Monomethylarsenic Diglutathione Transport by the Human Multidrug Resistance Protein 1 (MRP1/ABCC1)

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ABSTRACT:
The ATP-binding cassette (ABC) transporter protein multidrug resistance protein 1 (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 (MMA₃), monomethylarsonic acid (MMA₅), dimethylarsinous acid (DMA₃), and dimethylarsinic acid (DMA₅). The trivalent forms MMA₃ and DMA₃ are more reactive and toxic than their inorganic precursors, arsenite (AsO₃³⁻) and arsenate (AsO₅³⁻). The ability of MRP1 to transport methylated arsenicals is unknown and was the focus of the current study. HeLa cells expressing MRP1 (HeLa-MRP1) were found to confer a 2.6-fold higher level of resistance to MMA₃ than empty vector control (HeLa-vector) cells, and this resistance was dependent on GSH. In contrast, MRP1 did not confer resistance to DMA₃, MMA₅, or DMA₅. HeLa-MRP1 cells accumulated 4.5-fold less MMA₃ than HeLa-vector cells. Experiments using MRP1-enriched membrane vesicles showed that transport of MMA₃ was GSH-dependent but not supported by the nonreducing GSH analog, ophthalmic acid, suggesting that MMA₃(GS)₂ was the transported form. MMA₃(GS)₂ was a high-affinity, high-capacity substrate for MRP1 with an apparent Kₚᵣ and Vₘₐₓ values of 11 μM and 11 nmol mg⁻¹ min⁻¹, respectively. MMA₃(GS)₂ transport was osmotically sensitive and inhibited by several MRP1 substrates, including 17β-estradiol 17-β-D-glucuronide (E₂17βG). MMA₃(GS)₂ competitively inhibited the transport of E₂17βG with a Kᵢ 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 MMA₃ and have implications for environmental and clinical exposure to arsenic.

Introduction

Arsenic (As) is a multitarget human carcinogen; chronic exposure is associated with increased incidences of skin, lung, and bladder tumors (Straif et al., 2009). More than 100 million people worldwide are chronically exposed to unacceptable levels of nonanthropogenic sources of As [both arsenite (AsO₃³⁻) and arsenate (AsO₅³⁻)] in their drinking water, the most common exposure route for adverse health effects (Rahman et al., 2009). Arsenic compounds are also used in chemotherapy. Arsenic trioxide (As₂O₃) has been approved for treating acute promyelocytic leukemia with high remission rates (Emadi and Gore, 2010). Furthermore, As₂O₃ and another arsenical, dimethylarsenic glutathione, are in clinical trials for the treatment of multiple hematological and solid tumors (Mann et al., 2009; Emadi and Gore, 2010).

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ABBREVIATIONS:
As, arsenic; ABC, ATP-binding cassette; AsO₃³⁻, arsenite; AsO₅³⁻, arsenate; As(GS)₃, arsenic triglutathione; BSO, L-buthionine sulfoximine; DMA₃, dimethylarsinous acid; DMA₅, dimethylarsinic acid; E₂17βG, 17β-estradiol 17-β-D-glucuronide; ICP-MS, inductively coupled plasma mass spectrometry; MMA₃, monomethylarsonous acid; MMA₅(GS)₂, monomethylarsenic diglutathione; MMA₅, monomethylarsonic acid; MRP, multidrug resistance protein; VCR, vincristine; GSTP1, glutathione transferase P1; HEK, human embryonic kidney.

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clinical anticancer drug resistance (Deeley et al., 2006). In addition, MRP1 is expressed in nonmalignant tissues and transports a chemically diverse array of endogenous molecules, including GSH and GSSG conjugated organic anions, such as the cholestatic steroid 17β-estradiol 17-ß-glucuronide (E17ßG) and the cysteiny1 leukotriene 4 (Deeley et al., 2006). 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 (Cole and Deeley, 2006). Evidence suggests that at least some of these substrates are coexported with GSH across the plasma membrane.

In addition to anticancer 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 (Leslie et al., 2005). MRP1 is well known to play an important role in conferring cellular protection against inorganic arsenicals (Cole et al., 1994; Lorico et al., 2002) and transports AsIII in its trithiolate conjugate form, As(GS)3 (Leslie et al., 2004). MRP1 is expressed in most tissues throughout the body; however, its levels are almost undetectable in the healthy human liver (Deeley et al., 2006). 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. Therefore, MRP1 is unlikely to be directly involved in the elimination of arsenic from the body. However, MRP1 is potentially important for preventing the accumulation of arsenicals in certain cell types and tissues. The expression of MRP1 in tumors 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 MMAIII in a GSH-dependent manner and also transports MMAIII(GS)2. The transport of MMAIII was not supported by ophthalmic acid, a GSH protector against MMAIII in a GSH-dependent manner and also transports MMAIII(GS)2. The transport of MMAIII(GS)2 was then characterized extensively.

Materials and Methods

Chemicals and Reagents. GSH, GSSG, ATP, AMP, succrose, Tris base, AsIII, AsV, MMAIII, DMAIII, L-buthionine sulfoximine (BSO), and MgCl2 were purchased from Sigma-Aldrich (Oakville, ON, Canada). Carrier free [6,7-3H]estradiol 17βG (50.1 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Woodbridge, ON, Canada). Creatine kinase, glutathione reductase, creatine phosphate, NADPH, and protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). Nicotinic acid was purchased from Fisher Scientific (Ottawa, ON, Canada). E17βG was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Ophthacilic acid was purchased from Bachem (Torrance, CA). MMAIII and DMAIII in the form of diiodomethylarsine (CH2AsI2) and iodomethylarsine (CH3AsI) were gifts from Dr. William Cullen (University of British Columbia, Vancouver, BC, Canada).

Cell Lines. The HaLa-vector and HeLa-MRP1 cells were gifts from Dr. Susan P.C. Cole (Queen’s University, Kingston, ON, Canada) and generated and maintained as described previously (Ito et al., 2001). 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 (Ito et al., 2001). 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.

Cytotoxicity Testing. HeLa-MRP1 and HeLa-vector cells were seeded in 96-well plates at 1 × 104 cells/well and grown for 24 h. In quadruplicate, cells were then treated with AsIII (0.1–100 μM), MMAIII (0.1–100 μM), DMAIII (0.1–100 μM), AsV (0.001–10 mM), MMAV (0.3–100 mM), or DMAV (0.01–100 mM) for 72 h. To measure the influence of GSH depletion on AsIII and MMAIII 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 (Rahman et al., 2006). 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 CellTitre96 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 EC50 values were determined. Relative resistance values were calculated as the ratio of the HeLa-MRP1 EC50/HeLa-vector EC50.

Cellular Accumulation of Arsenic Species. HeLa-vector and HeLa-MRP1 cells were seeded in six-well plates at 5 × 105 cells/well and grown for 24 h. Cells were treated with 1 μM AsIII, MMAIII, or DMAIII 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, Hachioji, Japan) using the standard addition method as described previously (Kalivas, 1987). Samples were introduced directly into the nebulizer of the ICP-MS.

Transient Expression of MRP1 in HEK293 Cells. HEK293 cells, HEK293 cells were transfected with pcDNA3.1(−)MRP1 [constructed as described previously (Ito et al., 2001)] using the calcium phosphate method as described previously (Carew and Leslie, 2010). In brief, 3 × 105 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 NaHPO4, 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 800g for 10 min. Cell pellets were stored at −80°C until membrane vesicles were prepared.

Membrane Vesicle Preparation. Plasma membrane-enriched vesicles were prepared from MRP1 and empty pcDNA3.1(−)-transfected HEK293 cells, according to previously described methods (Carew and Leslie, 2010). In brief, cells were thawed and resuspended in buffer containing 250 mM sucrose, 50 mM Tris, pH 7.4, 0.25 mM CaCl2, 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 800g 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 100,000g 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 100,000g for 30 min at 4°C. The membranes were washed with Tris sucrose buffer and centrifuged at 100,000g 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.
physiological concentrations of GSH (3 mM) were maintained in all MMAIII(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 (0.1–30 μM) on the transport of MMAIII was measured at a 1-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 MMAIII(GS)₂ (0.1–30 μM). The modulation of MMAIII(GS)₂ transport by the MRP1 substrates E₂¹βG (25 μM), GSSG (500 μM), and VCR (100 μM) was measured at a 1-min time point.

**E₂¹βG Transport Inhibition Assays.** Inhibition of E₂¹βG transport by MMAIII(GS)₂ was performed essentially as described previously (Loe et al., 1996a). In brief, 5 μg of membrane vesicles were incubated with [³H]E₂¹βG (0.1–30 μM, 40–80 nCi), AMP or ATP (4 mM), MgCl₂ (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 MMAIII(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 Microbeta² (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.

### Results

**MRP1 Decreases the Cytotoxicity of MMAIII.** To determine whether 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 AsIII, MMAIII, AsV, MMAV, DMAIII, DMAV, and DMAV. The EC₅₀ value for each arsenical was determined, and the relative resistance was calculated from the ratio of the EC₅₀ values for HeLa-MRP1 and HeLa-vector (summarized in Table 1). Consistent with previously published work, HeLa-MRP1 cells conferred resistance to AsIII and AsV (Fig. 1A; Table 1) (Cole et al., 1994). HeLa-MRP1 cells were approximately 3-fold more resistant to MMAIII compared with the vector control cell line (Fig. 1B); however, no difference in the sensitivity of the cell lines was observed for MMAV, DMAIII, or DMAV (Table 1).

To determine whether resistance conferred by MRP1 to AsIII and MMAIII 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 are known to confer resistance to arsenicals partly through the neutralization of reactive oxygen species (Ducheler et al., 2008). Consistent with this, both arsenicals became significantly more toxic to HeLa-vector and HeLa-MRP1 cell lines after GSH depletion (Tables 1 and 2; Fig. 1). Furthermore, GSH depletion

### Table 1

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<tr>
<th>Arsenic Species</th>
<th>EC₅₀ (±S.E.)</th>
<th>Relative Resistance*</th>
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<tr>
<td></td>
<td>HeLa-Vector</td>
<td>HeLa-MRP1</td>
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<tr>
<td>AsIII (n = 11)</td>
<td>7 ± 0.7</td>
<td>22 ± 1.4</td>
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<tr>
<td>AsV (n = 5)</td>
<td>510 ± 70</td>
<td>1000 ± 100</td>
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<tr>
<td>MMAIII (n = 6)</td>
<td>3.1 ± 0.4</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td>MMAV (n = 5)</td>
<td>9800 ± 780</td>
<td>9400 ± 1000</td>
</tr>
<tr>
<td>DMAIII (n = 3)</td>
<td>6 ± 0.3</td>
<td>7 ± 1.3</td>
</tr>
<tr>
<td>DMAV (n = 5)</td>
<td>740 ± 50</td>
<td>710 ± 180</td>
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*Ratio of EC₅₀ HeLa-MRP1/EC₅₀ HeLa-vector.

* EC₅₀ for HeLa-MRP1 is significantly different from HeLa-vector, P < 0.05 (Student's t test).

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**Fig. 1.** Effect of AsIII and MMAIII on the viability of transfected HeLa cells. Vector control transfected HeLa cells (C) and MRP1-transfected HeLa cells (D) were incubated in the presence of AsIII (A), MMAIII (B), AsIII and BSO (100 μM) (C), or MMAIII and BSO (100 μM) (D) for 72 h. 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 four additional experiments.
MRP1 Reduces the Accumulation of As\textsuperscript{III} and MMA\textsuperscript{III}. To assess the effect of MRP1 on the cellular accumulation of As\textsuperscript{III}, MMA\textsuperscript{III}, and DMA\textsuperscript{III}, the accumulation of total arsenic was determined after HeLa-vector and HeLa-MRP1 exposure to As\textsuperscript{III}, MMA\textsuperscript{III}, or DMA\textsuperscript{III} (1 mM for 24 h). Consistent with the cytotoxicity data, HeLa-MRP1 cells accumulated As\textsuperscript{III} and MMA\textsuperscript{III} at 3.5- and 4.5-fold, respectively, less than HeLa-vector cells (Fig. 2). No significant difference in DMA\textsuperscript{III} accumulation between the HeLa-MRP1 and HeLa-vector cell lines was observed (Fig. 2).

MRP1 Transports MMA\textsuperscript{III} in the Presence of GSH or as the Presynthesized GSH Conjugate MMA\textsuperscript{III}(GS)\textsubscript{2}. To determine whether MMA\textsuperscript{III} and/or MMA\textsuperscript{III}(GS)\textsubscript{2} were substrates of MRP1, ATP-dependent transport of these compounds into MRP1-enriched and vector control membrane vesicles was measured (Fig. 3A). ATP-dependent transport of MMA\textsuperscript{III} (1 mM) 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\textsuperscript{III} was observed with an activity of 308 pmol mg\textsuperscript{-1} min\textsuperscript{-1}. In previous studies, it has been shown that ophthalmic acid and other GSH analogs lacking a free thiol group can substitute for GSH and support the transport of several GSH-dependent MRP1 substrates (Loe et al., 1996b, 1998; Renes et al., 1999). These data 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 the transport to occur. However, ATP-dependent transport of MMA\textsuperscript{III} in the presence of ophthalmic acid (3 mM) was extremely low and similar to −GSH conditions or empty vector control (Fig. 3A). ATP-dependent transport of MMA\textsuperscript{III}(GS)\textsubscript{2} was then measured and found to have an activity of 240 pmol mg\textsuperscript{-1} min\textsuperscript{-1}. These data suggest that the free thiol group of GSH is required for MMA\textsuperscript{III} transport by MRP1 and are consistent with MMA\textsuperscript{III}(GS)\textsubscript{2} being the transported chemical species.

Using similar experimental conditions, transport of DMA\textsuperscript{III} and DMA\textsuperscript{III}(GS)\textsubscript{2} 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\textsuperscript{III}(GS)\textsubscript{2} rapidly dissociates in the absence of physiological concentrations of GSH (1–10 mM) (Kala et al., 2000; Kobayashi et al., 2005; Yehiayan et al., 2009). Thus, as stated in Materials and Methods, 3 mM GSH was included in all transport reactions containing MMA\textsuperscript{III}(GS)\textsubscript{2}. Because GSH can stimulate the transport of certain conjugated and unconjugated substrates of MRP1 (Cole and Deeley, 2006), the influence of different concentrations of GSH on MMA\textsuperscript{III} transport was evaluated (Fig. 3B). No significant difference in MMA\textsuperscript{III}(GS)\textsubscript{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 (Loe et al., 1996b, 1998; Renes et al., 1999). These data suggest that GSH is not stimulating the transport of MMA\textsuperscript{III}(GS)\textsubscript{2} by MRP1.

Osmotic Sensitivity of MMA\textsuperscript{III}(GS)\textsubscript{2} Transport. To confirm that the ATP-dependent MMA\textsuperscript{III}(GS)\textsubscript{2} transport by the MRP1-enriched

| Arsenic Species | EC\textsubscript{50} (\pm S.E.) | Relative Resistance
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<tr>
<td>As\textsuperscript{III} (n = 5)</td>
<td>0.37 ± 0.12</td>
<td>0.3</td>
</tr>
<tr>
<td>MMA\textsuperscript{III} (n = 6)</td>
<td>0.02 ± 0.004</td>
<td>0.5</td>
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\( ^{a} \) Ratio of EC\textsubscript{50} HeLa-MRP1/EC\textsubscript{50} HeLa-vector.

Fig. 2. Accumulation of As\textsuperscript{III}, MMA\textsuperscript{III}, and DMA\textsuperscript{III} by transfected HeLa cells. Vector control transfected HeLa cells (open bars) and MRP1 transfected HeLa cells (closed bars) were treated with 1 mM As\textsuperscript{III}, MMA\textsuperscript{III}, or DMA\textsuperscript{III} for 24 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 (\( \pm S.E. \)). * accumulation is significantly different from HeLa-vector, \( P < 0.001 \) (Student’s t test).
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. MMAIII(GS)2 uptake was decreased as the concentration of sucrose in the transport buffer increased, indicating that the ATP-dependent MMAIII(GS)2 uptake by the vesicles is osmotically sensitive, as expected for a true transport process (Fig. 4A).

**Kinetic Analysis of MRP1-Mediated MMAIII(GS)2 Transport.** Time courses of MMAIII(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 maximal activity of ~900 pmol mg⁻¹ at 10 min (Fig. 4B). MRP1-mediated transport was further characterized by determining the initial rates of transport over several concentrations of MMAIII(GS)2 (Fig. 4B). According to Michaelis-Menten kinetic analysis (GraphPad Prism 5), the average apparent Kₘ and Vₘₐₓ values (±S.E., n=3) for MMAIII(GS)2 were 11 ± 2.3 μM and 11 ± 0.6 nmol mg⁻¹min⁻¹, respectively.

**Inhibition of MMAIII(GS)2 Transport by Various Substrates of MRP1.** Several MRP1 substrates, including E17βG, GSSG, and VCR (+GSH) have been shown previously to be competitive inhibitors of MRP1 transport (Leier et al., 1996; Loe et al., 1996a, 1998). Transport of MMAIII(GS)2 (1 μM) was inhibited by E17βG (25 μM), GSSG (500 μM), and VCR (100 μM) by 57, 75, and 79%, respectively (Fig. 5A). Inhibitors were used at concentrations shown in previous studies to be at least 5- to 10-fold above their respective Kₘ or Kᵢ values to ensure binding site saturation.
MMA\textsuperscript{III}(GS)\textsubscript{2} Inhibition of E\textsubscript{17βG} Transport by MRPI. To determine whether MMA\textsuperscript{III}(GS)\textsubscript{2} and E\textsubscript{17βG} have overlapping binding sites on MRPI, the ability of MMA\textsuperscript{III}(GS)\textsubscript{2} to competitively inhibit the transport of E\textsubscript{17βG} was evaluated. The inhibition of E\textsubscript{17βG} transport by MRPI-enriched membrane vesicles was characterized by measuring the effect of MMA\textsuperscript{III}(GS)\textsubscript{2} (50 µM) on E\textsubscript{17βG} (0.1–30 µM) transport (Fig. 5C). Michaelis-Menten analysis showed that MMA\textsuperscript{III}(GS)\textsubscript{2} behaved as a competitive inhibitor of E\textsubscript{17βG} transport with an average apparent $K_v$ value $(\pm$ S.E., $n = 7)$ of 16.3 $\pm$ 3.6 µM (Fig. 5B). These data indicate that MMA\textsuperscript{III}(GS)\textsubscript{2} and E\textsubscript{17βG}, at minimum, have overlapping binding sites on MRPI.

Discussion

The metalloid arsenic is a multitarget 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 (Rosen and Liu, 2009). Members of the ABC transporter superfamily subfamily “C” have been shown to be critical for protecting many organisms from arsenic, including Saccharomyces cerevisiae, Leishmania, Arabidopsis, Caenorhabditis elegans, and Danio rerio (Broeks et al., 1996; Dey et al., 1996; Ghosh et al., 1999; Long et al., 2011; Song et al., 2010). In all of these organisms, ABC proteins detoxify arsenic either by extrusion from cells or through sequestration within intracellular organelles as thiol conjugates. We have shown previously human MRPI can transport inorganic arsenic as the triglutathione conjugate As(GS)\textsubscript{3} (Leslie et al., 2004), but the ability of MRPI to confer cellular protection to methylated arsenicals was unknown. In the present study, we have found that a HeLa cell line stably expressing MRPI is capable of conferring a 2.6-fold higher resistance level to MMA\textsuperscript{III} than the HeLa cell line expressing empty vector. Consistent with previously published observations, MRPI also conferred resistance to MMA\textsuperscript{III} and As\textsuperscript{V} (Cole et al., 1994). MRI did not confer protection against the pentavalent methylated arsenicals MMA\textsuperscript{V} and DMA\textsuperscript{V} or the trivalent DMA\textsuperscript{III}.

GSH depletion has been shown to enhance arsenic trioxide-induced apoptosis (Duechler et al., 2008; Han et al., 2008; Bhalia et al., 2010). Consistent with this, the toxicities of As\textsuperscript{III} and MMA\textsuperscript{III} were increased upon GSH depletion with BSO for both the HeLa vector and HeLa-MRPI cell lines, most dramatically for MMA\textsuperscript{III} (Fig. 1; Table 2). In addition, MRPI resistance levels to As\textsuperscript{III} and MMA\textsuperscript{III} were dependent upon GSH, suggesting a GSH-dependent efflux pathway. We have previously determined that As\textsuperscript{III} is transported by MRPI as As(GS)\textsubscript{3} (Leslie et al., 2004), and the next step was to investigate the species of MMA\textsuperscript{III} being transported. Direct transport assays using MRPI-enriched membrane vesicles confirmed that MMA\textsuperscript{III} was GSH-dependent. MRPI is capable of transporting compounds covalently attached to GSH or through a cotransport pathway (Cole and Deeley, 2006). Thus, transport of MMA\textsuperscript{III} was measured in the presence of the nonreducing GSH analog ophthalmic acid, known to stimulate the transport of GSH-dependent MRPI substrates (Loe et al., 1998; Leslie et al., 2001, 2003; Qian et al., 2001; Peklak-Scott et al., 2005). In the case of MMA\textsuperscript{III}, ophthalmic acid did not substitute for GSH; thus, the free sulfur group of GSH was required for transport. These data suggest that MMA\textsuperscript{III} conjugated to GSH is the transported form. Although GSH is best characterized to detoxify arsenicals through neutralization of reactive oxygen species (Flora, 2011), our data provide further support that formation and efflux of arsenic GSH conjugates are potentially of equal importance.

We have shown previously that the MRPI-dependent transport of As\textsuperscript{III} requires GSH and the glutathione transferase P1 (GSTP1) (Leslie et al., 2004). It is currently unknown whether the formation of MMA\textsuperscript{III}(GS)\textsubscript{2} under physiological conditions requires catalysis by GSTP1 or a related transferase. In the current study, transport activities for MMA\textsuperscript{III}(GS)\textsubscript{2} and free MMA\textsuperscript{III} in the presence of GSH were very similar (Fig. 3A). Superficially, these results suggest that MMA\textsuperscript{III}(GS)\textsubscript{2} formation is spontaneous; however, high levels of plasma membrane-associated GSTP1 exist in the HEK293 vesicles used in this study (Qazi et al., 2011), and although further experimentation is required, GSTP1 could be involved in catalyzing the formation of MMA\textsuperscript{III}(GS)\textsubscript{2}.

MMA\textsuperscript{III}(GS)\textsubscript{2} has been isolated from rat bile and mouse urine and, therefore, is formed physiologically and excreted by the liver and kidney (Kala et al., 2000, 2004). Biliary excretion of MMA\textsuperscript{III}(GS)\textsubscript{2} and As(GS)\textsubscript{3} in rats is dependent on the MRPI-related multidrug resistance protein 2 (Mrp2/Abcc2), and these conjugates account for most of the arsenic in bile (Kala et al., 2000). In mice deficient in γ-glutamyl transpeptidase, an enzyme responsible for GSH and GSH-conjugate catabolism, approximately 60 to 70% of the urinary arsenic was present as a GSH conjugate (Kala et al., 2004). 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\textsuperscript{III}(GS)\textsubscript{2} and As(GS)\textsubscript{3}, these conjugates are unstable at bile pH resulting in free As\textsuperscript{III} and MMA\textsuperscript{III} and entering the enterohepatic circulation, and urinary excretion is the predominant elimination pathway (Kala et al., 2000, 2004; Yehiayan et al., 2009). Thus, it is critical to understand how extrahepatic tissues handle arsenicals through transport proteins such as MRPI.

We have previously determined that the transport of As(GS)\textsubscript{3} by MRPI-enriched membrane vesicles prepared from the small cell lung cancer cell line H69AR is of high affinity but low capacity with an apparent $K_m$ of 0.32 µM, $V_{max}$ of 17 pmol mg\textsuperscript{-1} min\textsuperscript{-1} and overall efficiency $(V_{max}/K_m)$ of 53 µl mg\textsuperscript{-1} min\textsuperscript{-1} (Leslie et al., 2004). In the current study, transport of MMA\textsuperscript{III}(GS)\textsubscript{2} was found to be of high affinity and high capacity with an apparent $K_m$ of 11 µM, $V_{max}$ of 11,000 pmol mg\textsuperscript{-1} min\textsuperscript{-1} and overall efficiency $(V_{max}/K_m)$ of 1000 µl µmol\textsuperscript{-1} mg\textsuperscript{-1} min\textsuperscript{-1}. Thus, the overall efficiency of MMA\textsuperscript{III}(GS)\textsubscript{2} transport is remarkably higher than that for As(GS)\textsubscript{3}. In tissues with sufficient GSH levels, as well as methylation capacity, MRPI is probably an important detoxification pathway for the highly toxic MMA\textsuperscript{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\textsubscript{2}O\textsubscript{3} have plasma levels in the low micromolar range and tissue concentrations would probably exceed this value (Yoshino et al., 2009).

Despite the 20-fold higher efficiency of transport of MMA\textsuperscript{III}(GS)\textsubscript{2} than As(GS)\textsubscript{3} by MRPI, little difference existed between the resistance level and cellular accumulation of MMA\textsuperscript{III} and As\textsuperscript{III} in the intact HeLa-MRPI cell line (Figs. 1 and 2; Table 1). The whole-cell assay conditions involved a 24- to 72-h exposure of cells to arsenicals, whereas the membrane vesicle experiments measured transport of these conjugates for 1 to 3 min; thus, experimental results are difficult to compare. The chemical instability of the arsenic glutathione conjugates make it probable that, under culture conditions, the cellular protection conferred by MRPI to either As\textsuperscript{III} or MMA\textsuperscript{III} is underestimated compared with 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\textsuperscript{III}(GS)\textsubscript{2} or As(GS)\textsubscript{3} transported by MRPI 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 MRPI, its overexpression in hematological and solid tumors could have deleterious consequences by conferring resistance to arsenic-based chemotherapeutic
tics, in particular As$_3$O$_3$. In aqueous solution at physiological pH, As$_3$O$_3$ exists as As(OH)$_3$ or As$^{3+}$ (Liu et al., 2004). Thus, As$_3$O$_3$ can be methylated to MMA$_3$ and DMA$_3$$_3$, and these metabolites have been shown to be more potent inducers of apoptosis in leukemia and lymphoma cells than As$_3$O$_3$ (Chen et al., 2003). MRP1 could reduce the cellular concentrations of both inorganic arsenic and MMA$_3$ through the efflux of As(GS)$_3$ and MMA$_3$GS$_2$, and reduce treatment efficacy.

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$_3$ through the high-capacity efflux of MMA$_3$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$_3$GS$_2$. MRP1 is highly polymorphic, and genetic variants could account for some of the well-established but poorly understood interindividual susceptibility to arsenic-induced carcinogenesis (Conseil et al., 2005; Hernández and Marcos, 2008).

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Authorship Contributions

Participated in research design: Carew, Naranmandura, Shukalek, and Leslie
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Contributed new reagents or analytic tools: Naranmandura and Le Performed data analysis: Carew, Naranmandura, Shukalek, and Leslie
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References


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