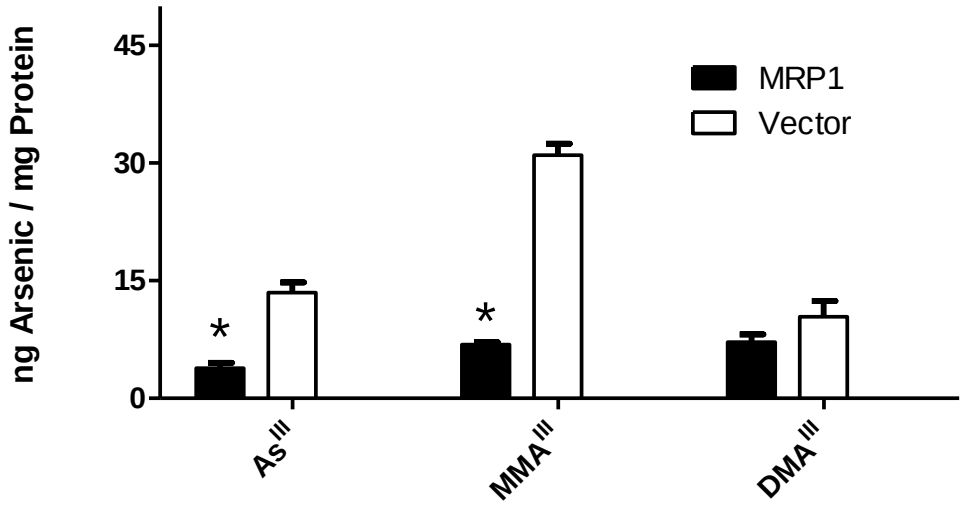

Fig 4.2. Accumulation of As^{III}, MMA^{III}, and DMA^{III} by transfected HeLa cells. Vector control transfected HeLa cells (open bars) and MRP1 transfected HeLa cells (closed bars) were treated with As^{III}, MMA^{III}, or DMA^{III} (1 μM) for 24 h. Cells were then harvested, digested with nitric acid and total arsenic determined using ICP-MS. Bars represent the means of 3 independent experiments (± S.E.). *P<0.001

4.4.3 *MRP1 transports MMA^{III} in the presence of GSH or as the presynthesized GSH conjugate $MMA(GS)_2$.*

To determine if MMA^{III} and/or $MMA(GS)_2$ were substrates of MRP1, ATP-dependent transport of these compounds into MRP1-enriched and vector control membrane vesicles was measured (Figure 4.3A). ATP-dependent transport of MMA^{III} (1 μM) by the MRP1-enriched membrane vesicles was extremely low and similar to transport observed in the presence of AMP or with the vector control membrane vesicles. However, in the presence of GSH (3 mM), ATP-dependent transport of MMA^{III} was observed with an activity of 308 pmol mg^{-1} min^{-1}. In previous studies ophthalmic acid and other GSH analogs lacking a free thiol group can substitute for GSH and supported the transport of several GSH-dependent MRP1 substrates [21-25]. These findings indicate that the thiol group of GSH is not required for transport of these substrates and rules out the possibility that formation of a GSH conjugate is critical for transport to occur. However, ATP-dependent transport of MMA^{III} in the presence of ophthalmic acid (3 mM) was extremely low and similar to minus GSH conditions or empty vector control (Figure 4.3A). ATP-dependent transport of $MMA(GS)_2$ was then measured and found to have an activity of 240 pmol mg^{-1} min^{-1}. These data suggest that the free thiol group of GSH is required for MMA^{III} transport by MRP1, and are consistent with $MMA(GS)_2$ being the transported chemical species.

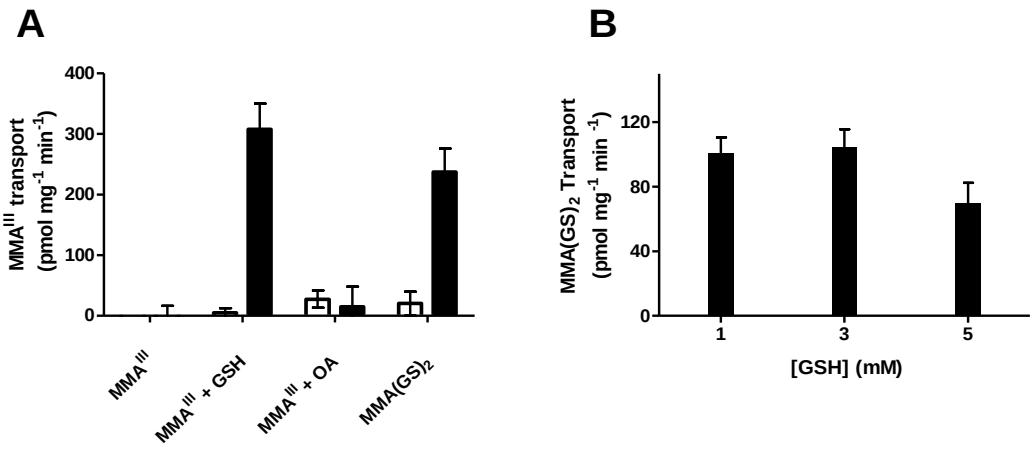

Fig 4.3. MRP1-mediated transport of $MMA(GS)_2$ or free MMA^{III} in the presence of GSH or OA. Membrane vesicles (20 µg of protein) prepared from MRP1-transfected (closed bar) or untransfected (open bar) HEK293 cells were incubated for 3 min at 37ºC in transport buffer in triplicate containing $MMA(GS)_2$ (1 µM) with 3 mM GSH or MMA^{III} (1 µM) ± GSH (3 mM) or OA (3 mM) (A), and $MMA(GS)_2$ (1 µM) with GSH (1, 3, and 5 mM) (B). Reactions were then pooled for analysis by ICP-MS. Bars represent the means of 3 independent experiments (± S.E.).

Using similar experimental conditions, transport of DMA^{III} and $DMA^{III}(GS)$ by MRP1-enriched vesicles was measured in the presence of GSH (3 mM). Consistent with the cytotoxicity and accumulation data, no MRP1-dependent transport was observed (data not shown).

$MMA(GS)_2$ is chemically unstable and is stabilized in the presence of physiological (1-10 mM) concentrations of GSH [26-28]. Thus, 3 mM GSH was included in all transport reactions containing $MMA(GS)_2$. Because GSH can stimulate the transport of certain conjugated and unconjugated substrates of MRP1 [11], the influence of different concentrations of GSH on $MMA(GS)_2$ transport was evaluated (Figure 4.3B). No significant difference in $MMA(GS)_2$ transport was observed in the presence of 1, 3 or 5 mM GSH, concentrations that have been shown previously to stimulate the transport of GSH-dependent substrates in a dose dependent manner [22]. These data suggest that GSH is not stimulating the transport of $MMA(GS)_2$ by MRP1.

4.4.4 *Osmotic sensitivity of $MMA(GS)_2$ transport.*

To assess whether the ATP-dependent $MMA(GS)_2$ transport by the MRP1-enriched membrane vesicles truly represents transport into the vesicle lumen rather than surface or intramembrane binding, the effect of changes in osmolarity on vesicular uptake was examined. $MMA(GS)_2$ (1 μM) uptake was decreased as the concentration of sucrose in the transport buffer increased, indicating that the ATP-dependent $MMA(GS)_2$ uptake by the vesicles is osmotically sensitive, as expected for a true transport process (Figure 4.4A).

4.4.5 *Kinetic analysis of MRP1-mediated $MMA(GS)_2$ transport.*

Time courses of $MMA(GS)_2$ (1 μM) transport by MRP1-enriched membrane vesicles were completed to determine the linear range of uptake. Transport was linear for up to 3 min with a maximum activity of ~900 pmol mg^{-1} at 10 min. (Figure 4.4B). MRP1-mediated transport was further characterized by determining the initial rates of transport over several concentrations of $MMA(GS)_2$ (Figure 4.4B). According to Michaelis-Menten kinetic analysis (Graphpad Prism™ 5), the average apparent K_m and V_{max} values (± S.E., $n=3$) for $MMA(GS)_2$ were 11 ± 2.3 μM and 11 ± 0.6 nmol mg^{-1} min^{-1}, respectively.

4.4.6 *Inhibition of $MMA(GS)_2$ transport by various substrates of MRP1.*

Several MRP1 substrates, including $E_2$17βG, GSSG, and VCR (+ GSH) are competitive inhibitors of MRP1 transport [10, 22, 29]. Transport of $MMA(GS)_2$ (1 μM) was inhibited by $E_2$17βG (25 μM), GSSG (500 μM), and VCR (100 μM) by 57, 75, and 79 %, respectively (Figure 4.5A). Inhibitors were used at concentrations shown in previous studies to be 5-10-fold above their respective K_m values to ensure binding site saturation.

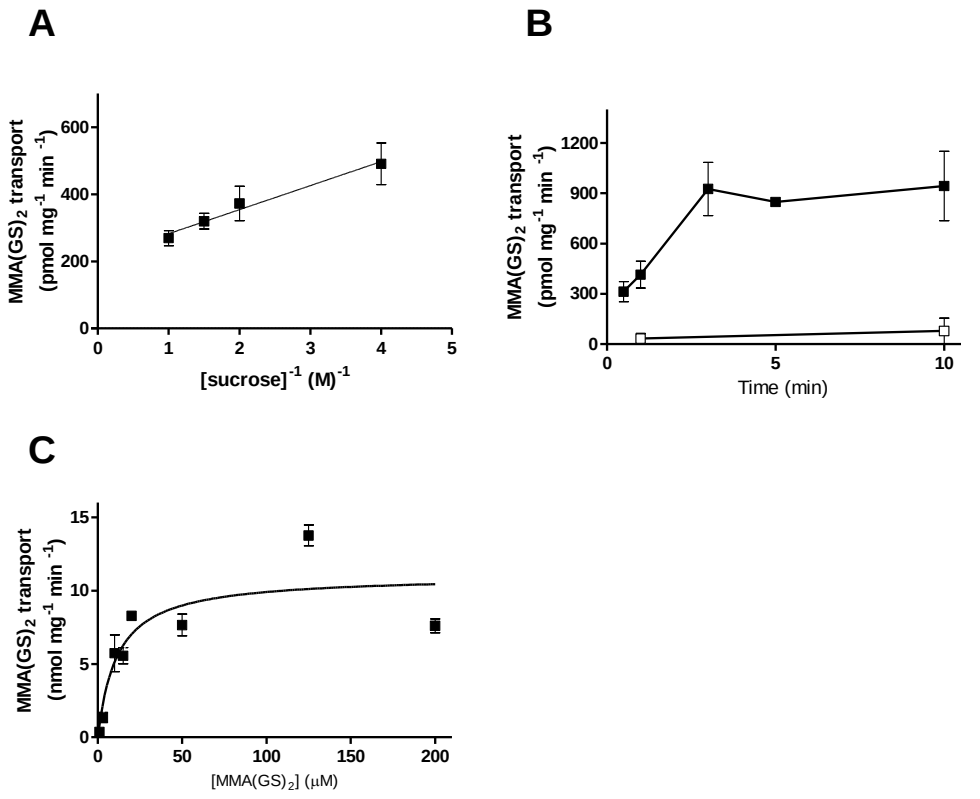

Fig 4.4. Osmotic sensitivity and kinetic analysis of MRP1-mediated ATP-dependent transport of $MMA(GS)_2$. (A) Membrane vesicles (20 µg of protein) prepared from MRP1-transfected HEK293 cells were incubated for 3 min at 37ºC with $MMA(GS)_2$ (1 µM) in transport buffer in triplicate containing increasing concentrations of sucrose (0.25, 0.5, 0.66, and 1 M). (B) Time course of ATP-dependent $MMA(GS)_2$ transport by membrane vesicles prepared from MRP1-transfected (■) and untransfected (□) HEK293 cells. Membrane vesicles (20 µg per time point) were incubated at 37ºC in transport buffer with $MMA(GS)_2$ (1 µM) in triplicate for the indicated time points, reactions were pooled for analysis by ICP-MS. (C) Membrane vesicles (20 µg of protein) prepared from MRP1-transfected HEK293 cells were incubated for 1 min at 37ºC in transport buffer in triplicate with various concentrations of $MMA(GS)_2$ (1-200 µM), reactions were pooled for analysis by ICP-MS. Kinetic parameters were determined using the Michaelis-Menten kinetic model in Graphpad Prism™ 5 software. Points represent the mean of 3 independent experiments (± S.E.).

4.4.7 *MMA(GS)$_2$ inhibition of E$_2$17βG transport by MRP1.*

In order to determine if MMA(GS)$_2$ and E$_2$17βG have overlapping binding sites on MRP1, the ability of MMA(GS)$_2$ to competitively inhibit the transport of E$_2$17βG was evaluated. The inhibition of E$_2$17βG transport by MRP1-enriched membrane vesicles was characterized by measuring the effect of MMA(GS)$_2$ (50 µM) on E$_2$17βG (0.1-30 µM) transport (Figure 4.5C). Michaelis-Menten analysis showed that MMA(GS)$_2$ behaved as a competitive inhibitor of E$_2$17βG transport with an average apparent K_i value (± S.E., *n=3*) of 16.3 ± 3.6 µM (Figure 4.5B). These data indicate that MMA(GS)$_2$ and E$_2$17βG, at minimum, have overlapping binding sites on MRP1.

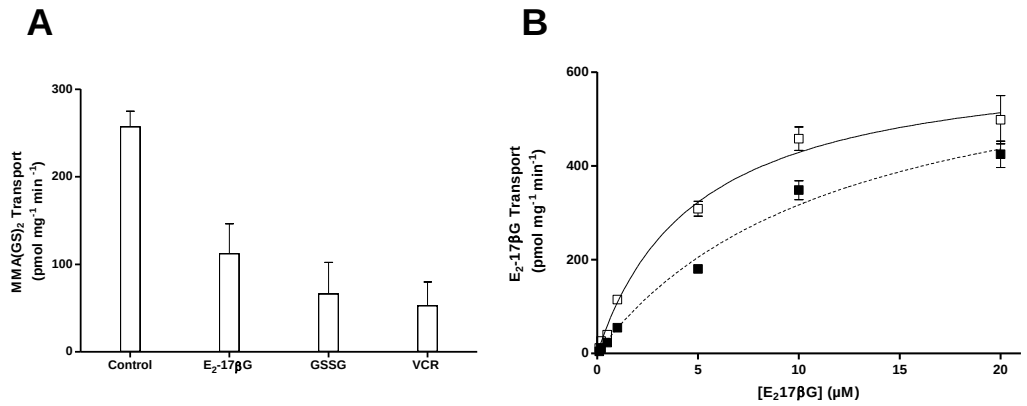

Fig 4.5. Inhibition of MRP1 mediated $MMA(GS)_2$ transport and $MMA(GS)_2$ inhibition of $E_2$17βG transport. (A) ATP dependent uptake of $MMA(GS)_2$ by membrane vesicles (20 µg of protein) prepared from MRP1-transfected HEK293 cells was measured at a concentration of 1 µM for 1 min at 37 ºC in the presence of $E_2$17βG (25 µM), GSSG (500 µM), and VCR (100 µM). Points represent the means of 3 independent experiments (± S.E.). (B) Membrane vesicles (20 µg of protein) prepared from MRP1-transfected HEK293 cells were incubated for 1 min at 37ºC in transport buffer with $E_2$17βG (0.1-30 µM) in the presence (■) or absence (□) of 50 µM $MMA(GS)_2$. Kinetic values were determined using the Michaelis-Menten kinetic model in Graphpad Prism™ 5 software. Points represent the mean of 3 independent experiments (± S.E.). Similar results were obtained in 2 additional experiments.

4.5 Discussion

The metalloid arsenic is a multi-target human carcinogen and a major concern as an environmental pollutant. The ubiquitous nature of arsenic in the environment has led to the evolution of arsenic adaptation mechanisms from bacteria to humans [30]. Members of the ABC transporter superfamily subfamily "C" have been shown to be critical for protecting many organisms from arsenic including *Saccharomyces cerevisae*, *Leishmania*, *Arabidopsis*, *Caenorhabditis elegans*, and *Danio rerio* [31-34]. In all of these organisms, ABCC proteins detoxify arsenic either by extrusion from cells or through sequestration within intracellular organelles as thiol conjugates. We have shown previously that human MRP1 can transport inorganic arsenic as the triglutathione conjugate $As(GS)_3$ [14], but the ability of MRP1 to confer cellular protection to methylated arsenicals was unknown. In the present study we have found that a HeLa cell line stably expressing MRP1 is capable of conferring a 2.6-fold higher resistance level to MMA^{III} than the HeLa cell line expressing empty vector. Consistent with previously published observations, MRP1 also conferred resistance to As^{III} and As^{V} [19]. MRP1 did not confer protection against the pentavalent methylated arsenicals MMA^{V} and DMA^{V} or the trivalent DMA^{III}, although data is presented in the Appendix (Figure 7.9) showing that MRP1 is capable of transporting DMA.

GSH depletion enhances arsenic trioxide-induced apoptosis [20, 35, 36]. Consistent with this, the toxicities of As^{III} and MMA^{III} were increased upon GSH depletion with BSO for both the HeLa-vector and HeLa-MRP1 cell lines, most dramatically for MMA^{III} (Figure 4.1 & Table 4.2). In addition, MRP1 resistance levels to As^{III} and MMA^{III} were dependent upon GSH, suggesting a GSH-

dependent efflux pathway. We have previously determined that As^{III} is transported by MRP1 as $As(GS)_3$ [14] and the next step was to investigate the species of MMA^{III} being transported. Direct transport assays using MRP1-enriched membrane vesicles confirmed that MMA^{III} transport was GSH-dependent. MRP1 transports compounds covalently attached to GSH or through a cotransport pathway [11]. Thus, transport of MMA^{III} was measured in the presence of the non-reducing GSH analog ophthalmic acid, known to stimulate the transport of GSH-dependent MRP1 substrates [21-25]. In the case of MMA^{III}, ophthalmic acid did not substitute for GSH, thus, the free sulphur group of GSH was required for transport. These data suggest that MMA^{III} conjugated to GSH is the transported form. Although GSH is best characterized to detoxify arsenicals through neutralization of reactive oxygen species [37], our data provide further support that formation and efflux of arsenic GSH conjugates is potentially of at least equal importance.

We have shown previously that the MRP1-dependent transport of As^{III} requires GSH and the glutathione transferase GSTP1 [14]. It is currently unknown if the formation of $MMA(GS)_2$ under physiological conditions requires catalysis by GSTP1 or a related transferase. In the current study, transport activities for $MMA(GS)_2$ and free MMA^{III} in the presence of GSH were very similar (Figure 4.3). Superficially these results suggest that $MMA(GS)_2$ formation is spontaneous; however, high levels of plasma membrane associated GSTP1 exist in the HEK293 vesicles used in this study [38], and although further

experimentation is required, GSTP1 could be involved in catalyzing the formation of $MMA(GS)_2$.

$MMA(GS)_2$ has been isolated from rat bile and mouse urine, and therefore is formed physiologically and excreted by the liver and kidney [39, 40]. Biliary excretion of $MMA(GS)_2$ and $As(GS)_3$ in rats is dependent on the MRP1 related multidrug resistant protein 2 (Mrp2/*Abcc2*) and these conjugates account for most of the arsenic in bile [39]. In mice deficient in γ-glutamyl transpeptidase, an enzyme responsible for GSH and GSH-conjugate catabolism, approximately 60–70% of the urinary arsenic was present as a GSH conjugate [40]. Overall, these observations suggest that arsenic-GSH conjugates are the transported forms and account for a major fraction of excreted arsenic. Although rat Mrp2 is important for biliary excretion of $MMA(GS)_2$ and $As(GS)_3$, these conjugates are unstable at biliary pH and free As^{III} and MMA^{III} undergo enterohepatic circulation, and urinary excretion is the predominant elimination pathway[39-41]. Thus, it is critical to understand how extrahepatic tissues handle arsenicals through transport proteins such as MRP1.

We have previously determined that the transport of $As(GS)_3$ by MRP1-enriched membrane vesicles prepared from the small cell lung cancer cell line H69AR, is high affinity but low capacity with an apparent K_m of 0.32 μM, V_{max} of 17 pmol mg^{-1} min^{-1}, and overall efficiency (V_{max}/K_m) of 53 μl mg^{-1} min^{-1} [14]. In the current study, transport of $MMA(GS)_2$ was high affinity and high capacity, with an apparent K_m of 11 μM, V_{max} of 11,000 pmol mg^{-1} min^{-1}, and overall efficiency (V_{max}/K_m) of 1000 μl mg^{-1} min^{-1}. Thus, the overall efficiency of

$MMA(GS)_2$ transport is remarkably higher than for $As(GS)_3$. In tissues with sufficient GSH levels, as well as methylation capacity, MRP1 is likely an important detoxification pathway for the highly toxic MMA^{III}. The apparent K_m value is of physiological relevance for clinical arsenic exposures. Pharmacokinetic studies of patients undergoing chemotherapy for acute promyelocytic leukemia with As_2O_3 have plasma levels in the low μM range and tissue concentrations would probably exceed this value [42].

Despite the twenty-fold higher efficiency of transport of $MMA(GS)_2$ than $As(GS)_3$ by MRP1 little difference existed between the resistance level and cellular accumulation of MMA^{III} and As^{III} in the intact HeLa-MRP1 cell line (Figures 4.1 and 4.2 and Table 4.1). The whole cell assay conditions involved 24 to 72 h exposure of cells to arsenicals while the membrane vesicle experiments measured transport of these conjugates for 1 to 3 min and thus, experimental results are difficult to compare. The chemical instability of the arsenic glutathione conjugates make it likely that under culture conditions, the cellular protection conferred by MRP1 to either As^{III} or MMA^{III} is underestimated compared to *in vivo*. Thus, As-GSH conjugates effluxed from the cell would rapidly dissociate in the culture media and the free arsenical taken back up into the cell where it could exert toxicity again. In the *in vivo* situation, $MMA(GS)_2$ or $As(GS)_3$ transported by MRP1 into the blood would be cleared from the tissue, preventing the reuptake of the intact conjugate or dissociated arsenical.

In contrast with a beneficial detoxification effect of MRP1, its over-expression in hematological and solid tumours could have deleterious

consequences by conferring resistance to arsenic based chemotherapeutics, in particular As_2O_3. In aqueous solution at physiological pH As_2O_3 exists as $As(OH)_3$ or As^{III} [43]. Thus, As_2O_3 can be methylated to MMA^{III} and DMA^{III} and these metabolites have been shown to be more potent inducers of apoptosis in leukemia and lymphoma cells than As^{III} [44]. MRP1 could reduce the cellular concentrations of both inorganic arsenic and MMA^{III} through the efflux of $As(GS)_3$ and $MMA(GS)_2$, and reduce treatment efficacy.

In order to prevent and treat arsenic induced toxicity and resistance to arsenic based therapeutics, it is critical to understand the cellular handling of this metalloid. In this study we have shown that MRP1 reduces the cellular toxicity and accumulation of MMA^{III} through the high capacity efflux of $MMA(GS)_2$. MRP1 could be essential for preventing toxicity after acute arsenic exposure as well as arsenic carcinogenesis during chronic exposure through the efflux $MMA(GS)_2$. MRP1 is highly polymorphic and genetic variants could account for some of the well-established, but poorly understood inter-individual susceptibility to arsenic-induced carcinogenesis [45, 46].

4.6 Bibliography

1. Straif K, Benbrahim-Tallaa L, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, Guha N, Freeman C, Galichet L, Cogliano V: **A review of human carcinogens--part C: metals, arsenic, dusts, and fibres**. *Lancet Oncol* 2009, **10**(5):453-454.
2. Rahman MM, Ng JC, Naidu R: **Chronic exposure of arsenic via drinking water and its adverse health impacts on humans**. *Environ Geochem Health* 2009, **31 Suppl 1**:189-200.
3. Emadi A, Gore SD: **Arsenic trioxide - An old drug rediscovered**. *Blood Rev* 2010, **24**(4-5):191-199.
4. Drobna Z, Naranmandura H, Kubachka KM, Edwards BC, Herbin-Davis K, Styblo M, Le XC, Creed JT, Maeda N, Hughes MF, Thomas DJ: **Disruption of the arsenic (+3 oxidation state) methyltransferase gene in the mouse alters the phenotype for methylation of arsenic and affects distribution and retention of orally administered arsenate**. *Chem Res Toxicol* 2009, **22**(10):1713-1720.
5. Drobna Z, Walton FS, Harmon AW, Thomas DJ, Styblo M: **Interspecies differences in metabolism of arsenic by cultured primary hepatocytes**. *Toxicol Appl Pharmacol* 2010, **245**(1):47-56.
6. Kumagai Y, Sumi D: **Arsenic: signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity**. *Annu Rev Pharmacol Toxicol* 2007, **47**:243-262.

7. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG: **Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line**. *Science* 1992, **258**(5088):1650-1654.

8. Deeley RG, Westlake C, Cole SP: **Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins**. *Physiol Rev* 2006, **86**(3):849-899.

9. Loe DW, Almquist KC, Cole SP, Deeley RG: **ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids**. *J Biol Chem* 1996, **271**(16):9683-9689.

10. Loe DW, Almquist KC, Deeley RG, Cole SP: **Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport**. *J Biol Chem* 1996, **271**(16):9675-9682.

11. Cole SP, Deeley RG: **Transport of glutathione and glutathione conjugates by MRP1**. *Trends Pharmacol Sci* 2006, **27**(8):438-446.

12. Leslie EM, Deeley RG, Cole SP: **Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense**. *Toxicol Appl Pharmacol* 2005, **204**(3):216-237.

13. Lorico A, Bertola A, Baum C, Fodstad O, Rappa G: **Role of the Multidrug Resistance Protein 1 in protection from heavy metal**

oxyanions: investigations in vitro and in MRP1-deficient mice. *Biochem Biophys Res Commun* 2002, **291**(3):617-622.

14. Leslie EM, Haimeur A, Waalkes MP: **Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required**. *J Biol Chem* 2004, **279**(31):32700-32708.

15. Ito K, Olsen SL, Qiu W, Deeley RG, Cole SP: **Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport**. *J Biol Chem* 2001, **276**(19):15616-15624.

16. Rahman I, Kode A, Biswas SK: **Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method**. *Nat Protoc* 2006, **1**(6):3159-3165.

17. Kalivas JH: **Evaluation of volume and matrix effects for the generalized standard addition method**. *Talanta* 1987, **34**(11):899-903.

18. Carew MW, Leslie EM: **Selenium-dependent and -independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/ABCC2): implications for the mutual detoxification of arsenic and selenium**. *Carcinogenesis* 2010, **31**(8):1450-1455.

19. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG: **Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells**. *Cancer Res* 1994, **54**(22):5902-5910.

20. Duechler M, Stanczyk M, Czyz M, Stepnik M: **Potentiation of arsenic trioxide cytotoxicity by Parthenolide and buthionine sulfoximine in murine and human leukemic cells**. *Cancer Chemother Pharmacol* 2008, **61**(5):727-737.

21. Qian YM, Song WC, Cui H, Cole SP, Deeley RG: **Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1**. *J Biol Chem* 2001, **276**(9):6404-6411.

22. Loe DW, Deeley RG, Cole SP: **Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione**. *Cancer Res* 1998, **58**(22):5130-5136.

23. Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, Cole SP: **Transport of the beta -O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog**. *J Biol Chem* 2001, **276**(30):27846-27854.

24. Leslie EM, Bowers RJ, Deeley RG, Cole SP: **Structural requirements for functional interaction of glutathione tripeptide analogs with the human multidrug resistance protein 1 (MRP1)**. *J Pharmacol Exp Ther* 2003, **304**(2):643-653.

25. Peklak-Scott C, Townsend AJ, Morrow CS: **Dynamics of glutathione conjugation and conjugate efflux in detoxification of the carcinogen,**

4-nitroquinoline 1-oxide: contributions of glutathione, glutathione S-transferase, and MRP1. *Biochemistry* 2005, **44**(11):4426-4433.

26. Yehiayan L, Pattabiraman M, Kavallieratos K, Wang X, Boise LH, Cai Y: **Speciation, formation, stability and analytical challenges of human arsenic metabolites**. *J Anal At Spectrom* 2009, **24**:1397-1405.

27. Raab A, Meharg AA, Jaspars M, Genney DR, Feldmann J: **Arsenic-glutathione complexes-their stability in solution and during separation by different HPLC modes**. *J Anal At Spectrom* 2004, **19**:183-190.

28. Kobayashi Y, Cui X, Hirano S: **Stability of arsenic metabolites, arsenic triglutathione [As(GS)3] and methylarsenic diglutathione [CH3As(GS)2], in rat bile**. *Toxicology* 2005, **211**(1-2):115-123.

29. Leier I, Jedlitschky G, Buchholz U, Center M, Cole SP, Deeley RG, Keppler D: **ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump**. *Biochem J* 1996, **314 (Pt 2)**:433-437.

30. Rosen BP, Liu Z: **Transport pathways for arsenic and selenium: a minireview**. *Environ Int* 2009, **35**(3):512-515.

31. Ghosh M, Shen J, Rosen BP: **Pathways of As(III) detoxification in Saccharomyces cerevisiae**. *Proc Natl Acad Sci U S A* 1999, **96**(9):5001-5006.

32. Dey S, Ouellette M, Lightbody J, Papadopoulou B, Rosen BP: **An ATP-dependent As(III)-glutathione transport system in membrane vesicles**

of Leishmania tarentolae. *Proc Natl Acad Sci U S A* 1996, **93**(5):2192-2197.

33. Broeks A, Gerrard B, Allikmets R, Dean M, Plasterk RH: **Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode Caenorhabditis elegans**. *EMBO J* 1996, **15**(22):6132-6143.

34. Long Y, Li Q, Cui Z: **Molecular analysis and heavy metal detoxification of ABCC1/MRP1 in zebrafish**. *Mol Biol Rep* 2011, **38**(3):1703-1711.

35. Bhalla S, Gordon LI, David K, Prachand S, Singh AT, Yang S, Winter JN, Guo D, O'Halloran T, Platanias LC, Evens AM: **Glutathione depletion enhances arsenic trioxide-induced apoptosis in lymphoma cells through mitochondrial-independent mechanisms**. *Br J Haematol* 2010, **150**(3):365-369.

36. Han YH, Kim SZ, Kim SH, Park WH: **Enhancement of arsenic trioxide-induced apoptosis in HeLa cells by diethyldithiocarbamate or buthionine sulfoximine**. *Int J Oncol* 2008, **33**(1):205-213.

37. Flora SJ: **Arsenic-induced oxidative stress and its reversibility**. *Free Radic Biol Med* 2011, **51**(2):257-281.

38. Qazi SS, Osoria Perez A, Sam M, Leslie EM: **Glutathione transferase P1 interacts strongly with the inner leaflet of the plasma membrane**. *Drug Metab Dispos* 2011, **39**(7):1122-1126.

39. Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW: **The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic**. *J Biol Chem* 2000, **275**(43):33404-33408.

40. Kala SV, Kala G, Prater CI, Sartorelli AC, Lieberman MW: **Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione**. *Chem Res Toxicol* 2004, **17**(2):243-249.

41. Yehiayan L, Pattabiraman M, Kavallieratos K, Wang X, Boise LH, Cai Y: **Speciation, formation, stability and analytical challenges of human arsenic metabolites**. *J Anal At Spectrom* 2009, **24**(10):1397-1405.

42. Yoshino Y, Yuan B, Miyashita SI, Iriyama N, Horikoshi A, Shikino O, Toyoda H, Kaise T: **Speciation of arsenic trioxide metabolites in blood cells and plasma of a patient with acute promyelocytic leukemia**. *Anal Bioanal Chem* 2009, **393**(2):689-697.

43. Liu Z, Boles E, Rosen BP: **Arsenic trioxide uptake by hexose permeases in Saccharomyces cerevisiae**. *J Biol Chem* 2004, **279**(17):17312-17318.

44. Chen GQ, Zhou L, Styblo M, Walton F, Jing Y, Weinberg R, Chen Z, Waxman S: **Methylated metabolites of arsenic trioxide are more potent than arsenic trioxide as apoptotic but not differentiation inducers in leukemia and lymphoma cells**. *Cancer Res* 2003, **63**(8):1853-1859.

45. Conseil G, Deeley RG, Cole SP: **Polymorphisms of MRP1 (ABCC1) and related ATP-dependent drug transporters**. *Pharmacogenet Genomics* 2005, **15**(8):523-533.

46. Hernandez A, Marcos R: **Genetic variations associated with interindividual sensitivity in the response to arsenic exposure**. *Pharmacogenomics* 2008, **9**(8):1113-1132.

Chapter 5

Human Multidrug Resistance Protein 4 (MRP4/*ABCC4*) Protects Cells from Arsenic by Transporting $MMA(GS)_2$ and DMA^V

Contributions: Swanlund D. generated the HEK293-MRP4-1E1-6 stable cell line. Lu X. performed the HPLC-ICP-MS analysis. Banerjee M. performed a portion of the MTS experiments.

5 Human Multidrug Resistance Protein 4 (MRP4/*ABCC4*) Protects Cells from Arsenic by Transporting $MMA(GS)_2$ and DMA^V

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5.1 Abstract

Millions of people world-wide are exposed to the environmental carcinogen arsenic (as inorganic arsenite [As^{III}] and arsenate [As^V]) in drinking water. Chronic exposure to arsenic is associated with increased incidence of skin, lung and bladder tumours. In humans, arsenic is extensively methylated to monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^V), dimethylarsinous acid (DMA^{III}) and dimethylarsinic acid (DMA^V), mainly in the liver. Arsenic elimination from the body occurs primarily through urinary excretion. The transport protein(s) responsible for arsenic efflux from the liver (into blood), ultimately for urinary elimination are unknown. MRP4 (*ABCC4*) is localized to the basolateral surface of hepatocytes and the apical surface of renal proximal tubule cells making it an ideal candidate for arsenic elimination. Determining the ability of MRP4 to transport arsenic species was the focus of the current study. Cytotoxicity assays using HEK293 cells expressing empty vector (HEK-Vector) or MRP4 (HEK-MRP4) showed that MRP4 increased the EC_{50} values for As^V, MMA^{III}, MMA^V, DMA^{III}, and DMA^V by 2.8-, 1.7-, 1.6, 2.0-, and 1.6-fold, respectively. In contrast, MRP4 did not protect cells from As^{III}.

Protection against As^{V} and MMA^{III} was GSH-dependent while resistance to MMA^{V}, DMA^{III}, and DMA^{V} was not. Consistent with the cytotoxicity data, HEK-MRP4 cells accumulated significantly less total arsenic after treatment with As^{V}, MMA^{V}, MMA^{III}, DMA^{III}, and DMA^{V} (but not As^{III}) than HEK-Vector cells. Arsenic speciation analysis of HEK293 cells treated with As^{III} or As^{V} revealed that only ~5% of arsenic remained as inorganic As, suggesting that MRP4 confers cellular protection through the export of methylated species. ATP-dependent transport of As^{III}, As^{V}, MMA^{V}, MMA^{III}, DMA^{III}, and DMA^{V} (±GSH) was measured using MRP4-enriched membrane vesicles and total arsenic quantified by inductively coupled plasma mass spectrometry. As^{III}, As^{V}, MMA^{V} and DMA^{III} were not MRP4 substrates. Transport of MMA^{III} (1 μM) was GSH dependent but not supported by the non-reducing GSH analog, ophthalmic acid, suggesting that $MMA(GS)_2$ was the transported form. DMA^{V} (1 μM) was transported by MRP4 and the addition of GSH (3 mM) had no effect. $MMA(GS)_2$ and DMA^{V} inhibited MRP4-dependent transport of $E_2$17βG. These results suggest that MRP4 could be critical for hepatic basolateral transport and ultimately urinary elimination of arsenic.

5.2 Introduction

Millions of people worldwide are exposed to arsenic (As) in drinking water at levels which exceed the World Health Organization guidelines of <10 ppb. This is a public health crisis because As is a type I (proven) human carcinogen and exposure is associated with skin, lung, and bladder tumors [1-3]. In addition to cancer, chronic exposure to As has been associated with a myriad of other adverse

health effects including peripheral vascular disease, neurological disorders, and diabetes mellitus [4].

Contamination of drinking water occurs primarily with the inorganic As species arsenate (As^{V}) and arsenite (As^{III}). As^{III} enters cells at a much faster rate than As^{V}, which may account for its higher level of toxicity [5, 6]. In solution at physiological pH, As^{V} (pKa values 2.19, 6.94, and 11.5), is predominantly in the form $HAsO_4^{-2}$ with some $H_2AsO_4^{-}$ and is chemically similar to inorganic phosphate. As^{V} enters cells through the sodium/phosphate cotransporter type IIb (SLC34A2) [7]. In solution, As^{III} (pKa 9.2) exists in the neutral form $As(OH)_3$, and passively enters cells through aquaglyceroporins (AQPs) [8, 9]. AQP9 is expressed at high levels in hepatocytes, and has been proposed to contribute to As^{III} accumulation in the liver [5].

As^{III} and As^{V} are methylated within cells through a series of consecutive methylation reactions resulting in the formation of monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^{V}), dimethylarsinous acid (DMA^{III}), and dimethylarsinic acid (DMA^{V}) [10]. The majority of As methylation is thought to occur in the liver [11], however; elimination occurs predominantly in urine (60-80%), and of this, 10-30% is eliminated as inorganic arsenic, 10-20% as monomethylated, and 60-80% as dimethylated forms [12-14]. In addition to As^{III}, AQP9 facilitates the passage of MMA^{III}, MMA^{V}, and DMA^{V} [5, 15]. Permeation of AQP9 by MMA^{III} occurs at a higher rate than As^{III} while MMA^{V} (pKa 3.6 and 8.2) and DMA^{V} (pKa 6.5) uptake was inefficient at pH 7.5, likely due to their predominantly negative charge at physiological pH [5]. In addition to cellular

uptake, AQP9 allows the export of neutral As species [5]. Although AQPs are likely important for the transport of neutral methylated and inorganic forms of arsenic they do not account for the transport of arsenic glutathione conjugates or other negatively charged arsenic species.

In vivo and *in vitro* data suggest that the ATP-binding cassette transporter proteins multidrug resistance protein 1 (MRP1/*ABCC1*) and MRP2 (*ABCC2*) are important for the cellular efflux of $As(GS)_3$ and $MMA(GS)_2$ [16-19]. Within cells, it is estimated that 99% of trivalent arsenic species are bound to thiols [20] and the most abundant low molecular weight thiol is GSH present at 0.5-10 mM. At these concentrations of GSH, As will form $As(GS)_3$, $MMA(GS)_2$, and DMA(GS), although DMA(GS) has never been detected *in vivo* [16, 21, 22]. The importance of As-GSH conjugates for the efflux of As has been studied extensively [16-18, 23]. In addition, depletion of GSH in cells is associated with an increased As cytotoxicity [19, 24-27].

Despite the important roles MRP1 and MRP2 play in the cellular handling of As, neither protein is localized to the basolateral surface of hepatocytes. Thus, MRP1 and MRP2 are not responsible for the transport of hepatic metabolites into sinusoidal blood ultimately for urinary elimination. Since As forms GSH conjugates and MRPs are the major transporters of these conjugates, we hypothesized that another MRP is responsible for the basolateral transport of As from the liver.

The localization of MRP4 differs depending on cell/tissue type. In hepatocytes and choroid plexus epithelium, MRP4 is localized to the basolateral

membrane, whereas in the kidney, it is expressed at the apical membrane of renal proximal tubule cells [28]. In a colonic epithelial cell line, MRP4 is localized to both apical and basolateral membranes [29]. The basolateral expression of MRP4 in the hepatocyte and the apical expression in the proximal tubule of the kidney make MRP4 an ideal candidate for involvement in As elimination, however, its ability to transport arsenic species is unknown. MRP4 is capable of transporting a wide variety of drugs, including antiviral, antibiotic, cardiovascular, and cytotoxic agents, and endogenous molecules such as cyclic nucleotides, ADP, eicosanoids, uric acid, conjugated steroid hormones, folic acid, bile acids, and the prostaglandins PGE1 and PGE2 [30-33]. In addition to the liver and the kidney, MRP4 is widely expressed in other cell types, including neurons, cardiovascular tissue, blood-brain-barrier, testis, prostate, and intestine [33, 34]. The purpose of this study was to determine if MRP4 was capable of conferring cellular protection against inorganic and/or methylated arsenic species, identify the species transported, and characterize the transport mechanism. MRP4 was found to confer cellular protection against As^{V} and MMA^{III} in a GSH-dependent manner and MMA^{V}, DMA^{III}, and DMA^{V} independently of GSH. Direct transport studies using MRP4-enriched membrane vesicles revealed that $MMA(GS)_2$ and DMA^{V} were substrates for MRP4.

5.3 Methods

5.3.1 *Materials*

GSH, GSSG, ATP, AMP, sucrose, Tris base, As^{III}, As^{V}, MMA^{V}, DMA^{V}, L-buthionine sulfoximine (BSO) and $MgCl_2$ were purchased from Sigma-Aldrich (Oakville, Ontario). Carrier free [6,7-^{3}H]$E_2$17βG (50.1 Ci/mmol) was purchased from Perkin Elmer (Woodbridge, Ontario). Creatine kinase, glutathione reductase, creatine phosphate, NADPH, and protease inhibitor cocktail tablets (Complete™, mini EDTA free) were purchased from Roche Applied Science (Torrance, CA). Nitric acid was purchased from Fisher Scientific (Ottawa, Ontario). $E_2$17βG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ophthalmic acid was purchased from Bachem (Torrance, CA). MMA^{III} and DMA^{III} in the form of diiodomethylarsine (CH_3AsI_2) and iododimethylarsine ($[CH_3]_2AsI$) were gifts from Dr. William Cullen (University of British Columbia) and Dr X. Chris Le (University of Alberta).

5.3.2 *Cell culture*

The human embyonic kidney cell line (HEK293) and the SV40-transformed human embryonic kidney cell like (HEK293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% fetal bovine serum.

5.3.3 *pcDNA3.1(+)MRP4-vector construction and generation of HEK-MRP4 stable cell line*

The pcDNA3.1Hygro-MRP4 vector encoding the full length human MRP4 was a gift from Dr. Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany) and was constructed as previously described [35]. The MRP4 cDNA

was excised from pcDNA3.1(+)Hygro-MRP4 vector using the restriction enzymes *NheI* and *XhoI*, and subcloned into pcDNA3.1(+)(neomycin) to generate pcDNA3.1(+)MRP4.

For stable expression of MRP4 and empty vector, HEK293 cells were seeded at 2.0 x 10^5 cells/well of a 6 well plate and after 24 hours transfected with pcDNA3.1(+)MRP4 or empty pcDNA3.1(+), using the calcium phosphate transfection method, as previously described [36, 37]. After 48 hours, cells were split 1:24 and reseeded onto 6 well plates in media containing 1000 µg/ml G418 (Invitrogen). Cells were grown for ~2 weeks with regular media replacement, and were monitored visually by light microscopy, once cell colonies were visible, they were individually removed by scraping and aspirating with a pipette tip as described [38]. Levels of MRP4 in G418 resistant cell populations were then determined by immunodot blotting with the MRP4 specific MAb M_4I-10 (1:2000) as described previously [39]. The MRP4 positive clones were tested for the proportion of cells expressing MRP4 by flow cytometry (BD FACS Calibur, Cross Cancer Institute) using the MAb $M4_I$-10 (1:40) as described previously [40]. Populations of less than 80% were further cloned by limiting dilution to obtain populations of >80% expressing MRP4. The clone with the highest expression was selected for experiments (HEK-MRP4-1E1-6) and is referred to as HEK-MRP4 throughout this chapter. The empty vector cell line HEK-V4 was generated in the same manner and is referred to as HEK-Vector. Cytotoxicity results obtained from an additional clone (HEK-MRP4-1E1-42) were consistent with results from HEK-MRP4-1E1-6.

5.3.4 *Cytotoxicity testing*

The influence of MRP4 expression on the cytotoxicity of As^{V}, As^{III}, MMA^{V}, MMA^{III}, DMA^{V}, and DMA^{III} was determined using HEK-MRP4 and HEK-Vector cell lines as previously described [19, 41]. Briefly, cells were seeded in 96-well plates at 1 x 10^4 cells/well and 24 h later were treated with As^{V} (0.001-10 mM), As^{III} (0.1-100 μM), MMA^{V} (0.3-100 mM), MMA^{III} (0.1-100 μM), DMA^{V} (0.01-100 mM) or DMA^{III} (0.1-100 μM) in quadruplicate for 72 h. To measure the influence of GSH depletion on As^{V}, As^{III}, MMA^{V}, MMA^{III}, DMA^{V}, and DMA^{III} cytotoxicity, cells were treated with buthionine sulfoximine (BSO, 100 μM), which inhibits the rate limiting step of GSH synthesis, at the time of seeding and for the remaining experimental duration. Cytotoxicity of DMA^{III}, which is highly unstable, was tested as described above and also with daily media changes (containing new DMA^{III}). Cell viability was determined using the CellTiter96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Data were analyzed and EC_{50} values determined using the sigmoidal dose response equation in Graphpad Prism (GraphPad Software, San Diego, CA, USA). Relative resistance values were calculated as the ratio of the EC_{50} values of HEK-MRP4 to HEK-Vector.

5.3.5 *Cellular accumulation of arsenic species*

HEK-MRP4 and HEK-Vector cells were seeded in 6-well plates at 5 x 10^5 cells/well and 24 h later treated with 1 μM As^{V}, As^{III}, MMA^{V}, MMA^{III}, DMA^{V}, or DMA^{III} in culture media for 72 h. Cells were then washed three times with ice-cold phosphate buffered saline, trypsinized, pelleted by centrifugation at 1000 x g

for 10 min at 4 ºC, and then digested with 250 µl concentrated nitric acid for >96 h. Digested cells were diluted 1:1 with deionized distilled water and filtered through 0.45-µm syringe filters (Whatman, Toronto, ON, Canada). The total concentration of arsenic in samples was determined by inductively couple mass spectrometry (ICP-MS) (Agilent 7500; Yokogawa Analytical Systems, Hacchiouji, Japan) using the standard addition method, as described previously [19, 42]. Samples were introduced directly into the nebulizer of the ICP-MS.

5.3.6 *Arsenic speciation in HEK-MRP4 and HEK-Vector cells*

HEK-MRP4 and HEK-Vector cells were seeded at $2x10^5$ cells/well of a 6-well plate and grown for 24 h and then treated with As^V (1 µM) or MMA^{III} (1 µM) for 72 hours. Cells were trypsinized, pelleted, and frozen at -80ºC. Pellets were thawed the day of analysis and resuspended in NH_4HCO_3 (50 mM, pH 8.5). Cell membranes were disrupted using a Tenbroeck homogenizer. Homogenate was centrifuged at 100,000 x g to remove cell membrane and nuclei and the supernatant analyzed for arsenic speciation using HPLC-ICP-MS according to a previously described method [43].

An Agilent 1100 series HPLC system consisting of a pump, degasser, autosampler, column temperature control, and reversed-phase C_{18} column (ODS-3, 150 mm × 4.6 mm, 3-µm particle size; Phenomenex, Torrance, CA, USA) was used for the separation of arsenicals. The mobile phase consisted of 5 mM tetrabutylammonium, 5 % methanol, and 3 mM malonic acid (pH 5.65). The column was equilibrated with the mobile phase for at least 0.5 h at a flow rate of 0.8 ml/min before sample injection (50 µl). Separation of samples was

performed at a flow rate of 1.2 ml/min; the column temperature was maintained at 50 °C. The effluent from HPLC was introduced directly into the nebulizer of a 7500ce ICP-MS instrument (Agilent Technologies, Japan) using PEEK tubing. The collision cell of the ICP-MS instrument was operated in helium mode. Helium (3.5 mL/min) was used in the octopole reaction cell to reduce isobaric and polyatomic interferences. The ICP operated at a radio-frequency power of 1,550 W, and the flow rate of argon carrier gas was 0.9–1.0 L/min. Arsenic was monitored at *m*/*z* 75. Chromatograms from HPLC separation were recorded by ICP-MS ChemStation (Agilent Technologies, Santa Clara, CA, USA). Quantification was performed from standard curves generated from 0.5, 1, 5, 10, and 20 ppb mixtures of As^{III}, DMA^{V}, MMA^{V}, and As^{V} in addition to standard solutions of $As(GS)_3$ and $MMA(GS)_2$ (~1000 ppb).

5.3.7 *Transient expression of MRP4 in HEK293T cells*

HEK293T cells were transfected in 150 mm culture dishes using the calcium phosphate method, as described previously [36, 37]. Seventy-two hours post-transfection, cells were washed twice with Tris (50 mM, pH 7.4) sucrose (250 mM) buffer, scraped into 10 ml/plate of Tris sucrose buffer and collected by centrifuging at 800 x g for 10 min. Cell pellets were stored at -80ºC until membrane vesicles were prepared. Plasma membrane vesicles were prepared from MRP4 transfected HEK293T cells, according to previously described methods [44]. Expression of MRP4 in membrane vesicles was confirmed by immunoblot analysis, using the rat anti-human MRP4 antibody M_4-I10 (Abcam, Cambridge, MA) (1:2000). MRP4 protein levels were approximately 2-fold higher after

transient expression compared to the HEK-MRP4 stable cell line and therefore transient were used for all membrane vesicle transport experiments.

5.3.8 *$MMA(GS)_2$ and DMA^V transport assays.*

$MMA(GS)_2$ was synthesized from MMA^{III} and GSH according to previously described methods for $As(GS)_3$ [18], with modifications. Briefly, MMA^{III} (final concentration of 50 µM) was combined with GSH (final concentration of 75 mM) in degassed Tris sucrose buffer and incubated under a nitrogen atmosphere for >30 min at 4ºC. Membrane vesicles were incubated at 37ºC in Tris-sucrose buffer, with As^V (10 µM) or MMA^{III} (1 µM), ATP or AMP (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), GSH reductase (5 µg/ml), and NADPH (0.35 mM). GSH dependence was assessed by adding GSH (3 mM) or the non-reducing GSH analog ophthalmic acid (3 mM). At 3 or 5 min, transport was stopped by diluting the transport reaction in 800 µl ice-cold Tris-sucrose buffer and pelleting vesicles by centrifugation at 100,000 x g for 20 min at 4 ºC. Pelleted membrane vesicles were washed twice with 1ml ice-cold Tris-sucrose buffer, digested in 250 µl concentrated nitric acid for 48 h, diluted 1:1 with deionized distilled water, and filtered through 0.45 µm syringe filters (Whatman, Toronto, ON). The total concentration of arsenic in each sample was determined by ICP-MS, using the standard addition method, as described previously [19, 42].

5.3.9 *$E_2 17\beta G$ inhibition assays*

Inhibition of $E_2 17\beta G$ transport by $MMA(GS)_2$ and DMA^V was performed essentially as previously described [19, 45, 46]. Briefly, 10 µg of membrane vesicles were incubated with [^{3}H]$E_2 17\beta G$ (1 µM, 40 nCi), AMP or ATP (4 mM),

$MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 µg/ml) GSH reductase (5 µg/ml) and NADPH (0.35 mM) at 37ºC for 3 min in the absence or presence of $MMA(GS)_2$ (100 or 500 µM) or DMA^V (0.1, 1, or 3 mM). $MMA(GS)_2$ inhibition assays were done in the presence of GSH (3 mM), to maintain conjugate stability. Samples were filtered through glass fiber filters and then radioactivity was quantified by liquid scintillation counting. ATP-dependent transport was determined by subtracting transport in the presence of AMP from the transport in the presence of ATP.

5.4 Results

5.4.1 *MRP4 confers resistance to As^V, MMA^V, MMA^{III}, DMA^V, and DMA^{III}*

To determine if MRP4 can confer resistance to inorganic and methylated species of arsenic, HEK-MRP4 and HEK-Vector cell lines were treated with increasing concentrations of As^{III}, As^V, MMA^V, MMA^{III}, DMA^V, and DMA^{III}. The EC_{50} value for each arsenical was determined and the relative resistance was calculated from the ratio of the EC_{50} values for HEK-MRP4 and HEK-Vector (summarized in Table 5.1). HEK-MRP4 cells conferred resistance to As^V, MMA^V, MMA^{III}, DMA^V, and DMA^{III} (Figure 5.1 and Table 5.1). HEK-MRP4 cells were 2.8-fold more resistant to As^V, 1.6-fold more resistant to MMA^V, 1.7-fold more resistant to MMA^{III}, 1.6-fold more resistant to DMA^V, and 2.0-fold more resistant to DMA^{III} compared to the vector control cell line, however, no difference between the sensitivity of the cell lines was observed for As^{III} (Table 5.1 and Figure 5.1). In some experiments with DMA^{III}, medium was exchanged every 24 h with medium

containing fresh DMA^{III} and under these conditions no difference in resistance between the HEK-MRP4 and HEK-Vector cell lines was observed (Table 5.1).

TABLE 5.1. Resistance of MRP4 Transfected HEK293 cells to inorganic and methylated arsenic

Arsenic Species	EC_{50} (± S.D.) (μM)		Relative Resistance[a]
	HEK-Vector	HEK-MRP4	
As^{III} (*n = 5)*	3.0 ± 1.0	2.7 ± 1.4	0.9
As^{V} (n = 5)	17.1 ± 9.8	47.3 ± 15.8 *	2.8
MMA^{III} (n = 5)	1.6 ± 0.5	2.6 ± 0.6 *	1.7
MMA^{V} (n = 3)	13370 ± 155	21093 ± 1366 *	1.6
DMA^{III} (n = 5)	0.5 ± 0.2	1.0 ± 0.3 *	2.0
DMA^{III} (n = 4)[1]	0.4 ± 0.2	0.7 ± 0.3	1.7
DMA^{V} (n = 3)	748 ± 28	1164 ± 26 *	1.6

[a]Ratio of EC_{50} HEK-MRP4/HEK-Vector.

*EC_{50} for HEK-MRP4 is significantly different from HEK-Vector, $P < 0.05$ (Student's *t* test).

[1]Medium containing fresh DMA^{III} was added every 24 h

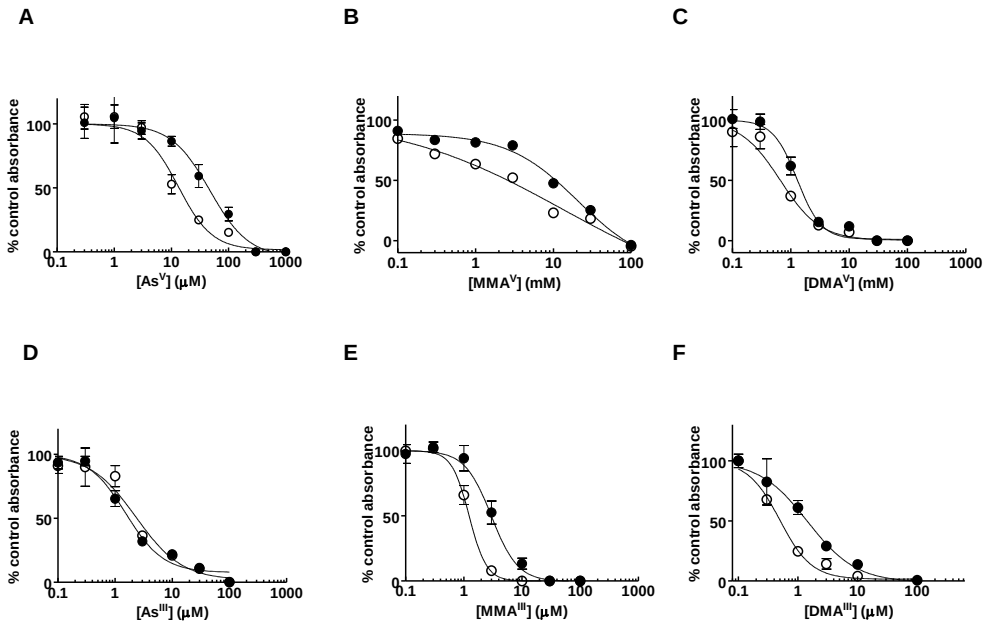

Figure 5.1. Effect of As^V, MMA^V, DMA^V, As^{III}, MMA^{III} and DMA^{III} on the viability of stably transfected HEK293 cells. Empty vector transfected HEK293 cells (○) and MRP4-transfected HEK293 cells (●) were incubated in the presence of As^V (A), MMA^V (B), DMA^V (C), As^{III} (D), MMA^{III} (E), or DMA^{III} (F). Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data points are means (±S.D.) of quadruplicate determinations in a representative experiment; similar results were obtained in at least two additional experiments.

To determine whether resistance conferred by MRP4 to As^{V}, MMA^{V}, MMA^{III}, DMA^{V}, and DMA^{III} was dependant on GSH, toxicity was tested after depleting HEK-Vector and HEK-MRP4 cells of GSH using BSO (100 µM). BSO alone had no effect on cell viability. High intracellular GSH levels are known to confer resistance to arsenicals partly through neutralization of reactive oxygen species [19, 26]. Consistent with this, As^{III}, As^{V}, MMA^{V}, MMA^{III} and DMA^{V} became significantly more toxic to both HEK-Vector and HEK-MRP4 cells although this wasn't the case for DMA^{III} (Table 5.2, Figure 5.2). GSH depletion resulted in the loss of MRP4-dependant cellular protection to As^{V} and MMA^{III} (Table 5.2, and Figure 5.2). Unexpectedly, MMA^{V}, DMA^{III}, and DMA^{V} resistance conferred by MRP4 was independent of GSH. Similar results were obtained with independently derived HEK-Vector and HEK-MRP4 cell clones.

TABLE 5.2. Resistance of MRP4 Transfected HEK293 cells to inorganic and methylated arsenic after cellular GSH depletion

Arsenic Species	EC_{50} (± S.D.) (μM)		Relative Resistance[a]
	HEK-Vector	HEK-MRP4	
As^{III} (*n = 5)*	0.2 ± 0.01	0.23 ± 0.006	1.1
As^{V} (n = 5)	2.3 ± 0.6	1.7 ± 0.5	0.8
MMA^{III} (n = 5)	0.06 ± 0.05	0.07 ± 0.03	1.3
MMA^{V} (n = 3)	85 ± 8	166 ± 3 *	2
DMA^{III} (n = 5)	0.6 ± 0.1	1.6 ± 0.1 *	2.7
DMA^{III} (n = 4)[1]	0.7 ± 0.3	0.6 ± 0.3	0.9
DMA^{V} (n = 3)	25.5 ± 1.0	60.9 ± 19 *	2.4

[a]Ratio of EC_{50} HEK-MRP4/HEK-Vector.

*EC_{50} for HEK-MRP4 is significantly different from HEK-Vector, $P < 0.05$ (Student's *t* test).

[1]Medium containing fresh DMA^{III} was added every 24 h

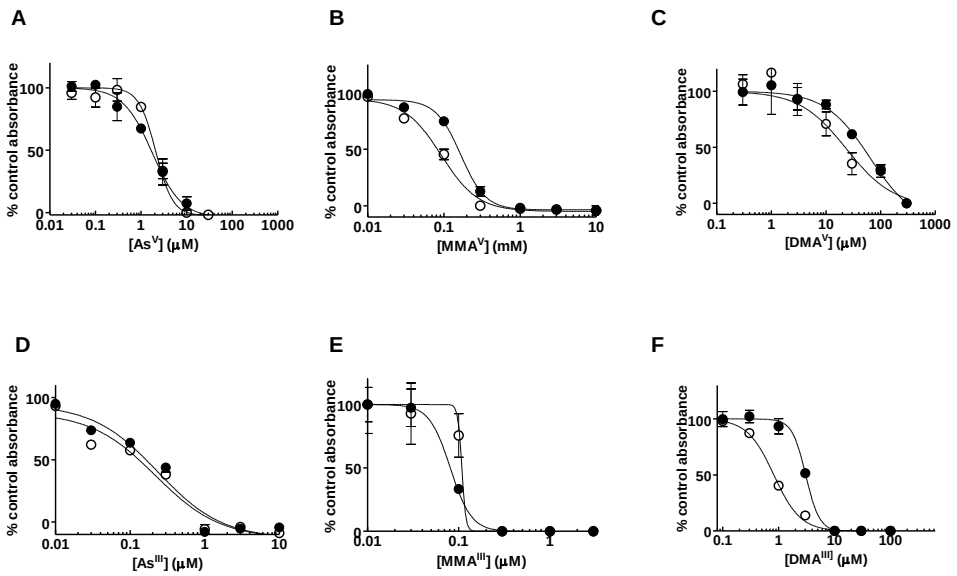

Figure 5.2. Effect of As^V, MMA^V, DMA^V, As^{III}, MMA^{III} and DMA^{III} on the viability of stably transfected HEK293 cells after glutathione depletion. Vector control transfected HEK293 cells (○) and MRP4-transfected HEK293 cells (●) were incubated in the presence of 100 μM BSO and As^V (A), MMA^V (B), DMA^V (C), As^{III} (D), MMA^{III} (E), or DMA^{III} (F). Cell viability was determined using a tetrazolium-based cytotoxicity assay. Data points are means (±S.D.) of quadruplicate determinations in a representative experiment; similar results were obtained in at least two additional experiments.

5.4.2 *Cellular accumulation of arsenic species*

To assess the effect of MRP4 on the cellular accumulation of different arsenic species, cellular total arsenic was determined after HEK-Vector and HEK-MRP4 exposure to As^{III}, As^{V}, MMA^{V}, MMA^{III}, DMA^{III}, and DMA^{V} (1 μM for 72 h). HEK-MRP4 cells accumulated 20% less As^{V}, 50% less MMA^{V}, 30% less MMA^{III}, 40% less DMA^{III}, and 55% less DMA^{V} than HEK-Vector (Figure 5.3). In addition, no significant difference in As^{III} accumulation between HEK-MRP4 and HEK-Vector was observed (Figure 5.3). Consistent with previous studies and the high EC_{50} values obtained for MMA^{V} and DMA^{V} (Tables 5.1 and 5.2), the accumulation of these pentavalent arsenicals was 5-30-fold lower than any of the other As species (Figure 5.3) [47, 48].

5.4.3 *Cellular arsenic metabolism*

Due to the long (72 h) exposure of cells to As in cytotoxicity and accumulation experiments it was important to determine the extent of As methylation by HEK293 cells over this time period. HEK-MRP4 and HEK-Vector stable cell lines were treated with As^{V} or As^{III} (1 μM) for 72 hours and the soluble cellular content was analyzed by HPLC-ICP-MS. Arsenic content of cells was 4.7 ± 3.5% inorganic arsenic (Figure 5.4). The remaining arsenic species were MMA^{III}, DMA^{V} and three unknown peaks that did not match retention times of standards. Taken together with cytotoxicity results, these data suggest that MRP4 is not protecting cells from inorganic arsenic but likely methylated species of As.

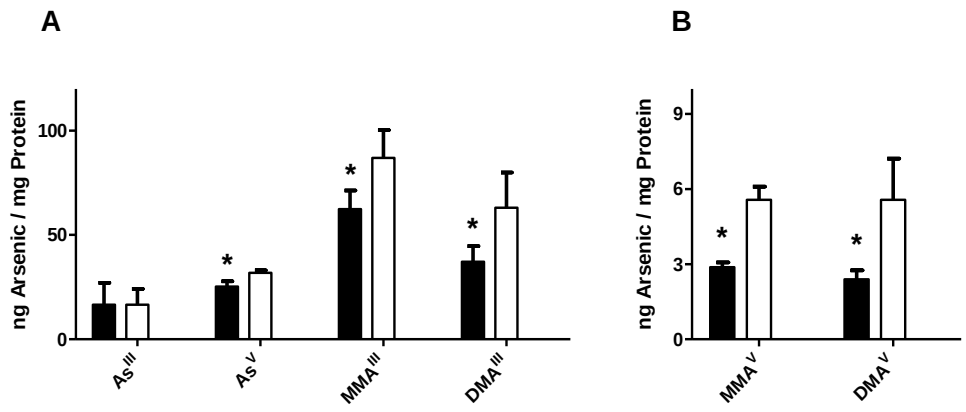

Figure 5.3. Accumulation of As^{III}, As^{V}, MMA^{III}, DMA^{III} by stably transfected HEK cells. Vector control transfected HEK cells (open bars) and MRP4 transfected HEK cells (closed bars) were treated with 1 μM of (A) As^{III}, As^{V}, MMA^{III}, DMA^{III}, (B) MMA^{V} or DMA^{V} for 72 h. Cells were then harvested and digested with nitric acid, and total arsenic was determined using ICP-MS. Bars represent the means of three independent experiments (±S.D.). *P<0.05

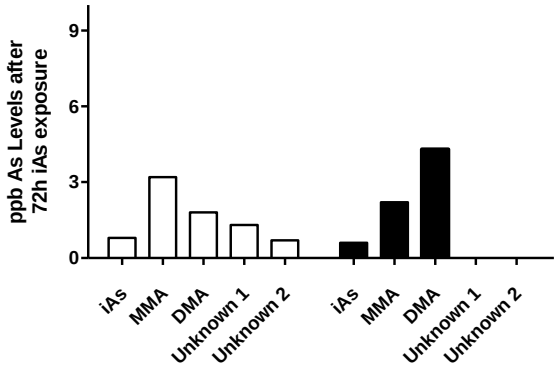

Figure 5.4 Methylation of As In HEK293 Cells. Vector control transfected HEK cells (open bars) and MRP4 transfected HEK cells (closed bars) were treated with 1 μM of As^{III} for 72 h. Cells were then harvested and lysed and arsenic speciation was immediately determined using HPLC-ICP-MS.

5.4.4 *MRP4 transports $MMA(GS)_2$ and DMA^V*

Although cytotoxicity and cellular accumulation studies provide important information for determining the role of MRP4 in protecting cells from As, the methylation and glutathionylation of As in intact cells prevents the identification of the transported species. To determine which species of As are transported by MRP4, ATP-dependent transport of individual As species by MRP4-enriched membrane vesicles prepared from HEK293T cells was measured (Figure 5.5). In contrast with the cytotoxicity and accumulation data that suggested As^V was potentially a GSH-dependent MRP4 substrate (Figure 5.1A and 5.2A, Table 5.1 and 5.2), MRP4 did not transport As^V under any conditions tested. Conditions included concentrations ranging from 0.1-10 μM, time points ranging from 1-10 min, and a pH range from 6-9 (Figure 5.6). Consistent with the MTS and accumulation data, As^{III} (0.1-10 μM) was not a substrate for MRP4 in the presence or absence of GSH (3 mM) or as the triglutathione conjugate, $As(GS)_3$ (0.1-10 μM) (Figure 5.6).

To determine whether MMA^{III}, $MMA(GS)_2$ and/or MMA^V were substrates of MRP4, ATP-dependent transport of these compounds into MRP4-enriched and vector control membrane vesicles was measured (Fig 5.5A). ATP-dependant transport of MMA^{III} by MRP4-enriched membrane vesicles was extremely low and similar to transport observed with the vector control membrane vesicles. In the presence of GSH (3 mM), ATP-dependant transport of MMA^{III} was observed with an activity of 110 pmol mg^{-1} min^{-1}. In previous studies, it was shown that ophthalmic acid and other GSH analogs lacking a free thiol group can substitute

for GSH and support the transport of several GSH-dependant MRP substrates [49-52]. These studies indicate that the thiol group of GSH and therefore the formation of a GSH conjugate are not necessary for transport. ATP-dependant transport of MMA^{III} in the presence of ophthalmic acid (3 mM) was extremely low and similar to that in the absence of GSH and vector control (Fig 5.5A). ATP-dependent transport of $MMA(GS)_2$ was then measured and found to have an activity of 113 pmol mg^{-1} min^{-1}. These data suggest that the free thiol group of GSH is required for MMA^{III} transport by MRP4 and are consistent with $MMA(GS)_2$ being the transported chemical species. Transport of MMA^{V} by MRP4 enriched membrane vesicles was measured, and no MRP4-dependent transport was detected (Figure 5.6).

To determine if DMA^{III} was a substrate of MRP4, ATP-dependent transport of DMA^{III} in the presence or absence of GSH by MRP4-enriched membrane vesicles was measured. In the presence of GSH (3 mM) transport was not detected and similar to transport observed in the presence of AMP. However, in the absence of GSH, ATP-dependent transport of DMA^{III} was observed with an activity of 40 pmol mg^{-1} min^{-1} (Figure 5.5B). This was consistent with the increased resistance conferred to DMA^{III} by MRP4 under GSH-depleted compared to GSH containing conditions (Table 5.1, Table 5.2, Figure 5.1F, and Figure 5.2F).

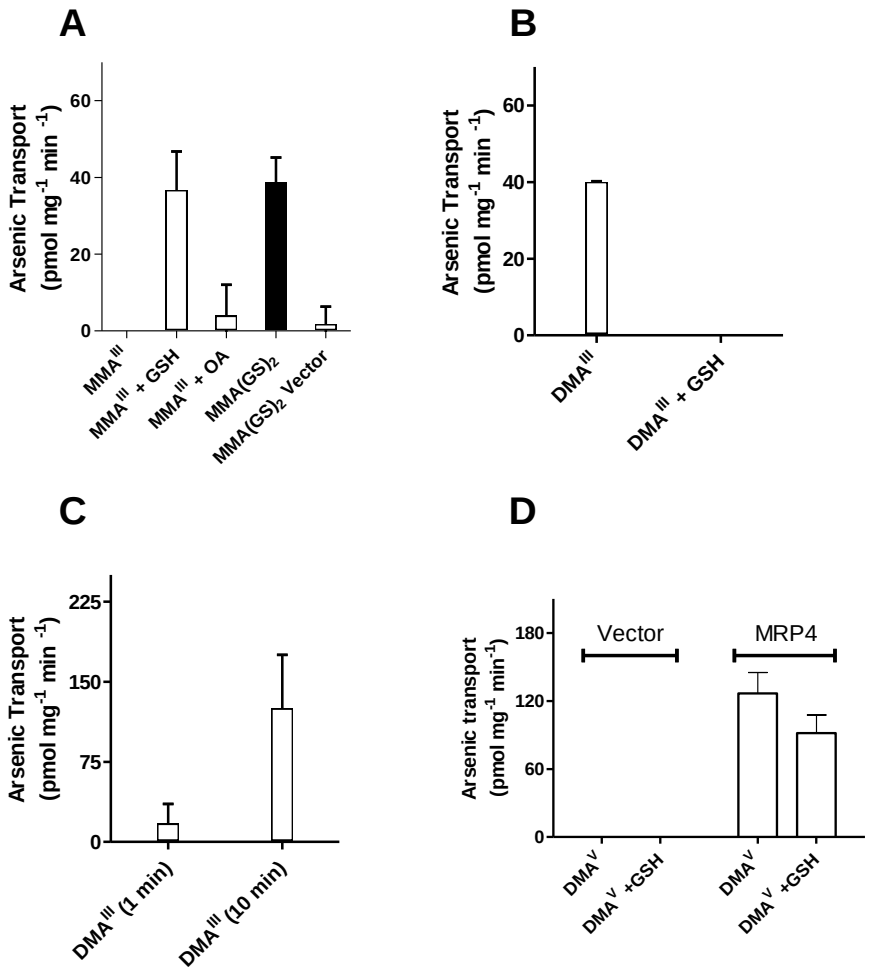

Figure 5.5. Transport of MMA^{III}, DMA^{III}, and DMA^{V} by MRP4-enriched membrane vesicles. Transport experiments were done with membrane vesicles (20 µg of protein) prepared from HEK cells transfected with pcDNA3.1(+)MRP4 (open bars) or empty pcDNA3.1(+) (closed bars) (A) Membrane vesicles were incubated for 3 min at 37ºC with $MMA(GS)_2$ (1 µM) or MMA^{III} (1 µM) in transport buffer, in the presence of GSH (3 mM) or ophthalmic acid (3 mM) (B) Vesicles were incubated for 5 min at 37ºC in transport buffer with DMA^{III} in the presence or absence of GSH (3 mM). (C) Vesicles were incubated for 1 min at 37^0C in transport buffer with DMA^{III}, prepared 1 min or 10 minutes prior to addition to transport reaction. (D) Vesicles were incubated with DMA^{V} (1 µM), in the presence or absence of GSH (3 mM). For all experiments transport was done in triplicate then reactions were pooled for analysis by ICP-MS. Bars represent the means of three independent experiments (±S.D.).

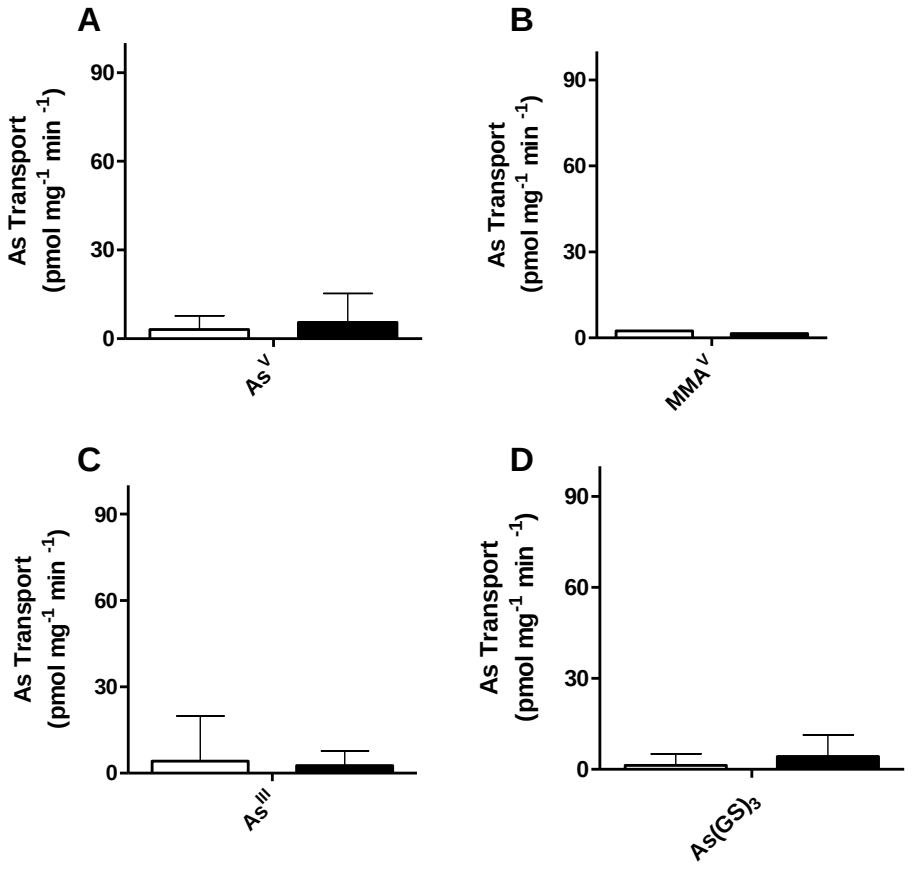

Figure 5.6. MRP4 does not transport As^V, As^{III}, or MMA^V. Transport experiments were done with membrane vesicles (20 µg of protein) prepared from HEK cells transfected with pcDNA3.1(+)MRP4 (open bars) or empty pcDNA3.1(+) (closed bars). Membrane vesicles were incubated for 3 min at 37ºC with (A) $^{73}As^V$ (1 µM), (B) MMA^V(1 µM), (C) $^{73}As^{III}$ (1 µM), or (D) $^{73}As(GS)_3$ (1 µM, +3 mM GSH) in transport buffer For all experiments transport was done in triplicate. For MMA^V, triplicate reactions were pooled for analysis by ICP-MS.

When dissolved at physiological pH, DMA^{III} spontaneously oxidizes to DMA^{V}, existing as ~55% DMA^{III} and 45% DMA^{V} [53]. It is possible that, in the presence of GSH, DMA^{III} was kept reduced and does not become a substrate for MRP4 until oxidized to DMA^{V}. In order to test this possibility, the transport of DMA^{III} was measured after incubating dissolved DMA^{III} for 1 or 10 min (to allow oxidation to DMA^{V}) before addition to the transport reaction mix, and the two conditions compared. DMA^{III} dissolved 10 min prior to addition to the transport reaction showed a 4-fold increase in transport activity compared with DMA^{III} dissolved 1 min prior (Figure 5.5C). These data suggest that DMA^{V} is the transported species. Direct transport studies of DMA^{V} (Figure 5.5D) showed that DMA^{V} was a substrate for MRP4 with a transport activity of 63 pmol mg^{-1} min^{-1}. GSH had no significant effect on the transport of DMA^{V}.

5.4.5 *Inhibition of $E_2$17βG by $MMA(GS)_2$ and DMA^V*

To determine whether $MMA(GS)_2$ and DMA^{V} were capable of inhibiting the transport of another MRP4 substrate, the ability of $MMA(GS)_2$ and DMA^{V} to inhibit the transport of $E_2$17βG was evaluated. $MMA(GS)_2$ (100 and 500 μM) inhibited $E_2$17βG transport by 64% and 70%, respectively (Fig 5.7A). DMA^{V} (1 and 3 mM) inhibited $E_2$17βG transport by 20% and 35%, respectively (Fig 5.7B).

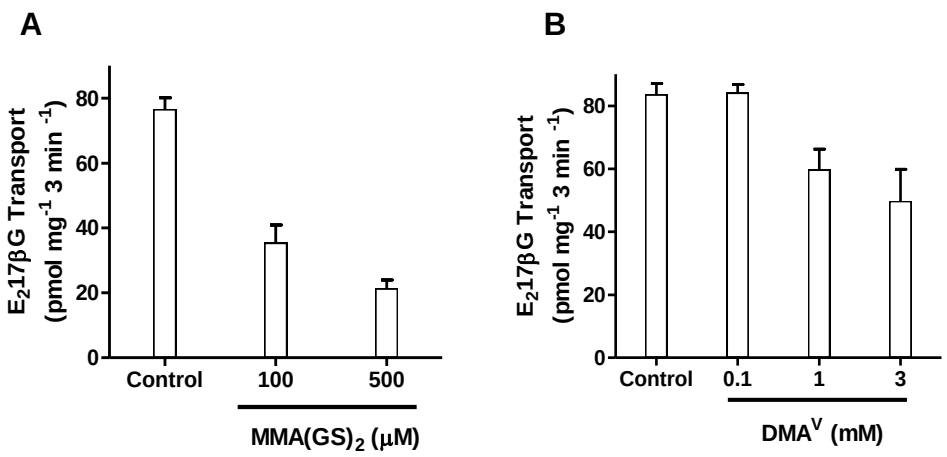

Figure 5.7. Inhibition of MRP4 mediated $E_2$17βG transport. Transport experiments were done with membrane vesicles (10 μg of protein) prepared from HEK cells transfected with pcDNA3.1(+)MRP4. Membrane vesicles were incubated for 3 min at 37ºC with $E_2$17βG (1 μM, 20 nCi) in transport buffer, in the presence of (A) $MMA(GS)_2$ (100 and 500 μM) and GSH (3 mM) or (B) DMA^V (0.1-3 mM). Bars represent the mean of triplicate determinations in a single experiment (± S.D.)

5.5 Discussion

Arsenic affects millions of people worldwide and has become a major concern as an environmental contaminant in the last 50 years. In addition to being a proven human carcinogen from chronic exposure in drinking water, the clinical use of arsenic trioxide in the treatment of APL has hastened the need for the understanding of the metabolism and elimination of this toxic metalloid. Arsenic occurs ubiquitously in the environment, as evidenced by the many mechanisms by which plants and bacteria have adapted to detoxify and sequester arsenic [8, 54, 55]. In the current study, the influence of MRP4 on the cytotoxicity, cellular accumulation, and membrane vesicle transport of inorganic (As^{III} and As^{V}), monomethylated (MMA^{III} and MMA^{V}) and dimethylated (DMA^{III} and DMA^{V}) arsenic species was characterized. Evidence supports an important role for MRP4 in the cellular export of $MMA(GS)_2$ and DMA^{V}. The basolateral localization of MRP4 at the surface of hepatocytes and apical localization in renal proximal tubule cells suggests MRP4 could be critical for urinary elimination of these hepatic metabolites [33].

GSH protects cells from many arsenic species through the efflux of GSH conjugates and the neutralization of reactive oxygen species [19, 26]. In the current study, depletion of cellular GSH caused an increase in toxicity to all arsenicals tested except for DMA^{III}. Initially it was unexpected that depletion of GSH would cause cells expressing MRP4 to be less sensitive to DMA^{III}, transport data suggests that DMA^{V} is transported by MRP4, and likely makes up a larger proportion of the dimethylated As within the cell when GSH is depleted. The

ability of MRP4 to transport $MMA(GS)_2$ in addition to DMA^V carries implications for cells to react to various situations of As exposure. Under acute As exposure with a high burden on the cell, when methylation pathways could become saturated, it is important for cells to be able to transport $As(GS)_3$ and $MMA(GS)_2$ as has been shown for MRP1 and MRP2. However, cells lose a significant amount of GSH in the process, as has been shown for cells expressing these proteins [25, 56]. MRP4 on the other hand, offers a less costly transport mechanism, which under lower As exposure scenarios, transports DMA^V out of the cell. If this notion of efficiency of transport is accurate, it would be expected that MRP4 would have a relatively higher affinity for DMA^V, with a lower relative affinity for $MMA(GS)_2$. The transport kinetics of $MMA(GS)_2$ and DMA^V warrant future determination.

Cytotoxicity assays showed that MRP4 resistance to As^V and MMA^{III} was GSH-dependent. Within the cell, As^V is reduced to As^{III} and when either MRP1 or MRP2 are present As^{III} can be effluxed as $As(GS)_3$. Initially, it was somewhat surprising that MRP4 did not confer resistance or reduce the accumulation of As^{III} when resistance was observed for all other species tested. These observations were consistent with a previous report that MRP4 expressed in NIH3T3 cells did not confer resistance to As^{III} [57]. In addition, in the current study, transport of As^{III} (±GSH) and $As(GS)_3$ by MRP4-enriched membrane vesicles was not detected. Taken together, these data raise the question: How can MRP4 confer resistance to As^V but not As^{III} when As^V is converted to As^{III} prior to methylation or GSH conjugation? As^{III} is well documented to be much more toxic to cells

than As^{V} and EC_{50} values in the current study are consistent with this (for example the EC_{50} value for HEK-MRP4 cells treated with As^{III} is 17-fold lower than for cells treated with As^{V} [4, 19, 58]. Cellular uptake of As^{III} occurs at a much faster rate than As^{V} and could result in the saturation of methylation pathways and a resulting accumulation of the more toxic As^{III}. Thus, when cells are exposed to As^{III} in the absence of a transporter that can efflux $As(GS)_3$, this likely results in more cell death compared to cells exposed to As^{V}.

The observation that in the absence of GSH MRP4 does not confer resistance to As^{V} or MMA^{III}, in combination with the speciation analysis and membrane vesicle transport data, suggests that MRP4 confers resistance to As^{V} and MMA^{III} by effluxing $MMA(GS)_2$. Currently, there are two general pathways proposed for the methylation of As. One involves oxidative methylation and is shown in Figure 5.6A. The other involves reductive methylation while As is complexed to GSH or protein (Figure 5.6B) [59-61]. Our results provide support for at least some portions of the latter pathway. Thus As^{V} and MMA^{III} are likely effluxed by MRP4 as $MMA(GS)_2$. If the formation of MMA^{III} occurred after the formation of MMA^{V}, as has been described previously by the Challenger pathway (Figure 5.6A), resistance to MMA^{V} should also be GSH dependent [59].

$MMA(GS)_2$ is a substrate for MRP1 and MRP2 [16, 19], but the observation that it is also a substrate for MRP4 carries a greater significance in regards to the elimination of As from the body. The widespread basolateral expression of MRP1 makes it an important transporter for providing cellular

protection against As exposure [18], but does not provide an excretory route. MRP2 on the other hand, has been shown to play an important role in transporting $MMA(GS)_2$ into bile [16]; however, urinary elimination is the predominant elimination pathway for As [12, 23]. This is explained by the relative instability of $MMA(GS)_2$ at biliary pH (6 minute half-life at pH 8.4) resulting in dissociation and entry into the enterohepatic circulation [53].

The observation that MRP4 does not confer resistance to DMA^{III} when cells were exposed to fresh media containing DMA^{III} every 24 h provides further evidence for the transport of DMA^{V}. Overall toxicity was not increased significantly, but the repeat exposure likely prevented MRP4 from conferring resistance through efflux of DMA^{V} that occurred over the 72 hours as DMA^{III} oxidized to DMA^{V}. DMA^{V} being the transported dimethylated As species provides support for the As reductive methylation pathway (Figure 5.6B), in which DMA^{V} is the end product of metabolism. Our previous work suggested that DMA^{V} is not a substrate for MRP1 since MRP1 does not confer resistance to $DMA^{III/V}$ [19]. Kala et al. did not identify DMA^{V} or DMA^{III} in the bile of WT Wistar rats making it unlikely that it is a substrate for Mrp2 [16]. To our knowledge, the observation that DMA^{V} is transported by MRP4 is the first pentavalent efflux pathway for arsenic identified for an active transport protein. The localization of MRP4 at both the basolateral surface of hepatocytes and apical surface of renal proximal tubule cells makes it an ideal transporter for urinary elimination of DMA^{V} and $MMA(GS)_2$ (Figure 5.8C).

To prevent and treat arsenic-induced toxicity, and to use arsenic and its various metabolites clinically, it is critical to understand the cellular handing of this metalloid. MRP4 single nucleotide polymorphisms (SNPs) that cause a reduction in $MMA(GS)_2$ and DMA^V transport could have serious implications for clinical and environmental exposure to As.

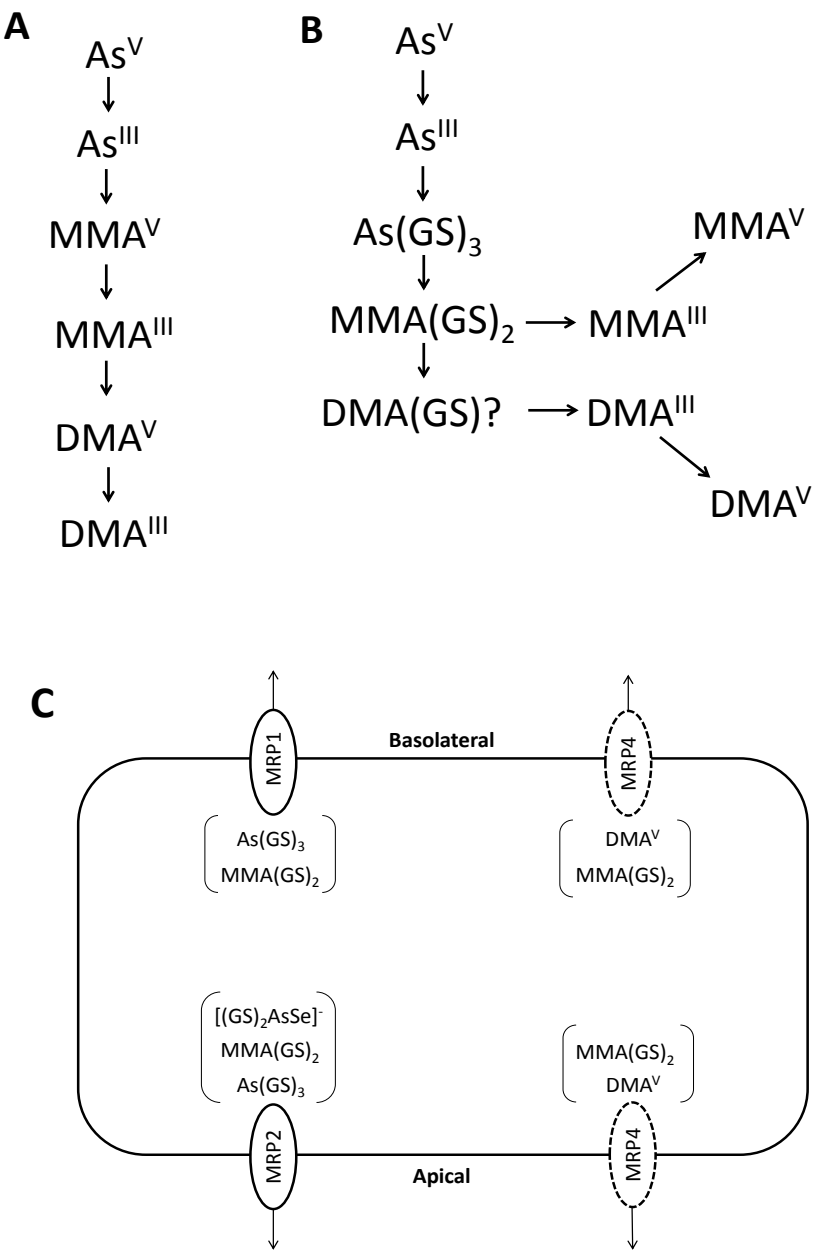

Figure 5.8 Summary of Arsenic Methylation Pathways and Efflux by MRPs. Adapted from [12, 59, 61]. A) The 'Challenger' pathway for arsenic methylation, with oxidative methylation. B) The alternative pathway containing reductive methylation. C) Polarized cell showing MRP4 localization dependent on tissue type.

5.6 Bibliography

1. Cui X, Kobayashi Y, Akashi M, Okayasu R: **Metabolism and the paradoxical effects of arsenic: carcinogenesis and anticancer**. *Curr Med Chem* 2008, **15**(22):2293-2304.
2. Straif K, Benbrahim-Tallaa L, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, Guha N, Freeman C, Galichet L, Cogliano V: **A review of human carcinogens--part C: metals, arsenic, dusts, and fibres**. *Lancet Oncol* 2009, **10**(5):453-454.
3. **IARC Monogrpahs on the Evaluation of Carcinogenic Risk to Humans**. 2012, **100**.
4. Roy P, Saha A: **Metabolism and toxicity of arsenic: A human carcinogen**. *Current Science* 2002, **82**(1):38-45.
5. McDermott JR, Jiang X, Beene LC, Rosen BP, Liu Z: **Pentavalent methylated arsenicals are substrates of human AQP9**. *Biometals* 2010, **23**(1):119-127.
6. Hirano S, Cui X, Li S, Kanno S, Kobayashi Y, Hayakawa T, Shraim A: **Difference in uptake and toxicity of trivalent and pentavalent inorganic arsenic in rat heart microvessel endothelial cells**. *Arch Toxicol* 2003, **77**(6):305-312.
7. Villa-Bellosta R, Sorribas V: **Arsenate transport by sodium/phosphate cotransporter type IIb**. *Toxicol Appl Pharmacol* 2010, **247**(1):36-40.

8. Rosen BP, Liu Z: **Transport pathways for arsenic and selenium: a minireview**. *Environ Int* 2009, **35**(3):512-515.

9. Liu Z, Carbrey JM, Agre P, Rosen BP: **Arsenic trioxide uptake by human and rat aquaglyceroporins**. *Biochem Biophys Res Commun* 2004, **316**(4):1178-1185.

10. Drobna Z, Walton FS, Harmon AW, Thomas DJ, Styblo M: **Interspecies differences in metabolism of arsenic by cultured primary hepatocytes**. *Toxicol Appl Pharmacol* 2010, **245**(1):47-56.

11. Lin S, Shi Q, Nix FB, Styblo M, Beck MA, Herbin-Davis KM, Hall LL, Simeonsson JB, Thomas DJ: **A novel S-adenosyl-L-methionine:arsenic(III) methyltransferase from rat liver cytosol**. *J Biol Chem* 2002, **277**(13):10795-10803.

12. Navas-Acien A, Umans JG, Howard BV, Goessler W, Francesconi KA, Crainiceanu CM, Silbergeld EK, Guallar E: **Urine arsenic concentrations and species excretion patterns in American Indian communities over a 10-year period: the Strong Heart Study**. *Environ Health Perspect* 2009, **117**(9):1428-1433.

13. Vahter M: **Genetic polymorphism in the biotransformation of inorganic arsenic and its role in toxicity**. *Toxicol Lett* 2000, **112-113**:209-217.

14. Loffredo CA, Aposhian HV, Cebrian ME, Yamauchi H, Silbergeld EK: **Variability in human metabolism of arsenic**. *Environ Res* 2003, **92**(2):85-91.

15. Liu Z, Styblo M, Rosen BP: **Methylarsonous acid transport by aquaglyceroporins**. *Environ Health Perspect* 2006, **114**(4):527-531.

16. Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW: **The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic**. *J Biol Chem* 2000, **275**(43):33404-33408.

17. Kala SV, Kala G, Prater CI, Sartorelli AC, Lieberman MW: **Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione**. *Chem Res Toxicol* 2004, **17**(2):243-249.

18. Leslie EM, Haimeur A, Waalkes MP: **Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required**. *J Biol Chem* 2004, **279**(31):32700-32708.

19. Carew MW, Naranmandura H, Shukalek CB, Le XC, Leslie EM: **Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1)**. *Drug Metab Dispos* 2011, **39**(12):2298-2304.

20. Kitchin KT, Wallace K: **Arsenite binding to synthetic peptides based on the Zn finger region and the estrogen binding region of the human estrogen receptor-alpha**. *Toxicol Appl Pharmacol* 2005, **206**(1):66-72.

21. Delnomdedieu M, Basti MM, Otvos JD, Thomas DJ: **Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study**. *Chem Biol Interact* 1994, **90**(2):139-155.

22. Gailer J, Lindner W: **On-column formation of arsenic-glutathione species detected by size-exclusion chromatography in conjunction with arsenic-specific detectors**. *J Chromatogr B Biomed Sci Appl* 1998, **716**(1-2):83-93.

23. Cui X, Kobayashi Y, Hayakawa T, Hirano S: **Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats**. *Toxicol Sci* 2004, **82**(2):478-487.

24. Chen ZS, Mutoh M, Sumizawa T, Furukawa T, Haraguchi M, Tani A, Akiyama S: **Reversal of heavy metal resistance in multidrug-resistant human KB carcinoma cells**. *Biochem Biophys Res Commun* 1997, **236**(3):586-590.

25. Liu J, Chen H, Miller DS, Saavedra JE, Keefer LK, Johnson DR, Klaassen CD, Waalkes MP: **Overexpression of glutathione S-transferase II and multidrug resistance transport proteins is associated with acquired tolerance to inorganic arsenic**. *Mol Pharmacol* 2001, **60**(2):302-309.

26. Duechler M, Stanczyk M, Czyz M, Stepnik M: **Potentiation of arsenic trioxide cytotoxicity by Parthenolide and buthionine sulfoximine in murine and human leukemic cells**. *Cancer Chemother Pharmacol* 2008, **61**(5):727-737.

27. Ong PS, Chan SY, Ho PC: **Differential augmentative effects of buthionine sulfoximine and ascorbic acid in As2O3-induced ovarian cancer cell death: oxidative stress-independent and -dependent cytotoxic potentiation**. *Int J Oncol* 2011, **38**(6):1731-1739.

28. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P: **Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines**. *Cancer Res* 1997, **57**(16):3537-3547.

29. Li C, Krishnamurthy PC, Penmatsa H, Marrs KL, Wang XQ, Zaccolo M, Jalink K, Li M, Nelson DJ, Schuetz JD, Naren AP: **Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia**. *Cell* 2007, **131**(5):940-951.

30. Chen ZS, Lee K, Kruh GD: **Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine**. *J Biol Chem* 2001, **276**(36):33747-33754.

31. Zelcer N, Reid G, Wielinga P, Kuil A, van der Heijden I, Schuetz JD, Borst P: **Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4)**. *Biochem J* 2003, **371**(Pt 2):361-367.

32. Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P: **The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs**. *Proc Natl Acad Sci U S A* 2003, **100**(16):9244-9249.

33. Russel FG, Koenderink JB, Masereeuw R: **Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules**. *Trends Pharmacol Sci* 2008, **29**(4):200-207.

34. Borst P, de Wolf C, van de Wetering K: **Multidrug resistance-associated proteins 3, 4, and 5**. *Pflugers Arch* 2007, **453**(5):661-673.

35. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D: **Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane**. *Hepatology* 2003, **38**(2):374-384.

36. Carew MW, Leslie EM: **Selenium-dependent and -independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/ABCC2): implications for the mutual detoxification of arsenic and selenium**. *Carcinogenesis* 2010, **31**(8):1450-1455.

37. Chen CA, Okayama H: **Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA**. *Biotechniques* 1988, **6**(7):632-638.

38. Karin NJ: **Cloning of transfected cells without cloning rings**. *Biotechniques* 1999, **27**(4):681-682.

39. Leggas M, Adachi M, Scheffer GL, Sun D, Wielinga P, Du G, Mercer KE, Zhuang Y, Panetta JC, Johnston B, Scheper RJ, Stewart CF, Schuetz JD: **Mrp4 confers resistance to topotecan and protects the brain from chemotherapy**. *Mol Cell Biol* 2004, **24**(17):7612-7621.

40. Leslie EM, Letourneau IJ, Deeley RG, Cole SP: **Functional and structural consequences of cysteine substitutions in the NH2 proximal region of the human multidrug resistance protein 1 (MRP1/ABCC1)**. *Biochemistry* 2003, **42**(18):5214-5224.

41. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG: **Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells**. *Cancer Res* 1994, **54**(22):5902-5910.

42. Kalivas JH: **Evaluation of volume and matrix effects for the generalized standard addition method**. *Talanta* 1987, **34**(11):899-903.

43. Chen B, Cao F, Yuan C, Lu X, Shen S, Zhou J, Le XC: **Arsenic speciation in saliva of acute promyelocytic leukemia patients undergoing arsenic trioxide treatment**. *Anal Bioanal Chem* 2013, **405**(6):1903-1911.

44. Loe DW, Almquist KC, Deeley RG, Cole SP: **Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport**. *J Biol Chem* 1996, **271**(16):9675-9682.

45. Loe DW, Almquist KC, Cole SP, Deeley RG: **ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids**. *J Biol Chem* 1996, **271**(16):9683-9689.

46. van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG: **The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP**. *J Am Soc Nephrol* 2002, **13**(3):595-603.

47. Kenyon EM, Hughes MF: **A concise review of the toxicity and carcinogenicity of dimethylarsinic acid**. *Toxicology* 2001, **160**(1-3):227-236.

48. Naranmandura H, Ibata K, Suzuki KT: **Toxicity of dimethylmonothioarsinic acid toward human epidermoid carcinoma A431 cells**. *Chem Res Toxicol* 2007, **20**(8):1120-1125.

49. Loe DW, Deeley RG, Cole SP: **Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione**. *Cancer Res* 1998, **58**(22):5130-5136.

50. Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, Cole SP: **Transport of the beta -O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog**. *J Biol Chem* 2001, **276**(30):27846-27854.

51. Leslie EM, Bowers RJ, Deeley RG, Cole SP: **Structural requirements for functional interaction of glutathione tripeptide analogs with the**

human multidrug resistance protein 1 (MRP1). *J Pharmacol Exp Ther* 2003, **304**(2):643-653.

52. Peklak-Scott C, Townsend AJ, Morrow CS: **Dynamics of glutathione conjugation and conjugate efflux in detoxification of the carcinogen, 4-nitroquinoline 1-oxide: contributions of glutathione, glutathione S-transferase, and MRP1**. *Biochemistry* 2005, **44**(11):4426-4433.

53. Yehiayan L, Pattabiraman M, Kavallieratos K, Wang X, Boise LH, Cai Y: **Speciation, formation, stability and analytical challenges of human arsenic metabolites**. *J Anal At Spectrom* 2009, **24**(10):1397-1405.

54. Finnegan PM, Chen W: **Arsenic toxicity: the effects on plant metabolism**. *Front Physiol* 2012, **3**:182.

55. Castillo-Michel H, Hernandez-Viezcas JA, Servin A, Peralia-Videa JR, Gardea-Torresdey JL: **Arsenic localization and speciation in the root-soil interface of the desert plant Prosopis juliflora-velutina**. *Appl Spectrosc* 2012, **66**(6):719-727.

56. Zaman GJ, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RP, Baas F, Borst P: **Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein**. *Proc Natl Acad Sci U S A* 1995, **92**(17):7690-7694.

57. Lee K, Klein-Szanto AJ, Kruh GD: **Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells**. *J Natl Cancer Inst* 2000, **92**(23):1934-1940.

58. Petrick JS, Ayala-Fierro F, Cullen WR, Carter DE, Vasken Aposhian H: **Monomethylarsonous acid (MMA(III)) is more toxic than arsenite in Chang human hepatocytes**. *Toxicol Appl Pharmacol* 2000, **163**(2):203-207.

59. Challenger F: **Biological methylation**. *Adv Enzymol Relat Subj Biochem* 1951, **12**:429-491.

60. Hayakawa T, Kobayashi Y, Cui X, Hirano S: **A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19**. *Arch Toxicol* 2005, **79**(4):183-191.

61. Naranmandura H, Suzuki N, Suzuki KT: **Trivalent arsenicals are bound to proteins during reductive methylation**. *Chem Res Toxicol* 2006, **19**(8):1010-1018.

Chapter 6

General Discussion

6 General Discussion

In this chapter the main findings of my thesis will be summarized and future directions described.

6.1 Chapter 3: Selenium dependent and independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/*ABCC2*): implications for the mutual detoxification of arsenic and selenium.

The ability of arsenic and selenium to prevent the toxicity of each other has been well documented since the early 1900s [1-3]. Advances in analytical chemistry had identified the formation of $[(GS)_2AsSe]^-$ in rabbit bile and erythrocyte lysate. With MRP2 being localized to the canalicular membrane of hepatocytes and capable of transporting $As(GS)_3$, we hypothesized that MRP2 would also be capable of transporting $[(GS)_2AsSe]^-$. As expected, $[(GS)_2AsSe]^-$ was transported by MRP2 with a 2.5-fold higher affinity than $As(GS)_3$ but with remarkably similar transport efficiencies (K_m/V_{max}).

These findings contributed to a strong mechanistic explanation for the cooperative detoxification of arsenic and selenium. Our data shows that in addition to transporting $As(GS)_3$, MRP2 is also likely responsible for transporting $[(GS)_2AsSe]^-$ into bile. The higher stability of $[(GS)_2AsSe]^-$ in bile compared with $As(GS)_3$ and $MMA(GS)_2$ is likely the critical component of As/Se cooperative detoxification by allowing arsenic to be eliminated in feces rather than being reabsorbed in the intestine.

Future epidemiological studies based around a better understanding of this interaction are necessary. Selenium is a ubiquitous element very similar to arsenic and enters the food chain from its presence in soil and in most areas of the world, people reach their daily requirement inadvertently. However, some areas in China have been shown to have very low levels of selenium in the soil, and thus dietary intake of selenium is greatly reduced. [4, 5]. In addition an individual's selenium status will be reduced by the depleting effect of As. Studies focusing on individuals in these areas with monitoring of dietary arsenic and selenium intake and the effect of selenium supplementation might reveal an effect of selenium.

In addition to the need for epidemiological studies on the effect of selenium on arsenic toxicity and carcinogenicity, a better understanding of the interaction between arsenic and selenium in extrahepatic tissue is needed. For example, we have made an unpublished observation that incubation of red blood cells with selenium greatly increased the accumulation of radiolabelled arsenic within red blood cells (Figure 7.1 Appendix). In addition to increasing biliary excretion of arsenic, it seems feasible that selenium aids in sequestering arsenic in red blood cells and thus aids in preventing As toxicity through both mechanisms. *In vivo* data has previously shown that injection of As and Se delays the hepatic accumulation and transport of each, into bile, by extending their time in blood [6, 7]. Further research on human red blood cell formation of $[(GS)_2AsSe]^-$ is needed, including an explanation of how As and Se are sequestered in red blood

cells and then are liberated in the liver to enter hepatocytes and be transported into bile by MRP2.

6.2 Chapter 4: Monomethylarsenic diglutathione [$MMA^{III}(GS)_2$] transport by the human multidrug resistance protein 1 (MRP1/*ABCC1*).

We explored the ability of MRP1 to protect cells from inorganic and methylated arsenic species using cytotoxicity, accumulation, and direct transport assays. By comparing the cell survival of HeLa-MRP1 with HeLa-vector cell lines treated with As^V, As^{III}, MMA^V, MMA^{III}, DMA^V, and DMA^{III} we found that expression of MRP1 protected cells from As^V, As^{III}, and MMA^{III} (As^V and As^{III} results were consistent with previously published data). We also showed that cells expressing MRP1 had a lower accumulation of total arsenic after a 24 h exposure to As^{III} and MMA^{III} supporting the hypothesis that in cells expressing MRP1, resistance is conferred through cellular efflux. Using membrane vesicles from cells expressing MRP1, we were able to show that MRP1 is capable of transporting MMA^{III} as its glutathione conjugate, $MMA^{III}(GS)_2$.

These findings demonstrated an exciting and newly understood role for MRP1 in the cellular protection against MMA^{III}. The methylated species of arsenic have increased toxicity compared to As^{III}, and have been shown to be more potent inducers of apoptosis in leukemia and lymphoma cells [8]. MRP1 is highly polymorphic and future studies should focus on polymorphic variants of MRP1 which may alter transport of either of these arsenic conjugates and could account for the established but poorly understood interindividual susceptibility to arsenic-induced carcinogenesis [9, 10].

6.3 Chapter 5: Human Multidrug Resistance Protein 4 (MRP4/*ABCC4*) Protects Cells from Arsenic by Transporting $MMA(GS)_2$ and DMA^V

Dimethylated arsenic is considered the end product of arsenic metabolism in humans, compared to bacteria, rodents, and marine organisms, which have been shown to produce trimethylated arsenic species [11, 12]. In humans, DMA^V accounts for 60-70% of total arsenic eliminated in urine. Thus, understanding the transport of $DMA^{III/V}$ is critical for understanding arsenic elimination. Due to the localization of MRP4 at the basolateral membrane of hepatocytes and the apical membrane of the proximal tubule in the kidney, MRP4 was the first candidate we investigated for its ability to confer resistance to and transport As^V, As^{III}, MMA^V, MMA^{III}, DMA^V, and DMA^{III}. Unlike MRP1 and MRP2, MRP4 was incapable of transporting $As(GS)_3$. It was however found to be able to transport $MMA(GS)_2$ and DMA^V.

The cellular proportions of DMA^V and DMA^{III} are unknown. I propose that the majority of DMA in the cell exists as DMA^V and under normal physiological conditions, the reducing environment of the cell is not strong enough to keep DMA^{III} reduced. The first piece of evidence to support my hypothesis would be further studies into the oxidative status of DMA in cell culture media, in addition to cytosol. Due to the time required to prepare samples and run on HPLC-ICP-MS this may prove difficult due to the rapid oxidation of DMA^{III} to DMA^V. MRP4 is also very polymorphic and future site directed mutagenesis studies will be performed with the aim of identifying polymorphic variants of MRP4 which may have altered ability to transport DMA^V. In addition, As speciation experiments of HEK293 cells demonstrated a high level of

methylation after As exposure and also revealed three unknown peaks. The identities of these unknown species (e.g. As-thiol compound) should be established through HPLC-ICP-MS.

These findings demonstrate a crucial role for MRP4 in the clearance of arsenic. In addition to the ability of MRP4 to transport $MMA(GS)_2$, it is also currently the only identified protein capable of transporting DMA^V. The basolateral localization of MRP4 in the liver and the apical localization in the proximal tubule also contribute to its significance by allowing transport across two crucial membranes necessary for the passage of arsenic methylated in the liver and ultimately eliminated in urine (Figure 6.1).

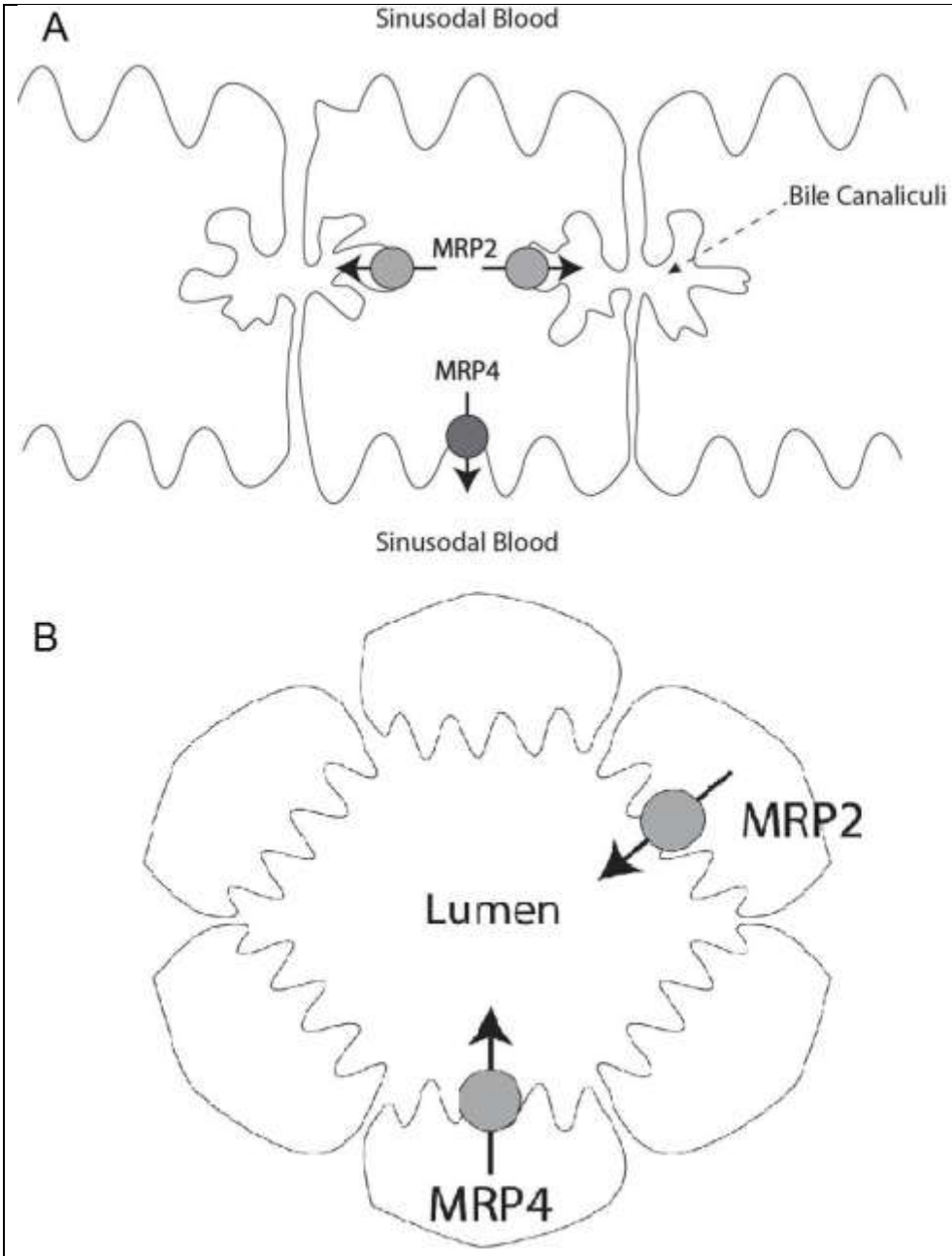

Figure 6.1 Localization of MRP2 and MRP4 in the Hepatocyte and the Proximal Tubule. A) Localization of MRP4 is shown at the basolateral surface of a hepatocyte and MRP2 is shown at the canalicular (apical) surface. B) Expression of both MRP2 and MRP4 at the apical surface of proximal tubular cells.

6.4 Bibliography

1. Moxon AL: **The Effect of Arsenic on the Toxicity of Seleniferous Grains**. *Science* 1938, **88**(2273):81.
2. Levander OA, Baumann CA: **Selenium metabolism. VI. Effect of arsenic on the excretion of selenium in the bile**. *Toxicol Appl Pharmacol* 1966, **9**(1):106-115.
3. Zeng H, Uthus EO, Combs GF, Jr.: **Mechanistic aspects of the interaction between selenium and arsenic**. *J Inorg Biochem* 2005, **99**(6):1269-1274.
4. Hatfield DL, Gladyshev VN: **The Outcome of Selenium and Vitamin E Cancer Prevention Trial (SELECT) reveals the need for better understanding of selenium biology**. *Mol Interv* 2009, **9**(1):18-21.
5. Fleet JC: **Dietary selenium repletion may reduce cancer incidence in people at high risk who live in areas with low soil selenium**. *Nutr Rev* 1997, **55**(7):277-279.
6. Gregus Z, Gyurasics A, Koszorus L: **Interactions between selenium and group Va-metalloids (arsenic, antimony and bismuth) in the biliary excretion**. *Environ Toxicol Pharmacol* 1998, **5**(2):89-99.
7. Palmer IS: **Modification of Selenite metabolism by arsenite.** *J Agric Food Chem* 1957, **5**:89-99.
8. Chen GQ, Zhou L, Styblo M, Walton F, Jing Y, Weinberg R, Chen Z, Waxman S: **Methylated metabolites of arsenic trioxide are more potent than arsenic trioxide as apoptotic but not differentiation**

inducers in leukemia and lymphoma cells. *Cancer Res* 2003, **63**(8):1853-1859.

9. Conseil G, Deeley RG, Cole SP: **Polymorphisms of MRP1 (ABCC1) and related ATP-dependent drug transporters**. *Pharmacogenet Genomics* 2005, **15**(8):523-533.

10. Hernandez A, Marcos R: **Genetic variations associated with interindividual sensitivity in the response to arsenic exposure**. *Pharmacogenomics* 2008, **9**(8):1113-1132.

11. Xi S, Jin Y, Lv X, Sun G: **Distribution and speciation of arsenic by transplacental and early life exposure to inorganic arsenic in offspring rats**. *Biol Trace Elem Res* 2010, **134**(1):84-97.

12. Jin Y, Xi S, Li X, Lu C, Li G, Xu Y, Qu C, Niu Y, Sun G: **Arsenic speciation transported through the placenta from mother mice to their newborn pups**. *Environ Res* 2006, **101**(3):349-355.

Chapter 7

Appendix

7 Appendix

7.1 Role of Cysteine Residues in the Human Multidrug Resistance Protein 1 (MRP1/*ABCC1*) for the transport of $As(GS)_3$.

7.1.1 *Introduction*

Arsenic (As) is a multi-target human carcinogen: chronic exposure is associated with increased incidences of skin, lung, and bladder tumours [1]. This metalloid occurs naturally in the earth's crust and it is estimated that over 100 million people worldwide are exposed to levels in drinking water that are capable of causing adverse health effects [2]. Despite its acute and chronic toxicity, arsenic trioxide (As_2O_3) is used successfully in the treatment of acute promyelocytic leukemia and is in clinical trials for the treatment of multiple haematological and solid tumors [3, 4].

The multidrug resistance protein 1 (MRP1/*ABCC1*) is an ATP-binding cassette transporter protein that was originally identified based on its ability to confer multidrug resistance in tumour cell lines through an ATP-dependent decrease in cellular drug accumulation [5]. In addition to expression in tumors, MRP1 is widely expressed in non-malignant tissue and transports a chemically diverse array of endogenous molecules including reduced and oxidized glutathione (GSH and GSSG, respectively), conjugated organic anions such as the cholestatic steroid 17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG) and the cysteinyl leukotriene LTC_4 [6]. In addition to anti-cancer agents and physiological substrates, MRP1 transports many other drugs, carcinogens and toxicants and is believed to play a protective role by preventing xenobiotic accumulation and

resulting toxicity [7]. MRP1 is a well characterized transporter of As^{III} in its triglutathione conjugate form $As(GS)_3$ [8], and is thought to be important for providing cellular protection against inorganic arsenic exposure.

The tripeptide glutathione (GSH) plays a diverse and complicated role in the transport of MRP1 substrates [9]. In addition to the substrates mentioned previously in which conjugation to GSH is required for transport, several substrates that do not form conjugates are transported in a GSH dependent manner and a cotransport mechanism has been proposed [6]. GSH is required for transport as both a requirement for substrates such as NNAL-*O*-glucuronide (4-(nitrosamino)-1-(3-pyridyl)-1-butanol-*O*-glucuronide) [10] and to stimulate substrates such as estrone sulphate [11], but in both cases GSH itself is not transported. In addition there are many organic anion substrates of MRP1 that are transported independently of GSH including methotrexate and estradiol glucuronide ($E_2$17βG) [12, 13].

MRP1 contains 25 cysteine residues and the function of these residues has been investigated previously for the purpose of creating a cysteine-less MRP1 mutant for cysteine scanning mutagenesis [14, 15]. Qin et al., demonstrated that a three cysteine MRP1 (Cys388, Cys 1439, Cys1479) could be expressed and maintained transport activity similar to wild-type [8]. Cysteine residues are involved in As binding for the bacterial arsenic ATPase, ArsA. Bhattacharjee et al. showed that the three crucial cysteine residues forming the substrate binding pocket work cooperatively, and the removal of any two of the three cysteine residues abolished transport function [16]. The metallochaperone ArsD has also

been shown to interact with As via three specific Cys residues [17, 18].

Cys residues at positions 43, 49, 85, 148, 190, 208, and 265 of MRP1 were previously mutated to alanine and serine and expressed in HeLa cells. With the exception of C43S, which showed a 2.5-fold decrease in cellular resistance to arsenite and C265S, which had a 3-fold increased resistance to arsenite, none of these residues showed an effect on arsenite resistance [19]. Cys residues at the positions 7 and 32 affect the structure of the protein [19, 20] and were also ruled out for the current study.

The purpose of this study was to determine if cysteine residues within MRP1 are involved in the transport of $As(GS)_3$. Following our observation that reducing agents dithiothreitol (DTT), β-mercaptoethanol, and the cysteine modifying compound N-ethylmaleimide (NEM) inhibited the transport of $As(GS)_3$, but not other MRP1 substrates, we hypothesized that there is at least one cysteine residue in the MRP1 binding site that is critical for $As(GS)_3$ binding and transport. Fourteen individual Cys to Ala mutants, one Cys to Ser mutant, and one double Cys mutant were created and the function of these MRP1 proteins was investigated (Figure 7.1).

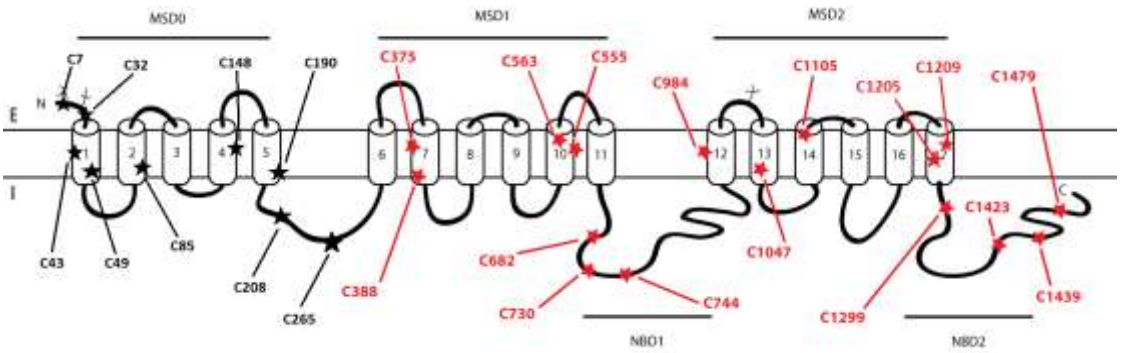

Figure 7.1. Predicted Topology Map of MRP1. Topology of the MRP1 protein indicating the location of all cysteine residues. Those selected for individual replacement with alanine are indicated with grey/red stars.

7.1.2 *Methods*

7.1.2.1 *Site directed mutagenesis*

MRP1 cysteine mutations (Cys375Ala, Cys388Ala, Cys555Ala, Cys563Ala, Cys682Ala, Cys730Ala, Cys744Ala, Cys984Ala, Cys1047Ala, Cys1047Ser, Cys1105Ala, Cys1205/1209Ala, Cys1299Ala, Cys1423Ala, Cys1439Ala, Cys1479Ala) were generated using the Stratagene Quikchange II site directed mutagenesis kit (Stratagene, La Jolla, CA). The template for mutagenesis was wild type (WT) MRP1 in pcDNA3.1(-) and was a gift from Dr. Susan P.C. Cole (Queen's University), constructed as described previously [21]. Mutagenesis was performed according to the manufacturer's instructions with the mutagenic primers listed in Table 1. Mutations were confirmed by sequencing (Macrogen, Maryland, USA).

7.1.2.2 *WT-MRP1 and Cys mutant expression in HEK293T cells and membrane vesicle preparation*

HEK293T cells were seeded on 150 mm culture dishes and transfected using the calcium phosphate method with modifications, as described previously [22]. Seventy-two hours post-transfection, cells were washed twice with Tris–sucrose buffer (50 mM Tris, pH 7.4, 250 mM sucrose), scraped into Tris–sucrose buffer and collected by centrifuging at 800*g* for 10 min. Membrane vesicles were prepared as previously described [22] and expression of MRP1 in membrane vesicles was confirmed by immunoblot analysis, using the rat monoclonal antibody (mAb), MRPr1 (1:10000) (Novus Biologicals, Littleton, CO). Relative

levels of MRP1 expression were estimated by densitometry using ImageJ software (NIH, Maryland).

7.1.2.3 *Chemical synthesis of $^{73}As^{III}$, $^{73}As(GS)_3$*

$^{73}As^{III}$ (50 µM, 50 nCi/µl) was prepared from $^{73}As^{V}$ with metabisulfite-thiosulfate reagent as described previously [23]. $^{73}As(GS)_3$ was prepared as described previously [24], with minor modifications. Briefly, $^{73}As^{III}$ (final concentration 25 µM) and GSH (final concentration 75 mM) were mixed under a nitrogen atmosphere in Tris–sucrose buffer and incubated at 4ºC for 30 min.

7.1.2.4 *Thin layer chromatography*

The formation of $^{73}As^{III}$ was monitored using a previously established thin-layer chromatography method [25]. The effects of NEM (0.01-100 mM) and DTT (0.1-100 µM) on $^{73}As(GS)_3$ stability were also determined using the same method. Prior to use, SigmaCell Type 100 cellulose plates (Sigma) were pre-run with H_20 and dried. Samples (1-10 µl) were loaded 1 µl at a time and allowed to dry between applications. Plates were developed with an isopropanol:acetic acid:water (10:1:5) solvent system in a sealed chamber until the solvent front reached ~2 cm from the top. The solvent front was marked, the plate dried and placed on film for ~16 hours.

7.1.2.5 *$^{73}As(GS)_3$ transport assays*

Transport studies were completed using the rapid filtration method [8, 26]. Briefly, membrane vesicles (20 µg) were incubated at 37°C in Tris–sucrose buffer, with ATP or AMP (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 µg/ml), GSH reductase (5 µg/ml), NADPH (0.35 mM), GSH

(3 mM) and $^{73}As(GS)_3$ (1 µM). At the indicated time point, transport was stopped by diluting the transport reaction with 800 µl of ice-cold Tris–sucrose buffer. Samples were filtered over glass fiber filters and radioactivity was quantified by liquid scintillation counting. ATP-dependent transport was determined by subtracting transport in the presence of AMP from the transport in the presence of ATP.

The effects of N-ethyl maleimide (NEM) (1 mM) and dithiothreitol (DTT) (0.1-100 µM) on transport were measured at a 3 min time point. Under certain NEM conditions, NEM was washed from membrane vesicles by centrifugation at 100,000 x g at 4 ºC for 15 min, followed by resuspension of membrane vesicles in tris sucrose buffer equal to the original volume.

Kinetic parameters of $^{73}As(GS)_3$ transport were determined by measuring the initial rate of uptake at eight different substrate concentrations (0.1–10 µM, 20–110 nCi) at a 1 min time point. K_m and V_{max} values were determined using the Michaelis-Menten kinetic analysis curve fit (Graphpad Prism™ 5).

7.1.3 *Results*

7.1.3.1 *Transport of $^{73}As(GS)_3$ is inhibited by DTT.*

DTT is commonly used at mM concentrations in MRP1 transport assays containing GSH to prevent its oxidation to GSSG and has no influence on MRP1 transport of LTC_4, $E_2$17βG, or estrone 3-sulphate [13, 26-28]. To confirm that this was the case in our hands, the influence of DTT (10 mM) on the MRP1 mediated transport of $E^2$17βG (400 nM) was tested. As expected, DTT had no effect of the transport of $E_2$17βG (Figure 7.2A). However, we unexpectedly

found that DTT abolished transport of $As(GS)_3$ (1 µM) at concentrations as low as 100 µM (Figure 7.2B). Inhibition was observed at DTT concentrations as low as 0.1 µM which reduced $As(GS)_3$ (1 µM) transport by 30% (Figure 7.2A).

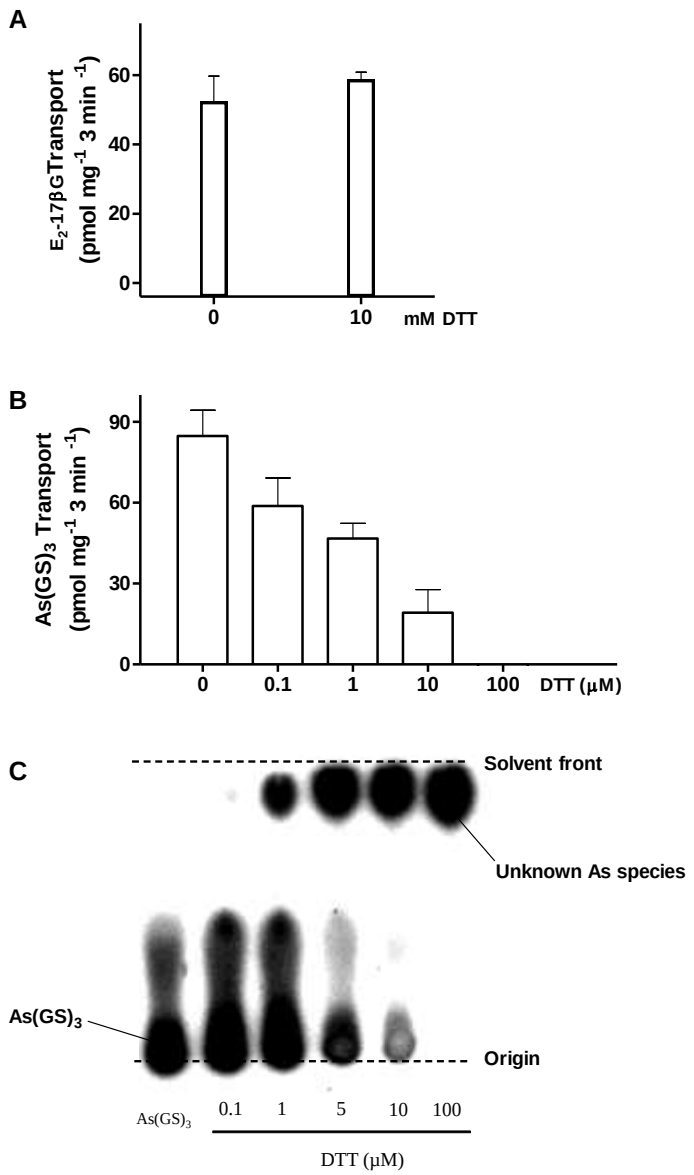

Figure 7.2. Effect of DTT on $^{73}As(GS)_3$ transport by MRP1. (A) Membrane vesicles (10 μg protein per point) prepared from MRP1 transiently transfected HEK293T cells were incubated for 3 min at 37^{0}C in transport buffer with $E_2$17βG (400 nM, 20 nCi) and in the presence and absence of DTT (10 mM). Membrane vesicles (20 μg protein per point) prepared from MRP1 transiently transfected HEK293T cells were incubated for 3 min at 37^{0}C in transport buffer with (B) $^{73}As(GS)_3$ (1 μM, 50 nCi) and DTT (0.1, 1, 10, or 100 μM). Columns represent the means (±SD) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments (C) Thin layer chromatograms of $^{73}As(GS)_3$ (1 μM, 1 μCi) incubated with DTT.

7.1.3.2 *DTT affects the stability of $^{73}As(GS)_3$.*

In order to assess the effect of DTT on the stability of $^{73}As(GS)_3$, a range of DTT concentrations were incubated with $^{73}As(GS)_3$ (1 µM) and analyzed by TLC. DTT caused a substantial breakdown of $^{73}As(GS)_3$ starting as low as 1 µM, and completely dissociated the conjugate at 100 µM (Figure 7.2B). Thus the, influence of DTT on MRP1 transport of $As(GS)_3$ is likely due to $^{73}As(GS)_3$ instability.

7.1.3.3 *Transport of $^{73}As(GS)_3$ is inhibited by NEM*

NEM is a thiol alkylating agent and thus a covalent modifier of free cysteine residues. NEM has no effect on $E_2$17βG transport and was confirmed in the present study (Figure 7.3). To determine the contribution of free Cys residues to $As(GS)_3$ transport, the influence of NEM on MRP1 transport of $As(GS)_3$ was measured. MRP1-enriched vesicles treated with NEM (1 mM) for 30 min on ice followed by 10 min at 37 ºC resulted in a reduction of $As(GS)_3$ transport by ~80% compared with untreated control. To rule out the possibility that NEM destabilizes $As(GS)_3$, NEM was incubated with MRP1-enriched membrane vesicles, then washed out (as described in methods), prior to the measurement of transport activity. Washing the membrane vesicles after incubation at 37 ºC for 10 minutes without NEM had no significant effect on $As(GS)_3$ transport. Incubation of vesicles at 4 ºC with 1 mM NEM followed by washing had no effect on $As(GS)_3$ transport. Incubation for 10 min at 37ºC in the presence of 1 mM NEM followed by washing the membrane vesicles resulted in a 60% reduction in transport (Figure 7.3A).

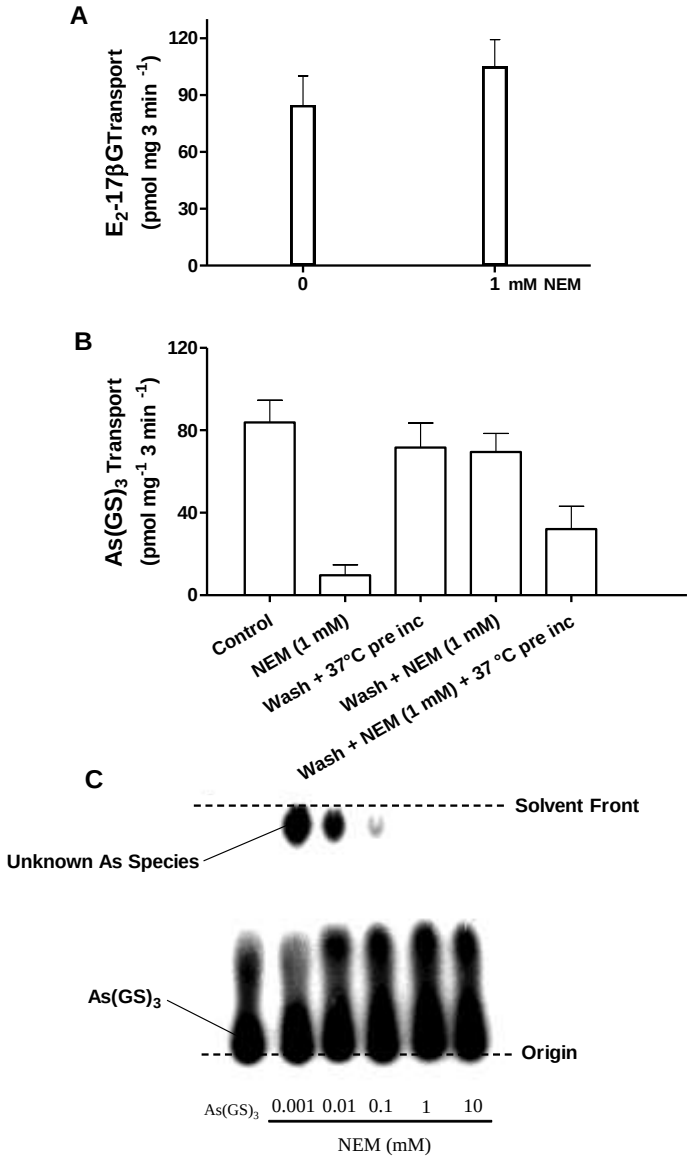

Figure 7.3. Effect of NEM on $^{73}As(GS)_3$ transport by MRP1. (A) Membrane vesicles (10 µg protein) prepared from MRP1 transiently transfected HEK293T cells were incubated on ice for 30 min in the presence or absence of NEM (1 mM) and then for 3 min at 37^0C in transport buffer with $E_2$17βG (400 nM, 20 nCi). (B) Membrane vesicles (20 µg protein) prepared from MRP1 expressing cells were incubated with NEM (1 mM) on ice for 30 min, at 37^0C for 10 min, and used for transport, or were washed by ultracentrifugation at 100,000 x g for 15 min and resuspended to remove all unbound NEM. (C) Thin layer chromatograms of $^{73}As(GS)_3$ (1 µM,1 µCi) incubated with NEM.

7.1.3.4 *NEM does not affect the stability of $^{73}As(GS)_3$.*

In order to assess the effect of NEM on the stability of $^{73}As(GS)_3$, NEM (0.01-100 mM) was incubated with $^{73}As(GS)_3$ (1 µM) and analyzed by TLC. In contrast to DTT, NEM showed no effect on $As(GS)_3$ stability at concentrations up to 10 mM. Conjugate breakdown was therefore observed at concentrations 100-fold higher than what was required to completely inhibit transport function (Figure 7.3B).

7.1.3.5 *All MRP1 cysteine mutants exhibit $E_2$17βG transport activity at levels similar to wild-type.*

Cys → Ala mutants were created, mutating 16 individual Cys residues in MRP1 in an attempt to identify a crucial residue necessary for the transport of $As(GS)_3$. Of the 16 Cys residues selected, seven mutants were Cys residues in the cytosolic loops (C730, C744, C682, C1299, C1423, C1439, C1479), and 9 were residues predicted to be in transmembrane helices (C375, C388, C563, C555, C984, C1047, C1105, C1205, C1209). In addition to the creation of a C1047A mutant, a C1047S mutant was also generated since it is a single nucleotide polymorphism of MRP1 [29]. All mutants were expressed at comparable levels to wild-type ranging from 0.5 to 1.3-fold WT (Figure 7.4). DTT and NEM had no effect on the transport of $E_2$17βG by wild type MRP1, suggesting $E_2$17βG binding and transport does not require a free Cys residue. Thus, $E_2$17βG was used as a control to ensure that individual Cys mutants were still transport-competent. All Cys mutants transported $E_2$17βG at a rate within ±50% of WT-MRP1, when corrected for protein expression, suggesting that individual Cys mutants retain transport function (Figure 7.5).

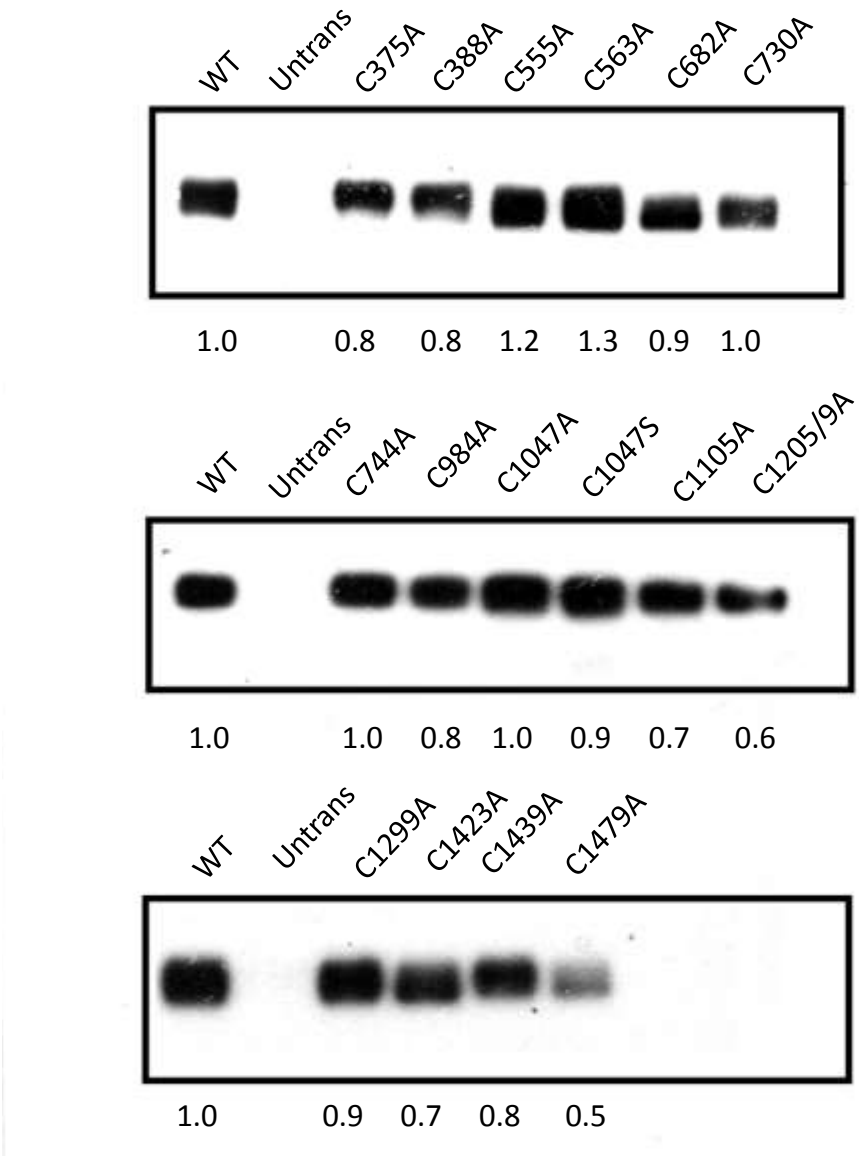

Figure 7.4. Expression of MRP1 Cysteine mutants. Immunoblot of membrane vesicles prepared from MRP1 WT and MRP1 Cys mutants transiently expressed in HEK293T cells. Plasma membrane enriched vesicle proteins (1 µg/lane) were resolved on a 7% acrylamide gel, transferred, and probed with the MRP1 monoclonal antibody, MRPr1, as described in "Methods". Relative expression levels of the Cys mutant proteins as compared with WT-MRP1 were determined by densitometric analysis using ImageJ software (NIH, Maryland).

7.1.3.6 *MRP1 Cys730Ala exhibits increased $^{73}As(GS)_3$ transport.*

In order to determine if an individual cysteine residue is crucial for the transport of $As(GS)_3$ by MRP1, the $As(GS)_3$ transport activity of all Cys → Ala mutants was determined. Unexpectedly, no mutants had $As(GS)_3$ transport activity reduced by more than 35% (Figure 7.6). Transport activity for C388A, C555A, and C744A was reduced by 28%, 33%, and 16% respectively. In contrast, C730A, C984A, C1105A, C1205/1209A, C1423A, and C1479A had 94%, 26%, 21%, 68%, 48%, and 70% increased levels of $As(GS)_3$ transport activity, respectively, compared with wild type-MRP1.

7.1.3.7 *Kinetic analysis of MRP1 Cys730Ala.*

To characterize the two-fold difference in transport activity for MRP1-Cys730Ala kinetic parameters were determined by measuring the initial rates of $As(GS)_3$ uptake over several concentrations of $As(GS)_3$. According to Michaelis-Menten kinetic analysis (Graph-pad Prism™ 5), the apparent K_m of MRP1-Cys730Ala for $As(GS)_3$ was found to be 10.6 µM and the V_{max} was 453 nmol/mg/min, compared to wild type-MRP1 with an apparent K_m of 8.3 µM and a V_{max} of 283 nmol/mg/min (Figure 7.7).

7.1.3.8 *Transport of $As(GS)_3$ by MRP1 cysteine mutants in the presence of NEM*

In order to test the possibility that NEM bound to a specific cysteine residue in the $As(GS)_3$ binding site of MRP1 and not interacting with $As(GS)_3$, all

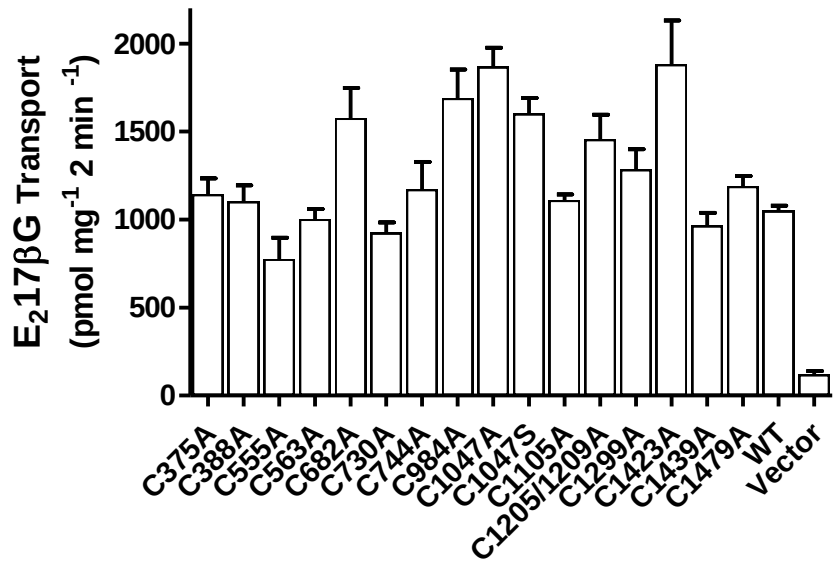

Figure 7.5. MRP1 cysteine mutant-mediated transport of $E_2$17βG. Membrane vesicles (10 µg protein per point) prepared from MRP1 cysteine mutant transiently transfected HEK293T cells were incubated for 3 min at 37^{0}C in transport buffer with $E_2$17βG (400 nM, 20 nCi). Columns represent the means (±SD) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments. Relative expression levels of the Cys mutant proteins as compared with WT-MRP1 were determined by densitometric analysis and transport activity was corrected for expression relative to WT.

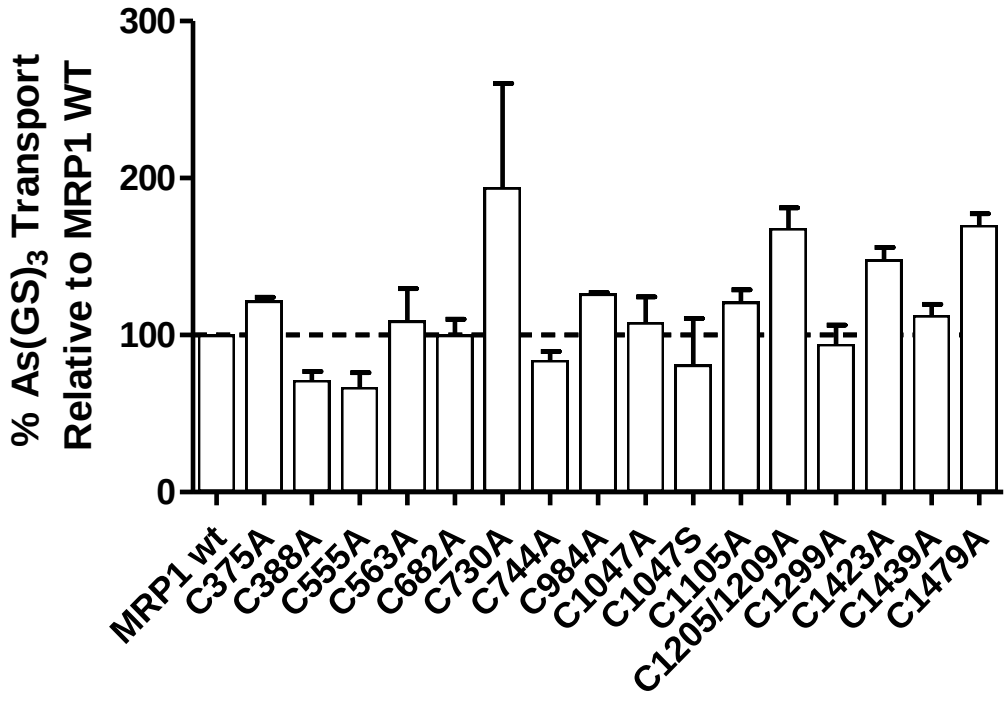

Figure 7.6. MRP1 cysteine mutant-mediated transport of $^{73}As(GS)_3$. Membrane vesicles (20 µg protein per point) prepared from MRP1 cysteine mutant transiently transfected HEK293T cells were incubated for 3 min at 37^0C in transport buffer with $^{73}As(GS)_3$ (1 µM, 50 nCi). Columns represent the means (±SD) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments. Relative expression levels of the Cys mutant proteins as compared with WT-MRP1 were determined by densitometric analysis, and used to correct transport activity..

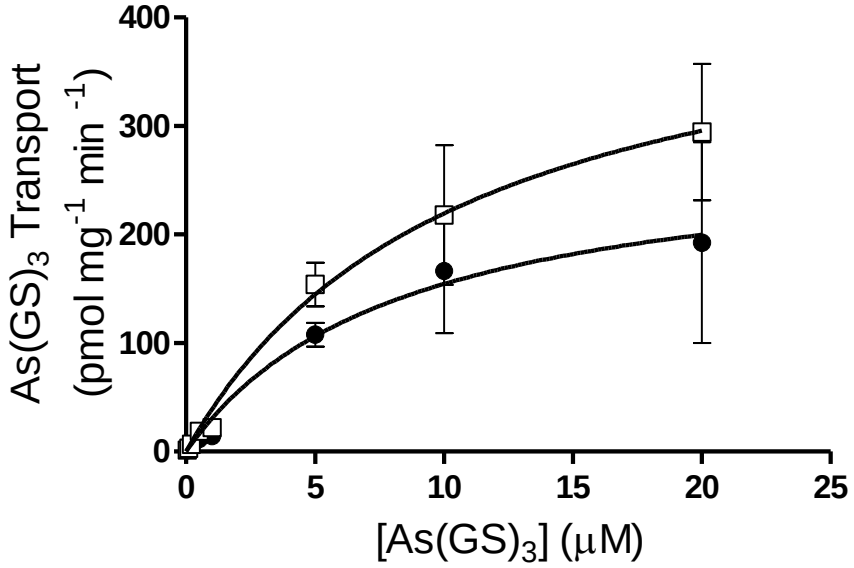

Figure 7.7. Kinetic analysis of MRP1-mediated $^{73}As(GS)_3$ transport. Membrane vesicles (20 μg of protein) prepared from MRP1 wt (●) and MRP1 C730A (□) transfected HEK293T cells were incubated for 1 min at 37^{0}C in transport buffer with various concentrations of $^{73}As(GS)_3$ (0.1-10 μM). Kinetic parameters were determined using the Michaelis-Menten kinetic model in Graphpad Prism™ 5 software. Points shown represent the means (±SD) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments.

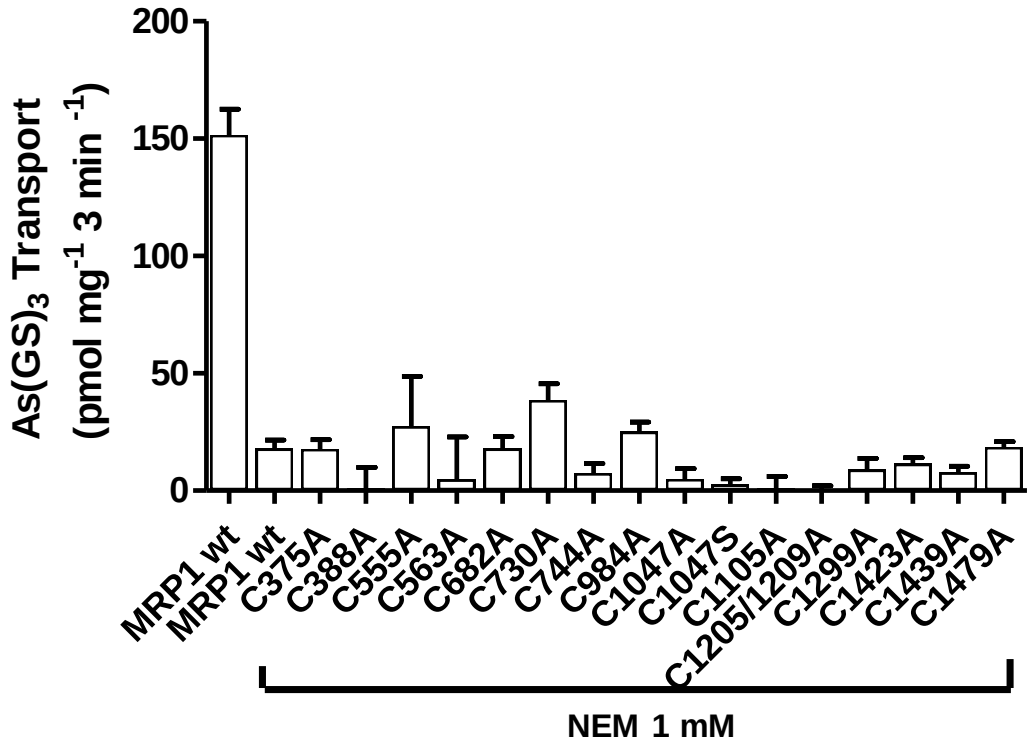

Figure 7.8. MRP1 cysteine mutant-mediated transport of $^{73}As(GS)_3$ in the presence of NEM. Membrane vesicles (20 µg protein per point) prepared from MRP1 cysteine mutant transiently transfected HEK293T cells were incubated on ice with 1 mM NEM for 30 min. Vesicles were then incubated for 3 min at 37^0C in transport buffer with $^{73}As(GS)_3$ (1 µM, 50 nCi). Data are normalized for MRP1 expression. Columns represent the means (±SD) of triplicate determinations in a single experiment; similar results were obtained in two additional experiments.

cysteine mutants were tested for transport function after incubation with NEM. However, all cysteine mutants were inhibited by NEM, suggesting that individually mutated cysteine residues are not critical for $^{73}As(GS)_3$ transport (Figure 7.8). This could suggest multiple cysteine residues are involved in $^{73}As(GS)_3$ transport, as has been observed previously with the bacterial ABC transporter ArsA [30].

Discussion

DTT has been used in the study of MRP1 transport to maintain GSH in its reduced form and has no influence on the transport of LTC_4, $E^2$17βG, or estrone-3-sulphate [13, 26-28]. Following the observation that DTT inhibited the transport of $As(GS)_3$ by MRP1, we examined the effect of the Cys modifying reagent NEM and found that preincubation of MRP1-enriched membrane vesicles with NEM also inhibited transport of $As(GS)_3$. In order to rule out the possibility that DTT and NEM were destabilizing $As(GS)_3$ instead of acting on MRP1, we examined the effect of a concentration range of each compound on the stability of $As(GS)_3$, analyzed by TLC. The inhibition of transport by DTT corresponded with a destabilization of $As(GS)_3$, leaving the effect of DTT on MRP1 as inconclusive. However, NEM has no effect on $As(GS)_3$ stability at concentrations that completely inhibited transport. This suggested that NEM inhibited transport by binding to one or more free Cys residues in MRP1. In order to determine if MRP1 was still functional in the presence of NEM, the transport of $E_2$17βG was measured. With no effect on the transport of $E_2$17βG, we hypothesized that there is one or more Cys residues in MRP1 crucial for the

transport of $As(GS)_3$ and that by substituting individual Cys residues we would be able to reveal information about the $As(GS)_3$ binding site of MRP1.

MRP1 contains 25 Cys residues and in this study, we examined the effects of one Ser and 14 Ala substitutions, in addition to one double Ala substitution of Cys residues, on the ability of MRP1 to transport $As(GS)_3$. All mutants were expressed at levels comparable to WT-MRP1, suggesting that none of these Cys residues play a critical role in protein synthesis.

A truncated Cys-less MRP1 protein lacking the amino acids (1-204) has been previously expressed in yeast and showed only a modest difference in transport affinity for leukotriene C4 [15]. Further studies revealed that this protein failed to traffic to the membrane in mammalian cells, and transport function was severely compromised when expressed in insect (Sf21) cells [14, 15]. Ultimately, a triple Cys MRP1 mutant (Cys388, Cys 1439, Cys 1479) was expressed and exhibited normal transport function [14]. Our study has shown that individual Cys→Ala mutations in Cys388, Cys 1439, and Cys 1479 have no effect on $As(GS)_3$ or $E_2$17βG transport.

Unexpectedly, we were unable to identify a single Cys substitution that reduced $As(GS)_3$ transport by greater than 35% compared to WT-MRP1. However, it is likely that there is overlap between adjacent Cys residues that interact with $As(GS)_3$. Studies on the bacterial arsenic transporter, ArsA, have previously identified an arsenic binding 'pocket' consisting of 3 Cys residues

[16]. Removal of any 1 of these 3 Cys residues did not affect the ability of ArsA to bind arsenic, yet removal of any 2 of the 3 Cys residues prevented binding.

Our results suggest that a group of Cys residues is responsible for the binding of As(GS)3, but without high resolution crystal stuctures for MRP1 it is difficult to predict which Cys residues may form a 3-Cys pocket. The generation of a functional 5-Cys mutant has identified Cys residues at positions 208, 375, 388, 1439, and 1479 necessary for expression and function [14]. Coupled with our observation that the latter 4 Cys residues can be individually substituted out without significantly affecting transport function supports the concept of Cys overlap within MRP1. Cys1439 and Cys1479 are highly conserved within the ABCC transporter family [14] and are likely not involved in the transport of $As(GS)_3$. Cys388, interestingly is shared between MRP1 and MRP2, the only MRPs currently known to transport $As(GS)_3$. Modest inhibition in transport of $As(GS)_3$ was demonstrated for Cys388Ala, Cys555Ala, Cys682Ala, and Cys744Ala and an increase in transport was shown in Cys730Ala. These Cys residues should serve as a starting place for future studies to create multiple Cys substitution mutants.

7.2 Transport of DMA^{III} by human multidrug resistance protein 1

Experimental design

Membrane vesicles were incubated at 37ºC in Tris-sucrose buffer, with DMA^{III} (1 µM) or DMA^{III}(GS) (1 µM), ATP or AMP (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), GSH reductase (5 µg/ml), and NADPH (0.35 mM). GSH dependence was assessed by adding GSH (3 mM) or the non-reducing GSH analog ophthalmic acid (3 mM). At 3 min, transport was stopped by diluting the transport reaction in 800 µl ice-cold Tris-sucrose buffer and pelleting vesicles by centrifugation at 100,000 x g for 20 min at 4 ºC. Pelleted membrane vesicles were washed twice with 1 ml ice-cold Tris-sucrose buffer, digested in 250 µl concentrated nitric acid for 48 h, diluted 1:1 with deionized distilled water, and filtered through 0.45 µm syringe filters (Whatman, Toronto, ON). The total concentration of arsenic in each sample was determined by ICP-MS, using the standard addition method, as described previously [31, 32].

Results and discussion

Unexpectedly, MRP1 was found to transport DMA^{III} only in the absence of GSH (Fig 7.9). The non-reducing GSH analog, OA, had no effect on transport. The DMA(GS) conjugate was also transported similarly to DMA^{III} alone, but when GSH was added (3 mM) transport was inhibited (Fig 7.9). When the experiment was performed, the transport activity was inexplicable and disregarded as an artifact of the newly developed method used. Subsequent work with MRP4 revealed that MRP4 was also transporting DMA^{III}, but only in the absence of GSH. This led us to the hypothesis that MRP4 was capable of transporting

DMA^{V} and when GSH was present was preventing DMA^{III} from oxidizing to DMA^{V}. In humans, $DMA^{III/V}$ is the end product of As metabolism and the possibility that MRP1 is capable of transporting DMA^{V} requires further testing.

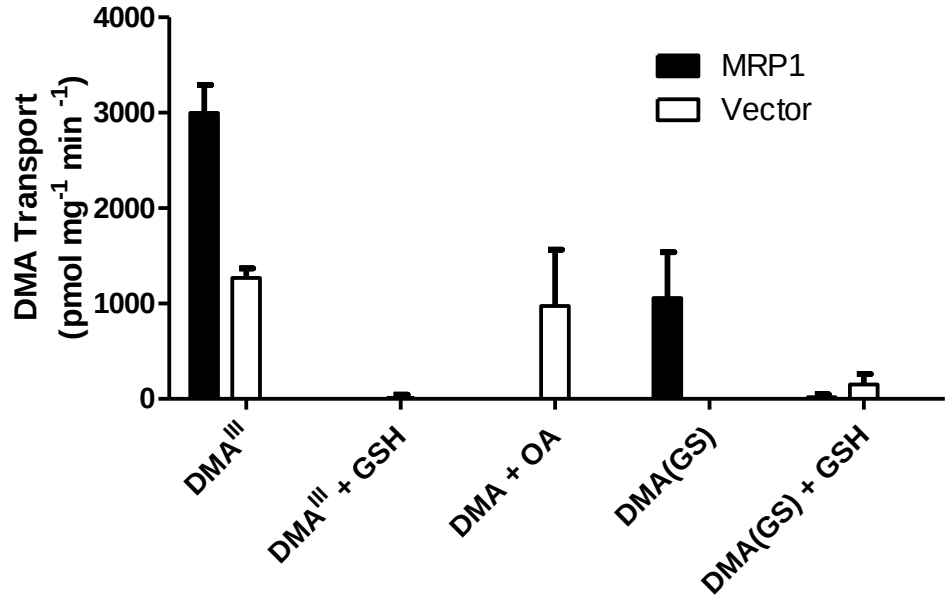

Figure 7.9 Transport of DMA by MRP1. Membrane vesicles (20 µg of protein) prepared from MRP1-transfected HEK293 cells were incubated for 3 min at 37ºC in transport buffer in triplicate containing DMA^{III} (1µM) and GSH (3 mM) or OA (3 mM), or $DMA^{III}(GS)$ (1µM). Reactions were then pooled for analysis by ICP-MS. Bars represent the means of 3 experiments.

7.3 The effect of selenium on the accumulation of arsenic in human erythrocytes.

Experimental design

$[(GS)_2AsSe]^-$ is assembled in rabbit erythrocyte lysate [33]. These experiments were performed to assess the ability of human erythrocytes to form this conjugate and thus affect the retention of arsenic, due to the increased stability of this conjugate over $As(GS)_3$. Additionally, MRP1 is expressed in erythrocytes, unlike MRP2, which is known to transport $[(GS)_2AsSe]^-$. Human blood samples (5 ml) were taken from volunteers and centrifuged at 5,000 x g for 5 min, plasma and white blood cells were removed and remaining erythrocytes were washed 3x by adding 10 ml isotonic saline solution (140 mM NaCl, 5 mM KCl, 10 mM MOPS, 5 mM glucose pH 7.4) and gently resuspending. After final wash, erythrocytes were resuspended to a final volume of 5 ml in isotonic saline. Erythrocytes were divided into 1 ml aliquots and incubated at 37 ºC. $^{73}As^{III}$ (1 µM, 100 nCi) was added to each tube in the presence or absence of Se^{IV} (1 µM), 200 µl aliquots were removed at 5, 15, 30, 45, and 60 minutes, and immediately filtered over glass fiber filters to remove extracellular arsenic. Radioactivity remaining was quantified by liquid scintillation counting and thus arsenic retained in erythrocytes was calculated.

Results and discussion

In the absence of selenium, arsenic accumulation in erythrocytes was low and did not significantly increase over time. Conversely, in the presence of selenium,

arsenic accumulation in erythrocytes was 2-fold higher than arsenic alone at 5 min and increased over time to 6-fold over arsenic alone at 60 min.

We have previously demonstrated the ability of MRP2 to transport $[(GS)_2AsSe]^-$ and thus shown a potential mechanism to explain the cooperative detoxification seen between arsenic and selenium. This data suggests a potential second mechanism by which selenium and arsenic decrease the toxicity of each other through sequestration in erythrocytes. Arsenic in erythrocytes is likely to form $As(GS)_3$ and would be transported out of the erythrocyte by MRP1. In the presence of selenium however, it has been shown in rabbit erythrocyte lysate that As, and Se form $[(GS)_2AsSe]^-$ and this conjugate is not transported by MRP1 and thus would be 'trapped' in the erythrocyte. It is unknown if this conjugate would form and later dissociate in the liver to allow for its biliary elimination as suggested in previous chapters. This mechanism presents a possible As 'sink' which reduces the As burden on the body in the presence of selenium.

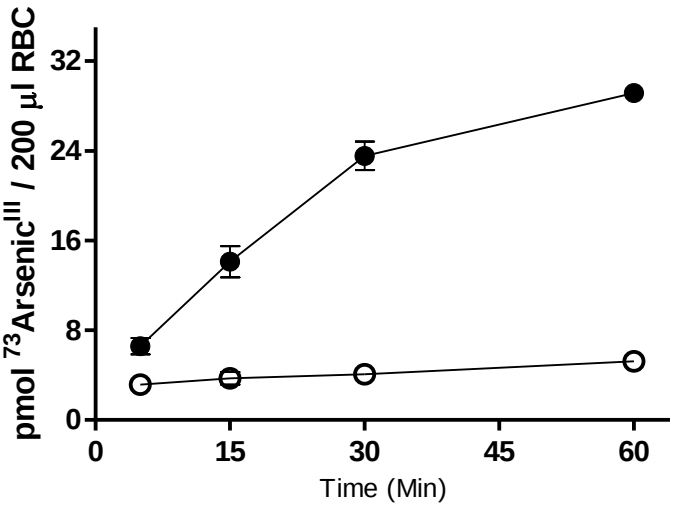

Figure 7.10. Accumulation of arsenic in red blood cells. Red blood cells were incubated with As^{III} (1µM, 100 nCi) in the presence (closed circles) or absence (open circles) of Se^{IV} (1 µM).

7.4 Bibliography

1. Straif K, Benbrahim-Tallaa L, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, Guha N, Freeman C, Galichet L, Cogliano V: **A review of human carcinogens--part C: metals, arsenic, dusts, and fibres**. *Lancet Oncol* 2009, **10**(5):453-454.
2. Rahman MM, Ng JC, Naidu R: **Chronic exposure of arsenic via drinking water and its adverse health impacts on humans**. *Environ Geochem Health* 2009, **31 Suppl 1**:189-200.
3. Emadi A, Gore SD: **Arsenic trioxide - An old drug rediscovered**. *Blood Rev* 2010, **24**(4-5):191-199.
4. Mann KK, Wallner B, Lossos IS, Miller WH, Jr.: **Darinaparsin: a novel organic arsenical with promising anticancer activity**. *Expert Opin Investig Drugs* 2009, **18**(11):1727-1734.
5. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG: **Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line**. *Science* 1992, **258**(5088):1650-1654.
6. Deeley RG, Westlake C, Cole SP: **Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins**. *Physiol Rev* 2006, **86**(3):849-899.
7. Leslie EM, Deeley RG, Cole SP: **Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense**. *Toxicol Appl Pharmacol* 2005, **204**(3):216-237.

8. Leslie EM, Haimeur A, Waalkes MP: **Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required**. *J Biol Chem* 2004, **279**(31):32700-32708.

9. Rothnie A, Conseil G, Lau AY, Deeley RG, Cole SP: **Mechanistic differences between GSH transport by multidrug resistance protein 1 (MRP1/ABCC1) and GSH modulation of MRP1-mediated transport**. *Mol Pharmacol* 2008, **74**(6):1630-1640.

10. Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, Cole SP: **Transport of the beta -O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog**. *J Biol Chem* 2001, **276**(30):27846-27854.

11. Rothnie A, Callaghan R, Deeley RG, Cole SP: **Role of GSH in estrone sulfate binding and translocation by the multidrug resistance protein 1 (MRP1/ABCC1)**. *J Biol Chem* 2006, **281**(20):13906-13914.

12. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D: **Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump**. *Cancer Res* 1996, **56**(5):988-994.

13. Loe DW, Almquist KC, Cole SP, Deeley RG: **ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance**

protein (MRP). Inhibition by cholestatic steroids. *J Biol Chem* 1996, **271**(16):9683-9689.

14. Qin L, Tam SP, Deeley RG: **Effect of multiple cysteine substitutions on the functionality of human multidrug resistance protein 1 expressed in human embryonic kidney 293 cells: identification of residues essential for function**. *Drug Metab Dispos* 2012, **40**(7):1403-1413.

15. Lee SH, Altenberg GA: **Transport of leukotriene C4 by a cysteine-less multidrug resistance protein 1 (MRP1)**. *Biochem J* 2003, **370**(Pt 1):357-360.

16. Bhattacharjee H, Rosen BP: **Spatial proximity of Cys113, Cys172, and Cys422 in the metalloactivation domain of the ArsA ATPase**. *J Biol Chem* 1996, **271**(40):24465-24470.

17. Ye J, Ajees AA, Yang J, Rosen BP: **The 1.4 A crystal structure of the ArsD arsenic metallochaperone provides insights into its interaction with the ArsA ATPase**. *Biochemistry* 2010, **49**(25):5206-5212.

18. Yang J, Rawat S, Stemmler TL, Rosen BP: **Arsenic binding and transfer by the ArsD As(III) metallochaperone**. *Biochemistry* 2010, **49**(17):3658-3666.

19. Leslie EM, Letourneau IJ, Deeley RG, Cole SP: **Functional and structural consequences of cysteine substitutions in the NH2 proximal region of the human multidrug resistance protein 1 (MRP1/ABCC1)**. *Biochemistry* 2003, **42**(18):5214-5224.

20. Yang Y, Chen Q, Zhang JT: **Structural and functional consequences of mutating cysteine residues in the amino terminus of human multidrug resistance-associated protein 1**. *J Biol Chem* 2002, **277**(46):44268-44277.

21. Ito K, Olsen SL, Qiu W, Deeley RG, Cole SP: **Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport**. *J Biol Chem* 2001, **276**(19):15616-15624.

22. Carew MW, Leslie EM: **Selenium-dependent and -independent transport of arsenic by the human multidrug resistance protein 2 (MRP2/ABCC2): implications for the mutual detoxification of arsenic and selenium**. *Carcinogenesis* 2010, **31**(8):1450-1455.

23. Reay PF, Asher CJ: **Preparation and purification of 74As-labeled arsenate and arsenite for use in biological experiments**. *Anal Biochem* 1977, **78**(2):557-560.

24. Delnomdedieu M, Basti MM, Otvos JD, Thomas DJ: **Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study**. *Chem Biol Interact* 1994, **90**(2):139-155.

25. Styblo M, Thomas DJ: **In vitro inhibition of glutathione reductase by arsenotriglutathione**. *Biochem Pharmacol* 1995, **49**(7):971-977.

26. Loe DW, Almquist KC, Deeley RG, Cole SP: **Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of**

glutathione-dependent vincristine transport. *J Biol Chem* 1996, **271**(16):9675-9682.

27. Loe DW, Deeley RG, Cole SP: **Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione**. *Cancer Res* 1998, **58**(22):5130-5136.

28. Leslie EM, Bowers RJ, Deeley RG, Cole SP: **Structural requirements for functional interaction of glutathione tripeptide analogs with the human multidrug resistance protein 1 (MRP1)**. *J Pharmacol Exp Ther* 2003, **304**(2):643-653.

29. Letourneau IJ, Deeley RG, Cole SP: **Functional characterization of non-synonymous single nucleotide polymorphisms in the gene encoding human multidrug resistance protein 1 (MRP1/ABCC1)**. *Pharmacogenet Genomics* 2005, **15**(9):647-657.

30. Ruan X, Bhattacharjee H, Rosen BP: **Cys-113 and Cys-422 form a high affinity metalloid binding site in the ArsA ATPase**. *J Biol Chem* 2006, **281**(15):9925-9934.

31. Carew MW, Naranmandura H, Shukalek CB, Le XC, Leslie EM: **Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1)**. *Drug Metab Dispos* 2011, **39**(12):2298-2304.

32. Kalivas JH: **Evaluation of volume and matrix effects for the generalized standard addition method**. *Talanta* 1987, **34**(11):899-903.

33. Manley SA, George GN, Pickering IJ, Glass RS, Prenner EJ, Yamdagni R, Wu Q, Gailer J: **The seleno bis(S-glutathionyl) arsinium ion is assembled in erythrocyte lysate**. *Chem Res Toxicol* 2006, **19**(4):601-607.