A Novel Pathway for Arsenic Elimination: Human Multidrug Resistance Protein 4 (MRP4/ABCC4) Mediates Cellular Export of Dimethylarsinic Acid (DMA\(\text{V}\)) and the Diglutathione Conjugate of Monomethylarsonous Acid (MMA\(\text{III}\))

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Received December 13, 2013; accepted May 28, 2014

ABSTRACT

Hundreds of millions of people worldwide are exposed to unacceptable levels of arsenic in drinking water. This is a public health crisis because arsenic is a Group I (proven) human carcinogen. Humans methylate arsenic to monomethylarsonous acid (MMA\(\text{III}\)), monomethylarsonic acid (MMA\(\text{V}\)), dimethylarsinous acid (DMA\(\text{III}\)), and dimethylarsinic acid (DMA\(\text{V}\)). Although the liver is the predominant site for arsenic methylation, elimination occurs mostly in urine. The protein(s) responsible for transport of arsenic from the liver (into blood), ultimately for urinary elimination, are unknown. Human multidrug resistance protein 1 (MRP1/ABCC1) and MRP2 (ABCC2) are established arsenic efflux pumps, but unlike the related MRP4 (ABCC4) are not present at the basolateral membrane of hepatocytes. MRP4 is also found at the apical membrane of renal proximal tubule cells, making it an ideal candidate for urinary arsenic elimination. In the current study, human MRP4 expressed in HEK293 cells reduced the cytotoxicity and cellular accumulation of arsenate, MMA\(\text{III}\), MMA\(\text{V}\), DMA\(\text{III}\), and DMA\(\text{V}\) while two other hepatic basolateral MRPs (MRP3 and MRP5) did not. Transport studies with MRP4-enriched membrane vesicles revealed that the diglutathione conjugate of MMA\(\text{III}\), monomethylarsonic diglutathione [MMA(GS)\(_2\)], and DMA\(\text{V}\) were the transported species. MMA(GS)\(_2\) and DMA\(\text{V}\) transport was osmotically sensitive, allosteric (Hill coefficients of 1.4 ± 0.2 and 2.9 ± 1.2, respectively), and high affinity (\(K_m\) of 0.70 ± 0.16 and 0.22 ± 0.15 \(\mu\text{M}\), respectively). DMA\(\text{V}\) transport was pH-dependent, with highest affinity and capacity at pH 5.5. These results suggest that human MRP4 could be a major player in the elimination of arsenic.

Introduction

It is estimated that 160 million people worldwide are exposed to arsenic through contaminated drinking water at levels above the World Health Organization guidelines of 10 ppb (Hubaux et al., 2013). Arsenic is classified as a Group I (proven) human carcinogen and causes skin, lung, and bladder tumors (International Agency for Research on Cancer, 2012). Chronic arsenic exposure is also associated with kidney, liver, and prostate cancers and a myriad of other adverse health effects (International Agency for Research on Cancer, 2012; Naujokas et al., 2013). Thus, arsenic is a major worldwide public health issue. In addition to environmental exposures, arsenic trioxide (\(\text{As}_2\text{O}_3\)) has been approved for treating both newly diagnosed and relapsed acute promyelocytic leukemia, and is under investigation for the treatment of multiple other hematologic and solid tumors (Kritharis et al., 2013).

Contamination of drinking water occurs primarily from leaching of mineral-bound arsenic and release of water-soluble arsenate (As\(\text{V}\)) and arsenite (As\(\text{III}\)). As\(\text{V}\) is chemically similar to inorganic phosphate and enters cells through the sodium/phosphate cotransporter type IIb (SLC34A2) (Villa-Bellosta and Sorribas, 2010). In solution, As\(\text{III}\) and \(\text{As}_2\text{O}_3\) exist as the neutral As(OH)\(_3\), which passively enters cells through aquaglyceroporins (Yang et al., 2012). Arsenic is methylated within most mammalian cells. The most generally well accepted methylation pathway involves the enzymatic reduction of pentavalent arsenic to arsenite (As\(\text{III}\)) and arsenate (As\(\text{V}\)), and an Alberta Innovates Health Solutions Scholar.

ABSTRACT

MOLECULAR PHARMACOLOGY Mol Pharmacol 86:168–179, August 2014

Supplemental material to this article can be found at: http://molpharm.aspetjournals.org/content/suppl/2014/05/28/mol.113.091314.DC1

This article has supplemental material available at molpharm.aspetjournals.org.
arsenic followed. These reactions result in the consecutive formation of monomethylarsonic acid (MMA), monomethylarsonous acid (MMAH), dimethylarsinic acid (DMA), and dimethylarsonious acid (DMAH). In addition to methylation, AsIII and MMAH form conjugates with glutathione (GSH/GS) (arsenic triglutathione [As(GS)3] and monomethylarsenic diglutathione [MMA(GS)2], respectively) in vivo, while dimethylarsonic glutathione (DMA(GS)) has never been isolated physiologically (Kala et al., 2000, 2004; Leslie, 2012).

Arsenic methylation occurs predominantly in the liver and increases the rate of arsenic clearance from the body (Drobna et al., 2010). Despite this, methylation is considered an activation pathway because MMAH and DMAH are substantially more toxic than AsIII (Petrick et al., 2000; Mass et al., 2001). Arsenic elimination occurs predominantly in urine (60%–80%), and of this, 10%–30% is eliminated as inorganic arsenic, 10%–20% as monomethylated forms, and 60%–80% as dimethylated forms (Vahter, 2000; Loffredo et al., 2003). How arsenic species traverse the basolateral membrane of the hepatocyte to enter sinusoidal blood (ultimately for elimination in urine) is not well understood. To prevent and treat arsenic-induced toxicity from either environmental or clinical exposures it is critical to understand underlying molecular mechanisms that render a person susceptible. This requires a thorough understanding of arsenic elimination pathways.

The multidrug resistance proteins (MRPs) are ATP-binding cassette (ABC) transporter proteins critical for the cellular efflux of a vast array of endogenous compounds, drugs, carcinogens, and environmental toxicants (Leslie et al., 2005; Deeley et al., 2006; Keppler, 2011; Cole, 2014). MRP1 (ABCC1) plays an important role in the cellular efflux of As(GS)2 and MMA(GS)2 (Leslie et al., 2004; Carew et al., 2011). In vivo studies of Mrp2 (Abcc2)-deficient (TR/−) Wistar rats show that Mrp2 is responsible for the biliary excretion of As(GS)2 and MMA(GS)2 (Kala et al., 2000). As(GS)2 and the seleno-bis(S-glutathionyl) arsinium ion [(GS)2AsSe]+ are substrates for human MRP2 in vitro (Carew and Leslie, 2010). Despite the important roles MRP1 and MRP2 play in protecting cells and tissues from arsenic, neither protein is localized to the basolateral surface of human hepatocytes. Thus, MRP1 and MRP2 are not responsible for the transport of hepatic arsenic metabolites into sinusoidal blood, ultimately for urinary elimination.

Candidate MRPs that are expressed at the basolateral surface of hepatocytes include MRP3 (ABCC3), MRP4 (ABCC4), and MRP5 (ABCC5). MRP3–5 are not capable of reducing the cytotoxicity of inorganic arsenic (Kool et al., 1999; McAleer et al., 1999; Lee et al., 2000; Wijnholds et al., 2000); however, their ability to protect cells from methylated arsenic species is unknown. MRP4 is an ideal candidate for the urinary elimination of arsenic because it is localized at the basolateral surface of hepatocytes and the apical surface of renal proximal tubule cells (Russel et al., 2008). The objective of the current study was to determine the ability of HEK293 cells stably expressing MRP3, MRP4, or MRP5 to confer resistance to AsIII, AsV, MMAH, MMAV, DMAH, or DMAV compared with cells expressing empty vector. MRP4 (but not MRP3 or MRP5) reduced the toxicity and accumulation of all arsenic species except for AsIII. MRP4-enriched membrane vesicles were then used to identify the transported species and determine transport characteristics. The results suggest that human MRP4 could be a major player in the elimination of arsenic from the human body.

Materials and Methods

Materials. [3,5,7,9-H11]Methotrexate (13.4 Ci/mmol) was purchased from Moravek Biochemicals Inc (Brea, CA). GSH, ATP, AMP, sucrose, Tris base, AsIII (>99% purity), AsV (>98% purity), MMAV (99.5% purity), DMAV (>99%), 1-buthionine sulfoximine (BSO), and MgCl2 were purchased from Sigma-Aldrich (Oakville, ON, Canada). 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, ON, Canada). Ophthalmic acid (γ-glutamyl-aminobutyryl-glycine) was purchased from Bachem (Torrance, CA). MMAH and DMAH in the form of diiodometharsine (CH3AsI2) and iododimethylarsine [(CH3)2AsI] were gifts from Dr. William Cullen (University of British Columbia, Vancouver, BC, Canada). NMR analysis at the University of British Columbia confirmed the identity of both chemicals and that the purity of each was at least 99%.

Antibodies and Cell Lines. Mouse monoclonal (M,II-9) anti-MRP3, rat monoclonal (M,1-10) anti-MRP4, and rat monoclonal (M,1-1) anti-MRP5 antibodies were purchased from Abcam Inc (Cambridge, MA). The rabbit polyclonal (H-300) anti-Na+/K+ ATPase antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). The mouse monoclonal (QCR1-1) anti-MRP1 antibody was a gift from Dr. Susan Cole (Queen’s University, Kingston, ON, Canada) (Hippen et al., 1994). The human embryonic kidney cell line (HEK293) and the SV40-transformed human embryonic kidney cell line (HEK293T) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4 mM l-glutamine and 7.5% fetal bovine serum in a humidified incubator with 95% air/5% CO2. Routine testing for Mycoplasma contamination of cell lines was performed using the ATCC Universal Mycoplasma Testing Kit (American Type Culture Collection, Manassas, VA).

Generation of Expression Vectors and Stable HEK293 Cell Lines Expressing MRP3, MRP4, and MRP5. The pcDNA3.1 (+)-MRP3 vector encoding the full-length human MRP3 cDNA was a gift from Drs. Susan Cole and Roger Deeley (Queen’s University), and its construction has been described previously (Oleschuk et al., 2003). The pcDNA3.1 (+)-Hygro-MRP4 vector encoding the full-length human MRP4 cDNA was a gift from Dr. Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany) and was constructed as previously described (Rius et al., 2003). 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. The pGEM5-MRP5 vector containing the full-length MRP5 cDNA was a gift from Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (Kool et al., 1997). The MRP5 cDNA was excised from pGEM5-MRP5 using the restriction enzymes NheI and XhoI, and subcloned into pcDNA3.1 (+)-vector to generate pcDNA3.1 (+)-MRP5. The pcDNA3.1(+)-Neomycin plasmid was transformed into E. coli DH5α and plated on Kanamycin (50 µg/mL) plates. The colonies were confirmed by PCR using primers specific for the vector (Invitrogen, Burlington, ON, Canada). Six-well plates and media were replaced with Dulbecco’s modified Eagle’s medium containing 1000 µg/mL G418 (Invitrogen) and 10% fetal bovine serum. Cells were grown for ~2 weeks with regular media replacement; once cell colonies were visible, they were individually removed by scraping and aspirating with a pipette tip as described.
previously (Karin, 1999). Levels of MRP3, MRP4, and MRP5 in G418-resistant cell populations were then determined by immunoblot analysis with the anti-MRP3 MAb M1I-9 (1:5000), anti-MRP4 MAb M1I-10 (1:2000), and anti-MRP5 MAB M4I-1 (1:2000). The positive clones (MRP3-18, MRP4-1E16, and MRP5-2-6) and empty vector (V4) were tested for the proportion of cells expressing the MRP of interest by flow cytometry (BD FACSCalibur; Cigny Cancer Institute Flow Cytometry Facility, Edmonton, AB, Canada) or confocal microscopy (Zeiss, 510 NLO; Cell Biology Imaging Facility, University of Alberta) as described previously (Leslie et al., 2003). Populations of $<$80% were further cloned by limiting dilution to obtain populations of $>$80%.

Independently derived pcDNA3.1 (+)/Hygro and pcDNA3.1 (+)/Hygro-MRP4 stable cell clones (HEK-MCV and HEK-MRP4-MC4, respectively) were generated using HEK293T cells. Procedures used were identical to those described above except cells were selected and cultured in 100 µg/ml hygromycin (Invitrogen) instead of G418.

**Cytotoxicity Testing.** The cytotoxicity of six arsenic species was measured using HEK-V4, HEK-MRP3, HEK-MRP4-1E16, and HEK-MRP5 (G418 selected) or HEK-MCV and HEK-MRP4-MC4 (hygromycin selected) stable cell lines as previously described (Carew et al., 2011). Briefly, cells were seeded in 96-well plates at $1 \times 10^3$ cells/well and grown for 24 hours. In quadruplicate, cells were treated with As(III) (0.1–100 µM), As(V) (0.001–10 mM), MMA(III) (0.1–100 µM), MMA(III) (0.3–100 mM), DMA(III) (0.1–100 µM), or DMA(III) (0.01–100 mM) for 72 hours. The pH of MMA(III) and DMA(III) was adjusted to pH 7.4 prior to treating cells. In parallel with arsenic species, the positive controls etoposide (0.1–100 µM) (for HEK-MRP3) and 6-mercaptopurine (6-MP) (0.1–100 µM) (for HEK-MRP4 and -MRP5) were run (Borst et al., 2007).

To measure the influence of GSH depletion on arsenic cytotoxicity, HEK-MRP4-1E16 and HEK-V4 cells were treated with BSO (100 µM), which inhibits the rate-limiting step of GSH synthesis, at the time of seeding and for the remaining experimental duration. Cell viability was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), 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, La Jolla, CA). Relative resistance values were calculated as the ratio of the EC$_{50}$ values of HEK-MRP to HEK-vector.

**Cellular Accumulation of Arsenic Species.** HEK-MRP4-1E16 and HEK-V4 cells were seeded in six-well plates at 5 × 10^5 cells/well and 24 hours later treated with 1 µM As(III), As(V), MMA(III), DMA(III), MMA(III) in culture media for 24 hours. Cells were washed three times with ice-cold phosphate-buffered saline, trypsinized, pelleted by centrifugation at 1000g for 10 minutes at 4°C, and then digested with 250 µl concentrated nitric acid for $>$48 hours. 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 coupled plasma mass spectrometry (ICP-MS) (Agilent 7500; Yokogawa Analytical Systems, Hachioji, Japan) using the standard addition method as described previously (Kalivas, 1987; Carew et al., 2011). Samples were introduced directly into the nebulizer of the ICP-MS.

**Influence of As(III), MMA(III), DMA(III), and DMA(IV) on MRP4 Protein Levels.** HEK-V4 and HEK-MRP4-1E16 cells were seeded in 75-cm$^2$ culture flasks at $2.5 \times 10^5$ cells/flask. Twenty-four hours later, cells were treated with As(III) (3.0 µM), MMA(III) (1.5 µM), DMA(III) (0.5 µM), or DMA(IV) (750 µM) (concentrations at or below the EC$_{50}$ value for HEK-V4 cells) for 72 hours. Cells were then harvested and crude membranes prepared. Briefly, cells were washed twice in ice-cold Tris (50 mM; pH 7.4) sucrose (250 mM) buffer and collected by centrifugation at 500g for 10 minutes. Cell pellets were snap-frozen in liquid nitrogen, thawed on ice, and disrupted by nitrogen cavitation (200 psi, 5 minutes), and the supernatant containing the membrane fraction was collected following centrifugation at 500g for 10 minutes. Crude membrane fraction was subsequently pelleted by centrifugation at 100,000g for 30 minutes, aliquoted, and stored at −80°C. MRP4 and MRP1 protein levels were then determined by immunoblot analysis using the rat anti-human MRP4 antibody M5I-10 (1:2000) and the mouse anti-human MRP1 antibody QCRL-1 (1:5000) after resolving at 10 µg of protein by SDS-PAGE as described previously (Hipfner et al., 1994).

**Transient Expression of MRP4 in HEK293T Cells and Membrane Vesicle Preparation.** HEK293T cells were transfected in 150-mm culture dishes using the calcium phosphate method as described previously (Carew and Leslie, 2010). Twenty-four hours post-transfection, culture medium was replaced with fresh. Seventy-two hours post-transfection, cells were washed once 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 800g for 10 minutes. Cell pellets were stored at −80°C until membrane vesicles were prepared. Plasma membrane–enriched vesicles were prepared from MRP4-transfected HEK293T cells, according to previously described methods (Carew and Leslie, 2010). MRP4 protein levels in membrane vesicles were determined by immunoblot analysis, using the rat anti-human MRP4 antibody M1I-10 (1:2000). Transiently expressed HEK293T cells were used for all membrane vesicle preparations because MRP4 protein levels were $\sim$2-fold higher after transient expression compared with the HEK-MRP4 stable cell lines (Supplemental Fig. 1).

**MMA(GS)$_2$ and DMA$^+$ Transport Assays.** MMA(GS)$_2$ was synthesized from MMA$^{III}$ and GSH as described previously (Kala et al., 2000; Carew et al., 2011). 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 $>$30 minutes at 4°C. Membrane vesicles were incubated at 37°C in Tris-sucrose buffer, with individual arsenic species (at concentrations indicated), ATP or AMP (3.4 mM), MgCl$_2$ (8.5 mM), creatine kinase (85 µg/ml), and creatine phosphate (8.5 mM). Conditions for the synthesis of MMA(GS)$_2$ resulted in the presence of 3 mM GSH in the transport reaction. When GSH was present, GSH reductase (4.25 µg/ml) and NADPH (0.30 mM) were also included to convert oxidized to reduced GSH. GSH dependence of MMA$^{III}$ transport was assessed by adding GSH (3 mM) or the nonreducing GSH analog ophthalmic acid (3 mM). At the indicated time point(s), transport was stopped by diluting the transport reaction in 800 µl ice-cold Tris-sucrose buffer and pelleting vesicles by centrifugation at 100,000g for 20 minutes at 4°C. Pelleted membrane vesicles were washed twice with 1 ml ice-cold Tris-sucrose buffer, then digested in 250 µl concentrated nitric acid for 48 hours, diluted 1:1 with deionized distilled water, and filtered with 0.45-µm syringe filters (Whatman). The total concentration of arsenic in each sample was determined by ICP-MS using the standard addition method as described previously (Kalivas, 1987; Carew et al., 2011). ATP-dependent transport was determined by subtracting transport in the presence of AMP from transport in the presence of ATP.

Kinetic parameters were determined by measuring the initial rate of MMA(GS)$_2$ and DMA$^+$ transport at eight different substrate concentrations (0.07–6.7 µM) at a single time point of 3 and 5 minutes, respectively. Curve fitting was done by nonlinear regression analysis (Michaelis-Menten curve fit or allosteric sigmoidal) with the computer program GraphPad Prism version 5.

**Methotrexate Transport Assay.** Methotrexate transport by MRP4 was performed as described previously (Chen et al., 2002; Li et al., 2011). Briefly, 20 µg of membrane vesicles was incubated with $[^{3}H]$methotrexate (1 µM, 200 nCi), AMP or ATP (4 mM), MgCl$_2$ (10 mM), creatine phosphate (10 mM), and creatine kinase (100 µg/ml) at 37°C for 5 minutes. Samples were filtered over glass fiber filters, and then radioactivity was quantified by liquid scintillation counting. ATP-dependent transport was determined as described above for MMA(GS)$_2$ and DMA$^+$.

**Results**

MRP4 Confers Resistance to As$^V$, MMA$^{III}$, MMA$^{V}$, DMA$^{III}$, and DMA$^+$. To determine if MRP3, MRP4, and MRP5 were capable of conferring resistance to inorganic and...
Resistance of human MRP4-transfected HEK293 cells to inorganic and methylated arsenic species

Data are mean ± S.D.

<table>
<thead>
<tr>
<th>Arsenic Species</th>
<th>HEK-V4</th>
<th>HEK-MRP4-1E16</th>
<th>Relative Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsIII (n = 6)</td>
<td>3.1 ± 0.8 µM</td>
<td>2.7 ± 1.5 µM</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>AsV (n = 9)</td>
<td>20 ± 8.1 µM</td>
<td>49 ± 15 µM</td>
<td>2.9 ± 1.8</td>
</tr>
<tr>
<td>MMAIII (n = 9)</td>
<td>1.7 ± 0.4 µM</td>
<td>2.9 ± 0.5 µM*</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>MMAV (n = 4)</td>
<td>0.5 ± 0.08 mM</td>
<td>0.8 ± 0.14 mM*</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>DMAIII (n = 7)</td>
<td>0.7 ± 0.1 µM</td>
<td>1.2 ± 0.2 µM*</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>DMAV (n = 6)</td>
<td>0.8 ± 0.1 mM</td>
<td>1.2 ± 0.2 mM*</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>6-MP (n = 6)</td>
<td>5.1 ± 1.9 µM</td>
<td>26 ± 5.8 µM*</td>
<td>5.4 ± 1.4</td>
</tr>
</tbody>
</table>

MRP4 Protects Cells from Arsenic by Exporting DMAV and MMA(GS)2

Data are mean ± S.D.

<table>
<thead>
<tr>
<th>Arsenic Species</th>
<th>HEK-V4</th>
<th>HEK-MRP5</th>
<th>Relative Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsIII (n = 5)</td>
<td>7.7 ± 2.8 µM</td>
<td>7.4 ± 3 µM</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>AsV (n = 3)</td>
<td>33 ± 7.8 µM</td>
<td>22 ± 5 µM</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>MMAIII (n = 5)</td>
<td>1.4 ± 0.5 µM</td>
<td>1.7 ± 0.9 µM</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>MMAV (n = 3)</td>
<td>0.6 ± 0.1 mM</td>
<td>0.5 ± 0.19 mM</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>DMAIII (n = 4)</td>
<td>0.6 ± 0.2 µM</td>
<td>0.8 ± 0.1 µM</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>DMAV (n = 6)</td>
<td>1.1 ± 0.3 mM</td>
<td>0.9 ± 0.3 mM</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>6-MP (n = 8)</td>
<td>3.3 ± 1.2 µM</td>
<td>6.4 ± 1.6 µM*</td>
<td>2.1 ± 0.8</td>
</tr>
</tbody>
</table>

*Ratio of EC50 of HEK-MRP5 to HEK-V4.

**P < 0.05 (Student’s t test) vs. HEK-V4.

To determine whether resistance conferred by MRP4 to AsV, MMAIII, MMAV, DMAIII, and DMAV was GSH-dependent, toxicity was tested after depleting HEK-V4 and HEK-MRP4-1E16 cells of GSH using BSO (100 µM) (Fig. 2 and Table 4). GSH is protective against many arsenic species (Leslie, 2012), and consistent with this, AsIII, AsV, MMAIII, MMAV, and DMAV became significantly more toxic to both HEK-V4 and HEK-MRP4-1E16 cells upon depletion (Table 1 compared with Table 4). In contrast, GSH depletion had no effect on the toxicity of DMAIII (Table 1 compared with Table 4). GSH depletion resulted in the loss of MRP4-dependent cellular protection to AsV and MMAIII but did not reduce the relative resistance for MMAV, DMAIII, or DMAV (Table 1 compared with Table 4).

MRP4 Reduces the Cellular Accumulation of Arsenic after Treatment with AsV, MMAIII, MMAV, DMAIII, and DMAV. To assess the effect of MRP4 on the cellular accumulation of different arsenic species, total arsenic levels were quantified after HEK-V4 and HEK-MRP4-1E16 cell treatment with AsIII, AsV, MMAIII, MMAV, DMAIII, and DMAV (1 µM for 72 hours). HEK-MRP4-1E16 cellular accumulation of arsenic was reduced by 20%, 30%, 50%, 40%, and 55% after treatment with AsV, MMAIII, MMAV, DMAIII, and DMAV, respectively, compared with HEK-V4 cells (Fig. 3). No significant difference in AsIII accumulation between HEK-MRP4-1E16 and HEK-V4 was observed (Fig. 3). The accumulation of MMAV and DMAV was 5- to 30-fold lower than that of any of the other arsenic species (Fig. 3), consistent with our high EC50 values, as well as previous studies suggesting that these pentavalent methylated species are inefficiently taken up by cells (Dopp et al., 2004; Naranmandura et al., 2007). Similar results were obtained using the independently derived HEK-MCV and HEK-MRP4-MC cell line pair.

MRP4 Protein Expression Is Increased by Certain Arsenic Species in HEK-V4 Cells. To determine if selected arsenicals increase MRP4 protein expression in HEK-V4 and HEK-MRP4-1E16 cell lines under conditions used for cytotoxicity and accumulation assays, MRP4 protein levels were measured after treating cells with concentrations of AsIII (3 µM), MMAIII (1.5 µM), DMAIII (0.5 µM), and DMAV (750 µM) at or below the EC50 value for the HEK-V4 cell line (Table 1). For the HEK-V4 cell line, MRP4 protein was increased 1.9- to 2.5-fold by AsIII, DMAIII, and DMAV (Fig. 4). In contrast, MRP4 protein levels were not significantly altered for the MRP4-overexpressing HEK-MRP4-1E16 cell line. The influence of these conditions on MRPl protein levels was also tested (Fig. 4A, Table 2).

<table>
<thead>
<tr>
<th>Arsenic Species</th>
<th>HEK-V4</th>
<th>HEK-MRP3</th>
<th>Relative Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsIII (n = 3)</td>
<td>5.5 ± 2.3 µM</td>
<td>4.3 ± 1.6 µM</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>AsV (n = 4)</td>
<td>22 ± 7.9 µM</td>
<td>34 ± 14 µM</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>MMAIII (n = 3)</td>
<td>1.5 ± 0.7 µM</td>
<td>1.3 ± 0.3 µM</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>MMAV (n = 3)</td>
<td>0.6 ± 0.1 mM</td>
<td>0.7 ± 0.03 mM</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>DMAIII (n = 2)</td>
<td>0.5 ± 0.6 µM</td>
<td>0.1 ± 0.7 µM</td>
<td>0.3 ± 1.1</td>
</tr>
<tr>
<td>DMAV (n = 3)</td>
<td>0.8 ± 0.1 µM</td>
<td>0.7 ± 0.1 µM</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Etoposide (n = 8)</td>
<td>0.2 ± 0.1 µM</td>
<td>1.3 ± 0.6 µM*</td>
<td>6.9 ± 1.6</td>
</tr>
</tbody>
</table>

*Ratio of EC50 of HEK-MRP3 to HEK-V4.

**P < 0.05 (Student’s t test) vs. HEK-V4.
middle panel), and no significant level of MRP1 was detected in either cell line. These results suggest that MRP4 is induced by AsIII, DMAIII, and DMAV when cellular MRP4 levels are low. Similar results have been observed in sandwich-cultured human hepatocytes (B. A. Roggenbeck and E. M. Leslie, unpublished observation).

MRP4 Does Not Transport Inorganic Arsenic. HEK293 cells endogenously express the enzyme responsible for the methylation of arsenic, arsenic (III oxidation state) methyltransferase (Sumi et al., 2011). Thus, cytotoxicity and cellular accumulation data (Figs. 1–3; Tables 1 and 4) provided evidence that MRP4 protects cells from arsenic through cellular efflux, but did not identify the transported species.

To identify the species of arsenic that are substrates for MRP4, ATP-dependent transport of AsIII, AsV, MMAIII, MMAV, DMAIII, and DMAV (under various conditions and in the presence and absence of 3 mM GSH) by MRP4-enriched and vector control membrane vesicles was measured. In contrast to the cytotoxicity and accumulation data that suggested AsV was a GSH-dependent MRP4 substrate (Fig. 1B; Fig. 2B; Tables 1 and 4), MRP4 did not transport AsV in the presence or absence of GSH (3 mM) (at AsV concentrations between 0.1 and 10 μM, time points ranging from 1–10 minutes, and a pH range from 6–9) (data not shown). Consistent with the cytotoxicity and accumulation data, AsIII (0.1–10 μM) was not a substrate for

Fig. 1. Effect of AsIII, AsV, MMAIII, MMAV, DMAIII, and DMAV on the viability of stably transfected HEK293 cells. (A) The relative levels of MRP4 in membrane vesicles (2 μg of protein) prepared from stably transfected HEK293 cells expressing pcDNA3.1(+)/Hygro (MCV), pcDNA3.1(+)/Hygro-MRP4 (MRP4-MC4), pcDNA3.1(+)-neomycin (V4), or pcDNA3.1(+)-MRP4 (MRP4-1E16) were determined after resolving protein by SDS-PAGE and immunoblotting with the MRP4 antibody M4I-10. Blots were then stripped and probed for either MRP1 (using the MRP1 antibody QCRL-1) or the Na+/K+ ATPase as a loading control (using the Na+/K+ ATPase antibody H-300). (B–H) Cells expressing empty vector (HEK-V4) (○) and MRP4 (HEK-MRP4-1E16) (●) were incubated in the presence of AsIII (B), AsV (C), MMAIII (D), MMAV (E), DMAIII (F), DMAV (G), or 6-MP (H) for 72 hours. 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 three additional experiments.

Fig. 2. Effect of AsIII, AsV, MMAIII, MMAV, DMAIII, and DMAV on the viability of stably transfected HEK293 cells after glutathione depletion. HEK-V4 (○) and HEK-MRP4-1E16 (●) cells were incubated with 100 μM BSO for 24 hours and then treated with 100 μM BSO and AsIII (A), AsV (B), MMAIII (C), MMAV (D), DMAIII (E), or DMAV (F) for 72 hours. 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 three additional experiments.

| Arsenic Species | EC50 (μM) | Relative Resistance
<table>
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<tbody>
<tr>
<td>AsIII (n = 5)</td>
<td>0.2 ± 0.01 μM</td>
<td>0.2 ± 0.006 μM</td>
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<tr>
<td>AsV (n = 8)</td>
<td>2.9 ± 0.7 μM</td>
<td>2.9 ± 0.5 μM</td>
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<tr>
<td>MMAIII (n = 7)</td>
<td>0.06 ± 0.04 μM</td>
<td>0.06 ± 0.03 μM</td>
</tr>
<tr>
<td>MMAV (n = 4)</td>
<td>85 ± 12 μM</td>
<td>169 ± 6 μM</td>
</tr>
<tr>
<td>DMAIII (n = 5)</td>
<td>0.6 ± 0.1 μM</td>
<td>1.9 ± 0.7 μM</td>
</tr>
<tr>
<td>DMAV (n = 4)</td>
<td>25 ± 1.1 μM</td>
<td>62 ± 16 μM</td>
</tr>
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*aRatio of EC50 of HEK-MRP4-1E16 to HEK-V4. *P < 0.05 (Student’s t test) vs. HEK-V4.
MRP4 in the presence or absence of GSH (3 mM) or as the triglutathione conjugate As(GS)3 (0.1–10 μM) (data not shown).

**MRP4 Transports MMAIII in the Presence of GSH or as the Presynthesized GSH Conjugate MMA(GS)2.** To determine whether MMAIII and/or MMA(GS)2 were substrates of MRP4, ATP-dependent transport of these compounds into MRP4-enriched and vector control membrane vesicles was measured (Fig. 5A). ATP-dependent transport of MMAIII 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-dependent transport of MMAIII was observed with an activity of 37 pmol mg−1 protein min−1. Ophthalmic acid and other GSH analogs lacking a free thiol group can substitute for GSH and support the transport of GSH-dependent MRP substrates (Cole and Deeley, 2006). Thus, the thiol group of GSH and therefore the formation of a GSH conjugate are not necessary for transport. ATP-dependent transport of MMAIII in the presence of ophthalmic acid (3 mM) was extremely low and similar to minus-GSH conditions and vector control (Fig. 5A). ATP-dependent transport of MMA(GS)2 was then measured and had an activity of 39 pmol mg−1 protein min−1. These data suggest that the free thiol group of GSH is required for MMAIII transport by MRP4 and are consistent with MMA(GS)2 being the transported chemical species. Using similar experimental conditions, transport of MMAV by MRP4-enriched membrane vesicles was measured, and ATP-dependent transport was not detected (data not shown).

**Osmotic Sensitivity of MMA(GS)2 Transport.** To assess whether ATP-dependent transport of MMA(GS)2 by MRP4-enriched membrane vesicles represents true transport into the vesicle lumen, rather than surface or intramembrane binding, the effect of changes in osmolality on vesicular uptake was examined. MMA(GS)2 (0.7 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 (Fig. 5B). Extrapolation of the line through x = 0 (representing infinite osmolality or zero intravesicular space) suggested that binding was ~20% for MRP4 under the usual transport conditions used.

**Kinetic Analysis of MRP4-Mediated MMA(GS)2 Transport.** To determine the linear range of MMA(GS)2 (0.7 μM) uptake by MRP4-enriched and vector control membrane vesicles, time courses were completed. Transport by MRP4 was linear for up to 5 minutes with a maximal activity of 250 pmol mg−1 protein at 5 minutes (Fig. 5C). ATP-dependent transport of MMAV by vector control membrane vesicles was very low and similar to transport observed in the presence of AMP. MRP4-mediated transport of...
Hill coefficient of 1.4
0.7
squares F test
(Michaelis-Menten equation, GraphPad Prism 5) (extra sum of squares was significantly better than a single-binding-site model interaction (allosteric sigmoidal equation, GraphPad Prism 5) if DMAIII was a substrate of MRP4, ATP-dependent transport of DMAIII (0.7 protein min
1) was observed with an activity of 20 pmol mg
1 protein min
1, while transport of DMAV by vector control vesicles was very low and similar to vector control membrane vesicles. MRP4 transported DMAV in an ATP-dependent manner with an activity of 18 pmol mg
1 protein min
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1 protein min
1, while transport of DMAV by vector control vesicles was very low and similar to vector control membrane vesicles.

Fig. 5. ATP-dependent transport of MMAIII (± GSH or ophthalmic acid) and MMA(GS)2 by MRP4-enriched membrane vesicles. Transport experiments were done with membrane vesicles (20 µg of protein) prepared from HEK293T cells transiently transfected with pcDNA3.1(+)-MRP4 (closed bars or symbols) or empty pcDNA3.1(+)- empty bars or symbols). (A) Membrane vesicles were incubated in transport buffer for 3 minutes at 37°C with MMAIII (1 µM) (either alone or in the presence of GSH (3 mM) or ophthalmic acid (OA) (3 mM) or MMA(GS)2 (1 µM). (B) Osmotic sensitivity of ATP-dependent MMA(GS)2 transport by MRP4 was determined by incubating HEK-MRP4 membrane vesicles with MMA(GS)2 (0.7 µM) for 3 minutes at 37°C in transport buffer containing increasing concentrations of sucrose (0.25, 0.33, 0.5, 0.67, and 1 M). (C) Time course of ATP-dependent MMA(GS)2 transport by MRP4 was determined by incubating HEK-MRP4 membrane vesicles with MMA(GS)2 (0.7 µM) in transport buffer at 37°C for the indicated time points. (D) HEK-MRP4 membrane vesicles were incubated for 3 minutes at 37°C with increasing concentrations of MMA(GS)2 (0.07–6.7 µM). Data were fit using a one-site Michaelis-Menten kinetic model (hatched line) or the allosteric sigmoidal model (solid line) with GraphPad Prism 5. For individual experiments, transport was done in triplicate, and bars and symbols represent the means (± S.D.) of three independent experiments, except in (C), where open symbols are means of two independent experiments.

MMA(GS)2 was further characterized by determining the initial rates of transport over several concentrations of MMA(GS)2 (Fig. 5D). MRP4 showed a positive cooperative allosteric interaction with MMA(GS)2. The curve fit for an allosteric interaction (allosteric sigmoidal equation, GraphPad Prism 5) was significantly better than a single-binding-site model (Michaelis-Menten equation, GraphPad Prism 5) (hatched line) or the allosteric sigmoidal model (solid line) with GraphPad Prism 5). For individual experiments, transport was done in triplicate, and bars and symbols represent the means (± S.D.) of three independent experiments, except in (C), where open symbols are means of two independent experiments.

MRP4 Transports DMAV but not DMAIII. To determine if DMAIII was a substrate of MRP4, ATP-dependent transport of DMAIII (0.7 µM) in the presence or absence of GSH (3 mM) by MRP4-enriched membrane vesicles was measured (Fig. 6). In the presence of GSH, transport was not detected and was similar to transport observed in the presence of AMP (Fig. 6A). However, in the absence of GSH, ATP-dependent transport of DMAIII was observed with an activity of 20 pmol mg
1 protein min
1 (Fig. 6B). This was consistent with the resistance conferred to DMAIII by MRP4 under GSH-depleted conditions (Fig. 2E; Table 4). When dissolved at physiologic pH, DMAIII is very unstable and rapidly oxidizes to DMAV (Gong et al., 2001; Yehiayan et al., 2009). We hypothesized that DMAIII was not transported by MRP4 (+ GSH) because DMAIII is the transported species and GSH stabilizes DMAIII in its reduced state. To test this hypothesis, transport was measured after preincubating DMAIII dissolved in transport buffer for 1 or 10 minutes, to allow oxidation to DMAV. Transport of DMAIII incubated for 10 minutes prior to addition to the transport reaction was 5-fold higher (25 pmol mg
1 protein min
1) than of DMAIII incubated for 1 minute prior (5.0 pmol mg
1 protein min
1) (Fig. 6B). These data suggested that DMAV, and not DMAIII, was the transported species. DMAV transport was then measured using MRP4-enriched and vector control membrane vesicles. MRP4 transported DMAV in an ATP-dependent manner with an activity of 18 pmol mg
1 protein min
1, while transport of DMAV by vector control vesicles was not detectable (Fig. 6C). Consistent with the GSH-independent resistance MRP4 conferred against DMAV in cytotoxicity assays (Fig. 2F; Table 4), the addition of GSH (3 mM) did not alter MRP4-dependent transport of DMAV (Fig. 6C).

Osmotic Sensitivity of DMAV Transport. To assess whether ATP-dependent transport of DMAV by MRP4-enriched membrane vesicles represents true transport into the vesicle lumen rather than surface or intramembrane binding, the effect of changes in osmolarity on vesicular uptake was examined. DMAV (0.7 µM) uptake was decreased as the concentration of sucrose in the transport buffer increased, indicating that the ATP-dependent DMAV uptake by the vesicles is osmotically sensitive, as expected for a true transport process (Fig. 7A). Extrapolation of the line through x = 0 (representing zero intravesicular space) suggested that very little ATP-dependent binding is occurring.

Kinetic Analysis of MRP4-Mediated DMAV Transport. To determine the linear range of DMAV (0.7 µM) uptake by MRP4-enriched and vector control membrane vesicles, time courses were completed. Transport by MRP4 was linear for up to 5 minutes with a maximal activity of 104 pmol mg
1 protein at 5 minutes (Fig. 7B). ATP-dependent transport of DMAV by vector control membrane vesicles was very low and similar to transport observed in the presence of AMP. MRP4-mediated transport of DMAV was further characterized by determining the initial rates of transport over several concentrations of DMAV (Fig. 7C). As for MMA(GS)2, MRP4 showed a positive cooperative allosteric interaction with DMAV. The curve fit for an allosteric interaction (allosteric sigmoidal equation, GraphPad Prism 5) was significantly better than a single-binding-site model (Michaelis-Menten equation, GraphPad Prism 5) (extra sum of squares F test, P < 0.0001), with average (± S.D.; n = 3) K0.5 of 0.70 ± 0.16 µM, Vmax of 112 ± 6.3 pmol mg
1 protein min
1, and Hill coefficient of 1.4 ± 0.23.

MRP4 Transport of DMAV Is pH-Dependent. DMAV [(CH3)2AsO2H] is a weak acid with a pH
1 of 6.3. To determine if pH influenced MRP4-dependent transport of DMAV, transport by MRP4-enriched membrane vesicles was measured at pH 5.5, 6.5, and 7.4, at which the dissociated anion [(CH3)2AsO2]
1− would exist at 14%, 61%, and 93%, respectively. DMAV transport was highest at pH 5.5, with an activity of 38 pmol mg
1 protein min
1, and similar at pH 6.5 and 7.5, with activities of 14 and 18 pmol mg
1 protein min
1.
respectively (Fig. 8A). In contrast with DMA\textsuperscript{V}, MMA(GS)\textsubscript{2} and methotrexate transport by MRP4 were not pH-dependent (Fig. 8, B and C). For MMA(GS)\textsubscript{2}, the dissociation constants for GSH (pK\textsubscript{a} for the carboxyl groups, 2.12 and 3.59) and the amino group (pK\textsubscript{a}, 8.75) determine a predominant (\(>95\%\)) net charge of \(-2\) over the pH range tested. For methotrexate, the dissociation constants of the \(\gamma\)-carboxyl (pK\textsubscript{a}, 6.9), \(\alpha\)-carboxyl (pK\textsubscript{a}, 3.4), and N(1) of the pteridine ring (pK\textsubscript{a}, 5.7) determine the net charge. Thus, at pH 5.5, 6.4, and 7.5, \(\approx 61\%, 86\%,\) and \(98\%\), respectively, of methotrexate would have a net charge of \(-2\) (with 39\%, 14\%, and 2\%, respectively, at a net negative charge of \(-1\) due to the protonation of the pteridine ring) (Breedveld et al., 2007).

To further characterize the high level of DMA\textsuperscript{V} transport at pH 5.5, kinetic parameters were determined (Fig. 8D). Data were fit to the allosteric sigmoidal equation (GraphPad Prism 5), with average (\(\pm\) S.D.; \(n = 3\)) \(K_{0.5} = 0.073 \pm 0.018 \mu\text{M}\), \(V_{\text{max}} = 50 \pm 2.3 \text{ pmol mg}^{-1} \text{ protein min}^{-1}\), and Hill coefficient value of 2.6 \(\pm 0.3\). Thus, the higher transport rate of DMA\textsuperscript{V} at pH 5.5, compared with pH 7.4, results from a 3-fold reduction in the \(K_{0.5}\) and a 1.6-fold increase in the \(V_{\text{max}}\), while the positively cooperative nature is similar.

**Discussion**

We report for the first time that MRP4 is a high-affinity transporter of the major urinary arsenic metabolite DMA\textsuperscript{V} and the diglutathione conjugate of the highly toxic MMA\textsuperscript{III} [MMA(GS)\textsubscript{2}]. These data along with the localization of MRP4 at the basolateral and apical surfaces of hepatocytes and renal proximal tubule cells, respectively, suggest that MRP4 could be a major player in the elimination of arsenic from the human body. Two other hepatic basolateral MRPs, MRP3 and MRP5, did not confer resistance to any of the arsenic species tested, suggesting that they are not involved in arsenic handling (summarized in Fig. 9).

Stable expression of MRP4 in HEK293 cells resulted in reduced toxicity and cellular accumulation of all arsenic species tested except for As\textsuperscript{III}. As\textsuperscript{V} is rapidly reduced to As\textsuperscript{III} once inside the cell, and prior to the first step of methylation; thus it was surprising that MRP4 conferred resistance to As\textsuperscript{V} but not As\textsuperscript{III}. This observation was consistent with a previous report that MRP4 expressed in NIH3T3 cells did not confer resistance to As\textsuperscript{III} (Lee et al., 2000) and our finding that As\textsuperscript{III} (\(\pm\) GSH) and As(GS)\textsubscript{2} were not substrates for MRP4. As\textsuperscript{III} is more toxic to cells than As\textsuperscript{V} (Tables 1–4) (Yang et al., 2012). Cellular uptake of As\textsuperscript{III} occurs at a faster rate than As\textsuperscript{V} (Yang et al., 2012), and this potentially results in the saturation of methylation pathways, impeding the formation of MMA(GS)\textsubscript{2} and DMA\textsuperscript{V} and preventing MRP4 from playing a protective role.

Although MRP4 reduced the toxicity and cellular accumulation of As\textsuperscript{V}, MMA\textsuperscript{V}, and DMA\textsuperscript{III}, they were not transported by MRP4. MRP4 resistance to As\textsuperscript{V} was completely GSH-dependent, suggesting that MRP4 reduced the toxicity and accumulation of As\textsuperscript{V} by effluxing MMA(GS)\textsubscript{2}. In contrast, MRP4 protected cells from MMA\textsuperscript{V} independently of GSH, likely due to further methylation and efflux as DMA\textsuperscript{V}. Similar to MMA\textsuperscript{V}, MRP4 likely decreased DMA\textsuperscript{III} toxicity and accumulation through the efflux of DMA\textsuperscript{V}. The relative resistance levels conferred by MRP4 to MMA\textsuperscript{V}, DMA\textsuperscript{III}, and DMA\textsuperscript{V} were increased after GSH depletion (Table 1 compared with Table 4), providing further support that MRP4 reduced the toxicity and cellular accumulation of MMA\textsuperscript{V} and DMA\textsuperscript{III} through the efflux of DMA\textsuperscript{V}. DMA\textsuperscript{V} can be reduced to DMA\textsuperscript{III} through an enzymatic and GSH-dependent process (Nemeti and Gregus, 2013), and GSH depletion would be protective because it would decrease the formation of highly toxic DMA\textsuperscript{III} and allow the efflux of DMA\textsuperscript{V}.

HEK-V4 cells treated with As\textsuperscript{III}, DMA\textsuperscript{III}, and DMA\textsuperscript{V} had a 2- to 2.5-fold-higher expression of MRP4 than untreated cells. HEK293 cells methylate arsenic, and the induction of MRP4 by As\textsuperscript{III} could be indirect, through the production of a methylated arsenic species. Alternatively, As\textsuperscript{III} could increase the expression of MRP4 in preparation for the export of MMA(GS)\textsubscript{2} and DMA\textsuperscript{V}. Arsenic is known to activate the arylhydrocarbon receptor and the nuclear factor (erythroid-derived 2)-like 2, transcription factors critical for protecting cells from oxidative stress (Kann et al., 2005; Lau et al., 2013). MRP4 mRNA and protein levels are known to be induced by these transcription factors in primary human
hepatocytes and HepG2 cells (Xu et al., 2010), offering a possible mechanism by which arsenic induces MRP4. The reduction in cellular toxicity and accumulation of arsenic species by MRP4 was low and in seeming contrast to the transport data showing that MMA(GS)2 and DMAV are excellent substrates for MRP4 (Fig. 5D; Fig. 7C). Induction of endogenous MRP4 in the HEK-V4 cell line by arsenic offers a partial explanation; however, the HEK-MRP4-1E16 cell line still had ~10-fold more MRP4 than the induced HEK-V4 (Fig. 4). It is likely that during these assays the arsenic species effluxed by MRP4 are taken back up by the cell and exert toxicity again prior to being re-effluxed. Thus, cytotoxicity and accumulation assays are likely an underestimation of the in vivo situation where MMA(GS)2 and DMAV effluxed into the blood or proximal tubule lumen would be cleared from the tissue and the reuptake of arsenicals prevented.

To our knowledge, DMAV transport by MRP4 is the first pentavalent arsenic efflux pathway to be reported. Despite the highly reducing environment of the cell, DMAV is detected in murine liver homogenates and human cell lines analyzed with oxidation state-specific hydride generation-cryotrapping-atomic absorption spectroscopy (Currier et al., 2011). Furthermore, the highly reactive nature of DMAIII combined with the lack of evidence for physiologic formation of dimethylarsenic glutathione [DMA(GS)] (Leslie, 2012) mean that DMAIII is highly bound to protein and not available for transport (Hippler et al., 2011; Shen et al., 2013). MRP4 transport of DMAIV was with high apparent affinity ($K_0.5$, 0.22 $\mu$M) and would allow for the efficient efflux of DMAIV at low cellular concentrations. Although DMAIV is often regarded as...
a “nontoxic” arsenic species, the efflux of DMA^V from the cell and ultimately the body is critical to prevent the formation of the highly reactive and toxic DMA^{III}. In addition, efflux of DMA^V from the cell/body by MRP4 could prevent product inhibition of arsenic (+3 oxidation state) methyltransferase, allowing the formation and elimination of more DMA^V.

Similar to the transport of DMA^V, MRP4-mediated MMA(GS)_2 transport was with high apparent affinity (K_{0.5} of 0.70 μM). These two newly identified MRP4 substrates have affinities comparable to the highest-affinity MRP4 substrates reported to date, including leukotriene C_4 (K_{0.5} 0.1–0.3 μM) (Russel et al., 2008). The high-affinity transport of DMA^V and MMA(GS)_2 is physiologically relevant for chronic environmental and clinical exposure to arsenic. Pharmacokinetic studies of patients undergoing chemotherapy for acute promyelocytic leukemia with As_2O_3 have plasma levels in the low-micromolar range (Yoshino et al., 2009). Environmental exposure would be approximately 100-fold lower, with expected tissue levels in the 100 nM range (Kritcharis et al., 2013).

The allostERIC nature of MMA(GS)_2 and DMA^V transport by MRP4 was not surprising because the transport of other MRP4 substrates (urate and GMP) is also through a positively cooperative mechanism (Van Aubel et al., 2005). The Hill coefficients for DMA^V of 2.9 ± 1.2 (pH 7.4) and 2.6 ± 0.3 (pH 5.5) are the highest reported to date for MRP4 [Hill coefficients for urate, cGMP, and MMA(GS)_2 are 1.7, 1.4, and 1.4, respectively], suggesting that DMA^V transport is mediated with strong positive cooperativity. Transport of toxic substances from cells through an allostERIC mechanism is extremely useful because it allows a rapid increase in the initial rate of transport with small increases in the concentration of toxicant.

In contrast with methotrexate and MMA(GS)_2, DMA^V was found to be transported by MRP4 in a pH-dependent manner. The highest transport rate of DMA^V was at pH 5.5, at which the predominant form of DMA^V is neutral. In contrast, the transport rate of DMA^V at pH 6.5 and 7.4 did not correlate with the concentration of neutral or negatively charged species. Thus, from the current data it is not possible to determine if the neutral, negatively charged, or both forms of DMA^V are the substrate(s) for MRP4. MRP4 generally transports negatively charged molecules (Russel et al., 2008; Keppler, 2011), and transport of a neutral molecule would be somewhat unexpected.

Rat Mrp2 transports As(GS)_3 and MMA(GS)_2 into bile, and this is potentially important for preventing hepatotoxicity; however, these GSH conjugates are not stable at the alkaline pH of bile (Kala et al., 2000; Gailer et al., 2002; Raab et al., 2004; YehiyaN et al., 2009). This leads to their rapid dissociation, reabsorption across the intestine, and cycling back to the liver through the portal circulation (Dietrich et al., 2001; Suzuki et al., 2001). Therefore, biliary excretion of As(GS)_3 and MMA(GS)_2 does not necessarily represent elimination of arsenic from the body (Leslie, 2012). In contrast, basolateral efflux of DMA^V and MMA(GS)_2 by MRP4 from the liver into sinusoidal blood could be a critical step in the pathway to eventual urinary elimination.

The localization of MRP4 to the apical surface of renal proximal tubule cells suggests that this protein might be important for the elimination of MMA(GS)_2 and DMA^V into urine. Mrp2 is also found at the apical surface of the renal proximal tubule; however, studies using Mrp2-deficient TR^- rats suggest that an Mrp in addition to Mrp2 is critical for urinary arsenic elimination (Kala et al., 2004). In fact, the urinary elimination of total arsenic was increased approximately 2-fold in the absence of Mrp2, likely due to the loss of Mrp2-dependent enterohepatic circulation of As(GS)_3 and MMA(GS)_2 (Kala et al., 2004). A further explanation for the increased urinary elimination is the increased expression of
Mrp4 in the kidney of the TR- rat (Chen et al., 2005). Overall, this evidence suggests that Mrp4 could play a more important role in urinary arsenic elimination than Mrp2.

To prevent and treat arsenic-induced toxicity, and to use arsenic effectively and safely in the clinic, it is critical to understand the cellular handling and elimination pathways of this metalloid. This work identifies Mrp4 as a novel transport pathway for DMA³ and MMA(GS)₂. Mrp4 is highly polymorphic (Abla et al., 2008; Krishnamurthy et al., 2008), and variants that alter DMA³ and/or MMA(GS)₂ transport could modulate an individual’s susceptibility to arsenic-induced toxicity. The contribution of Mrp4 to the well established but poorly understood interindividual susceptibility to arsenic is worthy of future investigation.

Acknowledgments

The authors thank Dr. Piet Borst (The Netherlands Cancer Institute) for the MRp5 cDNA, Dr. Susan P. Cole and Dr. Roger G. Deeley (Queen’s University) for the MRp3 cDNA and the mouse monoclonal anti-human MRp1 (QCRL-1) antibody, Dr. William R. Cullen (University of British Columbia) for the MMAIII and DMAIII, and Dr. Dietrich Keppler (German Cancer Research Center) for the MMAIII and DMAIII, monoclonal anti-human MRP1 (QCRL-1) antibody, Dr. William R. Cullen (University of British Columbia) for the MMAIII and DMAIII, and Dr. William R. Cullen (University of British Columbia) for the MMAIII and DMAIII. Excellent technical assistance from Diane Swland (cell culture) and Xiufen Lu (ICP-MS) is gratefully acknowledged.

Authorship Contributions

Participated in research design: Carew, Banerjee, Roggenbeck, Whitlock, Leslie. Conducted experiments: Banerjee, Carew, Roggenbeck, Whitlock. Contributed new reagents or analytic tools: Carew, Roggenbeck, Naranmandura, L. Performed data analysis: Banerjee, Carew, Roggenbeck, Whitlock, Leslie. Wrote or contributed to the writing of the manuscript: Carew, Banerjee, Roggenbeck, Leslie.

References


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A Novel Pathway for Arsenic Elimination: Human Multidrug Resistance Protein 4 (MRP4/ABCC4) Mediates Cellular Export of Dimethylarsinic Acid (DMA\text{V}) and the Diglutathione Conjugate of Monomethylarsonous acid (MMA\text{III}).

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Supplemental Figure 1. MRP4 and MRP1 protein levels in HEK293T transiently or HEK293 cells stably expressing MRP4.

Supplemental Figure 2. MRP3 and MRP5 protein levels after stable expression in HEK293 cells.
Supplemental Figure 1. MRP4 and MRP1 protein levels in HEK293T transiently or HEK293 cells stably expressing MRP4. MRP4 levels in membrane vesicles (2 µg of protein) prepared from HEK293T cells transiently expressing empty pcDNA3.1(+)neomycin (Vector) or pcDNA3.1(+)-MRP4 (MRP4) and HEK293 cells stably expressing pcDNA3.1(+)-neomycin (V4) or pcDNA3.1(+)-MRP4 (MRP4-1E16) were determined after resolving protein by SDS-PAGE and immunoblotting with the MRP4 antibody M1-10. Blots were then stripped and probed for either MRP1 (using the MRP1 antibody QCRL-1) or the Na⁺/K⁺ ATPase as a loading control (using the Na⁺/K⁺ ATPase antibody H-300).
Supplemental Figure 2. MRP3 and MRP5 protein levels after stable expression in HEK293 cells. The relative levels of A) MRP3 or B) MRP5 in membrane vesicles (2 µg of protein) prepared from HEK293 cells stably expressing empty vector pcDNA3.1(+)neomycin (V4), pcDNA3.1(+)MRP3 (HEK-MRP3) or pcDNA3.1(-)MRP5 (HEK-MRP5). Protein was resolved by SDS-PAGE and blotted with either the MRP3 antibody M II-9 or the MRP5 antibody M I-1. Blots were then stripped and probed for the Na+/K+ ATPase as a loading control (using the Na^+/K^- ATPase antibody H-300).