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Glutathione-dependent interaction of heavy metal compounds with multidrug resistance proteins MRP1 and MRP2

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ABSTRACT

The interactions of three heavy metal-containing compounds, cisplatin (CDDP), arsenic trioxide (As_2O_3), and mercury dichloride ($HgCl_2$), with the multidrug resistance transporters MRP1 and MRP2 and the involvement of glutathione (GSH)-related processes herein were investigated. In Madin–Darby canine kidney cells stably expressing MRP1 or MRP2, viability, GSH content, calcein efflux and polarized GSH efflux were measured as a function of exposure to CDDP, As_2O_3 and $HgCl_2$. In isolated *Sf*9-MRP1 and *Sf*9-MRP2 membrane vesicles, the interaction with MRP-associated ATPase activity was measured. In the latter model system adduct formation with GSH is not an issue.

The data show that (1) CDDP interacts with both MRP1 and MRP2, and GSH appears to play no major role in this process, (2) As_2O_3 interacts with MRP1 and MRP2 in which process GSH seems to be essential, and (3) $HgCl_2$ interacts with MRP1 and MRP2, either alone and/or as a metal–GSH complex.

1. Introduction

Heavy metal compounds, like cisplatin (*cis*-diammineplatinum(II) dichloride, CDDP) and arsenic trioxide (As_2O_3) are commonly used chemotherapeutics, but resistance towards this class of chemotherapeutics has been associated with enhanced glutathione levels and enhanced multidrug transporter proteins (MRPs) (Ishikawa et al., 1996; Borst et al., 2000). The membrane-embedded proteins MRP1 (ABCC1) and MRP2 (ABCC2) are members of the ATP-binding cassette (ABC) transporter protein superfamily and mediate the unidirectional cell efflux of glutathione-, glucuronide- and sulphate-conjugates of lipophilic substances. But also unconjugated amphiphilic anions have been shown to be substrates of these transport proteins, in which process the tripeptide glutathione appears to be essential (Leslie et al., 2001; Borst and Elferink, 2002; Borst et al., 2006). In many tumor cells, an overexpression is often accompanied with high levels of intracellular reduced glutathione (GSH) and glutathione-*S*-transferase (GST) resulting in an enhanced detoxification and efflux of the anticancer agent and finally unwanted multidrug resistance. Although it has been shown by many researchers that resistance of tumor cells to either cisplatin or arsenic trioxide is associated with enhanced levels of multidrug resistance proteins and/or glutathione (Ishikawa et al., 1996; Sharp et al., 1998; Cui et al., 1999; Vernhet et al., 2000; Kauffmann et al., 2002), the interactions of heavy metal-containing compounds with these GSH-related processes remains unclear. Next to these chemotherapeutics, also other heavy metal compounds, such as $HgCl_2$, are known to interact with MRP and GSH (Zalups, 2000; Aleo et al., 2005).

The role of GSH in the transport process of MRP1- and MRP2-mediated transport is diverse (Cnubben et al., 2005; Deeley and Cole, 2006; Ballatori et al., 2005). Except for the fact that MRP1 is known as a typical glutathione-*S*-conjugate (GS-X) pump, compounds that are not metabolized to a glutathione conjugate can also be substrates of MRP1 (see rev. Haimeur et al., 2004). For some of these compounds (such as the chemotherapeutic agents vincristine and daunomycin) it has been shown that in their transport GSH is co-transported out of the cell (Loe et al., 1996, 1998; Rappa et al., 1997). Glutathione can also stimulate the transport of compounds without being transported itself, as was demonstrated for estrone-3 sulphate and the glucuronide of the nitrosamine

Abbreviations: As_2O_3, arsenic trioxide; calcein-AM, calcein acetoxymethylester; CDDP, cisplatin (*cis*-diammineplatinum(II) dichloride); GSH, reduced glutathione; GST, glutathione *S*-transferase; HBSS, Hanks' balanced salt solution; $HgCl_2$, mercury dichloride; LTC_4, cysteinyl leukotriene; MDCK cells, Madin–Darby canine kidney cells; MDR, multidrug resistance protein; Pgp, P-glycoprotein; MRP, multidrug resistance protein; *Sf*9, *Spodoptera frugiperda* insect cells.

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metabolite (Qian et al., 2001; Leslie et al., 2001), indicating that an allosteric interaction of glutathione is needed for the transport of these compounds. In addition, it has been shown that some compounds (e.g. verapamil and some flavonoids) can stimulate the export of glutathione without being transported themselves (Loe et al., 2000a,b; Leslie et al., 2001; Leslie et al., 2003). A model of cross-stimulated transport for MRP1 has recently been described by Borst et al. (2006) to explain the complexity to proof processes of either co-transport or stimulation of transport by GSH.

In the present study, the interaction of three heavy metal-containing compounds, i.e. CDDP, As_2O_3, and $HgCl_2$ with MRP1 and MRP2, and the involvement of GSH-related processes herein was investigated. CDDP and As_2O_3 were used as model compounds for metal-containing chemotherapeutic agents and $HgCl_2$ was included as a model compound of a metal-containing environmental pollutant. From literature, the affinity for GSH and the stability of the metal–SG adducts appears to be CDDP $\ll$ As_2O_3 $\ll$ $HgCl_2$ (Dabrowiak et al., 2002; Hagrman et al., 2004; Delnomdedieu et al., 1994; Gailer and Lindner, 1998; Csanaky and Gregus, 2005; Ballatori, 2002). As a result of the different affinity for GSH, different levels of interaction with GSH and MRP-mediated transport for these three metal-containing compounds are to be expected. The aim of the present study was to examine these interactions of the heavy metal-containing compounds with GSH-related processes and MRP1- and MRP2-mediated transport. Due to the difficulty in accurately determining the heavy metal compound in its right chemical form either as a heavy metal ion or as a heavy metal complex several model systems and assays were used. Hereto, intact Madin–Darby canine kidney (MDCKII) cells stably transfected with human MRP1 and MRP2 were used to study the effect of CDDP, $HgCl_2$, As_2O_3 exposure on GSH content, MRP1- and MRP2-mediated efflux of model substrate calcein and GSH. In addition, in isolated membrane vesicles containing high levels of either MRP1 or MRP2, the direct interaction of the heavy metal-containing compound with MRP1 and MRP2 in the absence and presence of glutathione was studied. The data show that all three heavy metal compounds, CDDP, As_2O_3, and $HgCl_2$ interact with the MRP1 and MRP2 transporter proteins and GSH, although through different mechanisms which seem to be dependent on their affinity for GSH at physiological levels.

2. Materials and methods

2.1. Chemicals and cell lines

Cisplatin was obtained from Sigma Chemical Co. (St. Louis, MO), $HgCl_2$ from J&M Laboratories (Louisville, KY) and As_2O_3 from Baker (Deventer, The Netherlands). Dulbecco's minimum essential medium (DMEM) with GlutaMax, Grace's insect medium, fetal calf serum, penicillin/streptomycin and gentamycin were all from Gibco (Paisley, Scotland). The leukotriene D4 receptor antagonist MK-571, used as a typical MRP inhibitor, was obtained from BioMol (Plymouth Meeting, PA). The Pgp inhibitor PSC833 (Valspodar) was a kind gift from Novartis Pharma AG (Basel, Switzerland). Calcein acetoxymethylester was obtained from Molecular Probes (Eugene, OR). Other chemicals were from Sigma–Aldrich Chemical Co. (St. Louis, MO), unless stated otherwise.

*Sf*9 cells were obtained from Invitrogen (Groningen, The Netherlands). Recombinant baculoviruses containing either the human *MRP1* cDNA or the human *MRP2* cDNA were a kind gift from Prof. Dr. B. Sarkadi, National Institute of Haematology and Immunology, Research Group of the Hungarian Academy of Sciences, Budapest, Hungary. The Madin–Darby Canine Kidney (MDCKII) cell lines, stably expressing either human *MRP1* cDNA (hereafter called MRP1 cells) or *MRP2* cDNA (hereafter called MRP2 cells), or transfected with an empty vector (hereafter called control cells) were kindly provided by Prof. P. Borst (NKI, Amsterdam). Cells were previously described and characterized (Evers et al., 1998, 2000; Wortelboer et al., 2003). In these studies, Western blotting revealed very limited background levels of endogenous canine Pgp, and canine Mrp2, and no background of endogenous canine Mrp1. Cells were cultured in DMEM with GlutaMax (4.5 g glucose/l), 10% fetal calf serum and 1% penicillin/streptomycin (each 10,000 U/ml), and grown in a humidified atmosphere of 5% CO_2 at 37 °C. It was shown that in these polarized cell lines MRP1 routes to the basolateral side, whereas MRP2 routes to the apical side (Evers et al., 2000).

2.2. Cell viability assay

A difference in sensitivity towards the heavy metal compounds between the three MDCKII cell lines was expected. To establish an appropriate exposure concentration in the transport studies in all three cell lines, the effect of CDDP, As_2O_3, and $HgCl_2$ on cell viability in the MDCKII cells was determined using the neutral red uptake assay, which is based on the ability of viable cells to incorporate neutral red in the lysosomes (Borenfreund and Peurner, 1985). MDCKII-control, MRP1 and MRP2 cells (10^5 cells/well) were exposed to a concentration range 0–100 μM of either CDDP, As_2O_3, or $HgCl_2$ for 6 and 24 h. A stock solution of As_2O_3 (10 mM) was prepared in 0.2 M NaOH. Stock solutions of CDDP (1 mM) and $HgCl_2$ (10 mM) were prepared in distilled water. The pH value of each medium was checked before use. Triton X-100 was used for 100% lysis control. The EC_{50} values were determined with non-linear regression curve fit using the Hill equation with Sigmaplot version 9.0 (Systat Software Inc., Richmond, CA, USA).

2.3. Studies in transfected MDCKII cells: intracellular GSH and efflux assays

The effect of CDDP, As_2O_3 and $HgCl_2$ on the intra- and extracellular GSH concentrations was determined in control, MRP1 and MRP2 cells. In brief, 4×10^5 cells/cm^2 were seeded on microporous polycarbonate filters (0.4 μm pore size, 12 mm, Costar Corp. Cambridge, MA) and grown to confluence in 3 days. Confluent monolayers of cells were incubated in DMEM containing 0.5 mM acivicin, 0.1 μM PSC833, and either 10 μM CDDP, 5 μM As_2O_3 or 5 μM $HgCl_2$, for 1 and 20 h at 37 °C. Acivicin was added as inhibitor of γ-glutamyltranspeptidase to prevent false detection of unconjugated GSH due to degradation of possible formed GSH adducts by γ-glutamyltranspeptidase. PSC833 was added as Pgp inhibitor. After exposure, aliquots of 200 μl of both apical and basolateral medium were collected, 10 μl 6.5% (w/v) sulfosalicic acid was added and samples were stored at −20 °C upon further analysis. Intracellular GSH concentrations were determined at the end of each incubation period. Cells were washed twice with ice-cold PBS. Filters with cells were cut out and collected in 0.5 ml HBSS. Cells were disrupted by sonication on ice (10 s), the cell lysate centrifuged at 2800 × *g* for 5 min, and stored frozen at −20 °C until further analysis. Aliquots of the supernatant were used to determine the GSH concentration using the method of Anderson (1985).

2.4. Calcein efflux studies

Interactions of CDDP, As_2O_3, and $HgCl_2$ with MRP1- and MRP2-mediated transport were studied using a calcein efflux assay as described by Wortelboer et al. (2005). In brief, 4×10^5 cells/cm^2 were seeded on microporous polycarbonate filters (0.4 μm pore size, 24.5, Costar Corp. Cambridge, MA) and grown to confluence in 3 days. Thereafter, cells were loaded with calcein by culturing the cells in DMEM without phenol red containing calcein-AM (1 μM) and PSC833 (0.1 μM) for 2 h at 7 °C. At this temperature, premature MRP-dependent efflux of calcein could be reduced without the use of a MRP inhibitor during loading time. Because calcein is also known to be a substrate for the endogenous canine transporters in the MDCKII cells, such as P-glycoprotein, the Pgp inhibitor PSC833 was included. Loaded cells were washed with DMEM without phenol red and exposed to fresh medium (37 °C) containing 0.1 μM PSC833 and either 10 μM CDDP, 5 μM As_2O_3, 5 μM $HgCl_2$ or 25 μM MK571. Control cells received fresh medium (37 °C) with 0.1 μM PSC833. The efflux of calcein was measured in media samples from either the basolateral (MRP1) or the apical (MRP2) compartment after 25 and 45 min. It was shown before (Wortelboer et al., 2003), that calcein efflux in the control cells was very low. Fluorescence of calcein was determined using a Cytofluor 2300 (Millipore) with excitation at 485 nm and emission at 530 nm. The fluorescence of the samples was corrected for the minor changes in background fluorescence caused by the test compounds.

2.5. ATPase activity assay in isolated MRP1 and MRP2 containing *Sf*9-membrane vesicles

Membrane vesicles were prepared from *Spodoptera frugiperda* (*Sf*9) insect cells infected with recombinant baculoviruses containing either *MRP1* cDNA or *MRP2* cDNA as described (Wortelboer et al., 2003). A full characterisation of the MRP1 and MRP2 expression and their activities is given in Wortelboer et al. (2003).

The basal ATPase activity of MRP1 and MRP2 was measured essentially as described (Sarkadi et al., 1992) by the colorimetric detection of the liberation of inorganic phosphate from ATP. Either MRP1 or MRP2 containing *Sf*9 membranes (40 μg protein) were incubated at 37 °C in a 50 mM MOPS–Tris/KCl buffer containing 5 mM Na-azide, 2 mM DTT, 0.1 mM EGTA, and 1 mM ouabain for 30 min in the presence of low physiological concentrations of the metal-containing compounds (1 and 10 μM). The reaction was started with the addition of 5 mM Mg-ATP. Parallel incubations containing also 1 mM Na-orthovanadate (ATPase inhibitor) were used to determine the vanadate-sensitive fraction. The influence of the metal-containing compounds on the ATPase activities was determined in the absence and presence of 2 mM GSH in order to study possible co-transport or cross-stimulated transport of either GSH or the metal-containing compound.

Table 1
Intracellular GSH levels in MDCKII-control, MDCKII-MRP1 and MDCKII-MRP2 cells exposed to non-cytotoxic levels of CDDP (10 μM), As_2O_3 (5 μM), and $HgCl_2$ (5 μM), for 1 h

	Intracellular GSH (nmol/monolayer)		
	MDCKII-control	MDCKII-MRP1	MDCKII-MRP2
Control	9.11 ± 1.66 (100%)	1.10 ± 0.42 (100%)	8.30 ± 0.67 (100%)
CDDP	9.00 ± 1.03 (99%)	0.48 ± 0.41 (44%)	7.42 ± 0.49 (89%)
As_2O_3	11.6 ± 1.14 (127%)	0.50 ± 0.23 (45%)	6.56 ± 0.63 **(79%)
$HgCl_2$	8.78 ± 1.23 (96%)	0.27 ± 0.47 * (25%)	6.74 ± 0.44 ** (81%)

Data are means ± S.D. ($n = 3$). *$P < 0.1$, **$P < 0.05$ significant different from corresponding control.

2.6. Statistical analysis

Data are presented as mean ± S.D. where appropriate. Sigmaplot Version 9.0 (Systat Software Inc., Richmond, CA, USA) was used to plot dose–response curves using the Hill equation and to determine EC_{50} values in neutral red cytotoxicity experiments. The data were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test ($P < 0.05$).

3. Results

3.1. Effect of CDDP, As_2O_3 and $HgCl_2$ on the viability of control, MRP1 and MRP2 transfected MDCKII cells

To establish an appropriate exposure concentration in the transport studies in all three cell lines, the effect of CDDP, As_2O_3, and mercurychloride ($HgCl_2$) on cell viability in the MDCKII cells was determined. The neutral red uptake assay was used, which is based on the ability of viable cells to incorporate neutral red, a supravital dye into its lysosomes. The effect of CDDP, As_2O_3 and $HgCl_2$, all tested at a concentration range of 0–100 μM, in the control, MRP1 and MRP2 cells was determined after an exposure of 6 and 24 h, respectively. Exposure to CDDP, As_2O_3 and $HgCl_2$ for 6 h revealed no effect on cell viability in all three cell lines up to 33.3 μM, and EC_{50} values appeared to be >100 μM (data not shown). Cell viability curves of 24 h exposure are presented in Fig. 1. As expected, the control cells, lacking high levels of either MRP1 or MRP2, appeared to be the most sensitive for the heavy metal compounds. Exposure of cells to CDDP for 24 h resulted in EC_{50} values of 9.5, 32.1, and 21.4 μM in control, MRP1 and MRP2 cells, respectively. Exposure of the cells to As_2O_3 for 24 h resulted in EC_{50} values of 4.5 μM (control cells), 6.3 μM (MRP1 cells) and 33.8 μM (MRP2 cells), whereas an 24 h exposure to $HgCl_2$ resulted in EC_{50} values of 2.1 μM (control), 8.1 μM (MRP1) and 11.4 μM (MRP2 cells), respectively.

3.2. Effect of CDDP, As_2O_3 and $HgCl_2$ on intracellular GSH levels and GSH efflux

To study the effect of the heavy metal compounds on intracellular GSH levels and the efflux of unconjugated GSH, control, MRP1 and MRP2 cells were exposed to CDDP (10 μM), As_2O_3 (5 μM) and $HgCl_2$ (5 μM) for 1 h and 20 h. Acivicin was added to prevent enzymatic breakdown of possible formed glutathione–metal complexes by γ-glutamyltransferase. Table 1 presents the intracellular GSH data as measured after 1 h exposure. At this time point no efflux of free GSH could be detected. In the MRP1 cells, the intracellular GSH level is low compared to the level in MRP2 and control cells, a phenomenon which was described earlier (Wortelboer et al., 2003). In the control cells, no significant effect of the heavy metal-containing compounds on intracellular GSH levels could be detected, whereas in MRP1 and significantly in MRP2 cells a decrease in intracellular GSH was observed in cells exposed to especially As_2O_3 and $HgCl_2$, pointing at complex formation with GSH. Table 2 presents the intra- and extracellular GSH levels as measured in the MDCKII cell assay after an exposure for 20 h. As described before (Evers et al., 2000; Wortelboer et al., 2003), in the MRP1 cells, the high expression levels of MRP1 at the basolateral side resulted in an enhanced basal efflux of GSH to the basolateral side, whereas in the MRP2 cells the high MRP2 expression at the apical side resulted in an enhanced basal efflux of GSH to the apical side. In the control cells, no efflux of GSH could be detected (Table 2). Exposure of the control, MRP1 and MRP2 transfected MDCKII cells to the heavy metal-containing compounds did show a different effect on the intracellular GSH levels and efflux of free GSH. CDDP (10 μM) exposure did not result in significantly increased GSH levels or enhanced transport of free GSH out of the cells. Exposure of the MDCKII cells to As_2O_3 (5 μM),

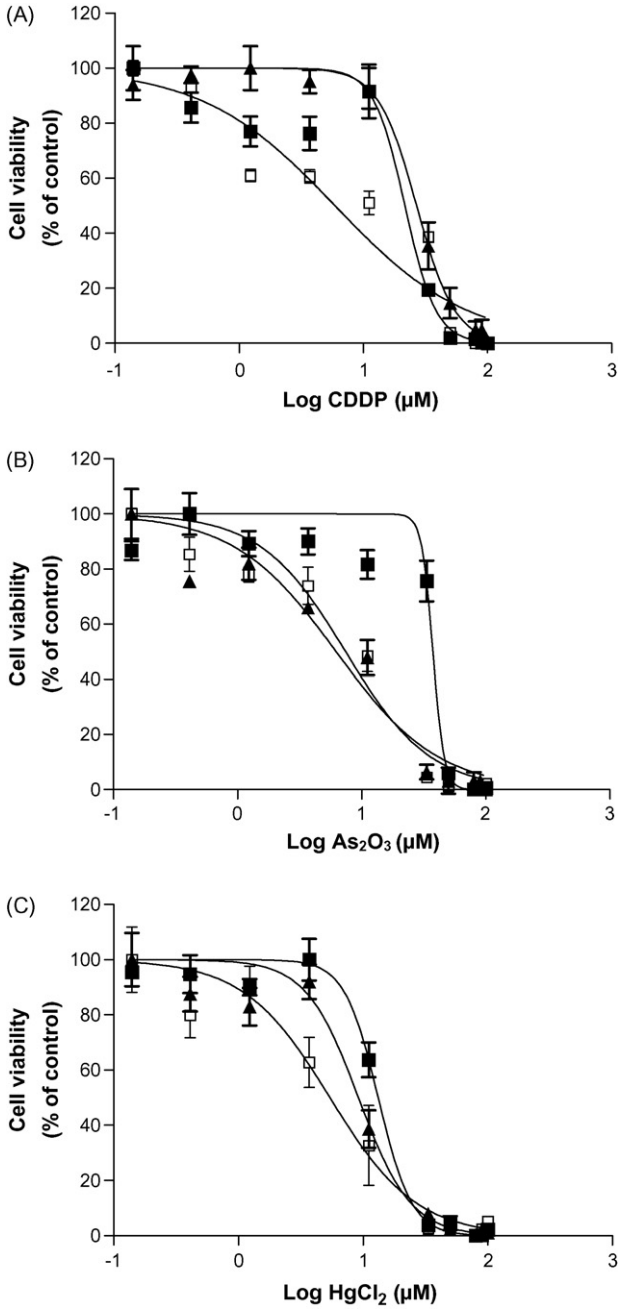

Fig. 1. Cell viability curves of MDCKII-control (open squares), MDCKII-MRP1 (black squares) and MDCKII-MRP2 (black triangles) cells after 24 h exposure to either CDDP (A), As_2O_3 (B) or $HgCl_2$ (C). Cell viability was determined using the neutral red uptake assay. The mean absorbance for each concentration tested was expressed as the percentage of the value obtained for the blank wells and plotted against the concentration of the test compound. Six observations were made for each concentration tested.

Table 2
Intracellular GSH levels and directional efflux of unconjugated GSH to apical and basolateral compartment in MDCKII-control, MDCKII-MRP1, and MDCKII-MRP2 cells exposed to non-cytotoxic levels of CDDP (10 μM), As_2O_3 (5 μM), and $HgCl_2$ (5 μM) for 20 h

Cells	Compound	Unconjugated GSH (nmol/monolayer)			
		Intracellular	Apical	Basolateral	Total
MDCKII-control	Control	4.81 ± 1.53	<0.001	<0.001	4.84 (100%)
	CDDP	6.57 ± 0.82	<0.001	<0.001	6.57 (136%)
	As_2O_3	9.96 ± 1.13*	1.86 ± 0.25*	0.52 ± 0.49*	12.33 (255%)
	$HgCl_2$	11.90 ± 0.65*	<0.001	< 0.001	11.90 (246%)
MDCKII-MRP1	Control	1.29 ± 0.24	0.08 ± 0.09	1.55 ± 0.20	2.92 (100%)
	CDDP	1.14 ± 0.17	0.09 ± 0.03	2.79 ± 0.23	4.01 (137%)
	As_2O_3	2.53 ± 0.44*	1.86 ± 0.25*	12.52 ± 2.43*	16.91 (579%)
	$HgCl_2$	2.28 ± 0.53*	<0.001	<0.001	2.28 (78%)
MDCKII-MRP2	Control	5.76 ± 0.33	0.98 ± 0.20	<0.001	6.74 (100%)
	CDDP	5.76 ± 0.99	0.86 ± 0.75	<0.001	6.62 (98%)
	As_2O_3	9.83 ± 0.47*	5.33 ± 0.25*	<0.001	15.16 (225%)
	$HgCl_2$	8.11 ± 0.64*	<0.001	<0.001	8.11 (120%)

Data are means ± S.D. ($n = 3$). *$P < 0.05$ significantly different from corresponding control.

resulted in significantly enhanced GSH levels in all three MDCKII cell lines. In the control cells, a low efflux of GSH was observed upon As_2O_3 (5 μM) exposure. In the MRP1 cells, exposure to As_2O_3 resulted in a clear enhanced efflux of free GSH to the basolateral side (up to 12.5 nmol GSH/monolayer), whereas in the MRP2 cells a clear efflux of free GSH to the apical side was measured (up to 5.3 nmol GSH/monolayer).

Exposure of MDCKII cells to $HgCl_2$ resulted in an increase in intracellular GSH levels in all three cell lines, but no GSH was detected in either the apical or basolateral compartment from the control, MRP1 and MRP2 cells.

3.3. Effect of CDDP, As_2O_3, and $HgCl_2$ on calcein efflux in MRP1 and MRP2 transfected MDCKII cells

The interaction of CDDP, $HgCl_2$ and As_2O_3 with MRP1- and MRP2-mediated transport was investigated using the calcein efflux method as described before (Wortelboer et al., 2003). Confluent monolayers of polarized MDCKII cells, cultured on transwells, were first loaded with calcein at a temperature of 7 °C to inhibit premature ATP-dependent efflux of calcein. Under these conditions intracellular calcein levels reached comparable levels in all three cell lines within a time span of 2 h. After loading the cells with calcein, the efflux of calcein to the basolateral (MRP1 cells) and apical (MRP2 cells) compartments was followed in the presence of CDDP (10 μM), As_2O_3 (5 μM), and $HgCl_2$ (5 μM) in fresh medium for 45 min at 37 °C. In non-treated cells, a clear polarized efflux of calcein – in MRP1 cells to the basolateral side, and in MRP2 cells to the apical side – was observed as described before (Wortelboer et al., 2003). Exposure to MK571 (25 μM), the known inhibitor of MRP1 and to a lesser extent of MRP2 resulted in a clear inhibition of the calcein efflux to the basolateral side in the MDCKII-MRP1 cells, and to the apical side in the MDCKII-MRP2 cells. Exposure to CDDP, As_2O_3 and $HgCl_2$ decreased the MRP1-mediated calcein efflux to 56, 66 and 62% of the non-treated MRP1 cells, whereas MRP2-mediated efflux of calcein was decreased to 65, 67, and 59%, of the non-treated MRP2 cells (Fig. 2). MK571 was included as a positive control. MK571 decreased the calcein efflux to a level of 9 and 47% of the corresponding non-treated cells in MRP1 and MRP2 cells, respectively.

3.4. ATPase activity in isolated MRP1- and MRP2-expressing Sf9 membrane vesicles

Multidrug resistance ABC transporters such as MRP1 and MRP2 utilize the energy of ATP for their drug transport activity. Stimulation of the MRP-associated ATPase activity indicates that the compound is a substrate for the transporter protein. The known MRP1 and MRP2 substrate LTC_4 resulted in an increase of the MRP1- and MRP2-transporter-associated ATPase activity by 235 and 204%, respectively (Table 3). CDDP stimulated both MRP1- and MRP2 associated ATPase activity (31 and 24% for MRP1, and 12 and 46% for MRP2 at 1 and 10 μM CDDP, respectively). As_2O_3 did not affect MRP1-associated ATPase activity, and even inhibited MRP2-

Table 3
Effect of CDDP, As_2O_3, $HgCl_2$ and the reference substrate LTC_4 on the vanadate-sensitive ATPase activity as measured in *Sf*9-MRP1 and *Sf*9-MRP2 membrane vesicles

Vanadate-sensitive ATPase activity (% of control)			
Compound	μM	*Sf*9–MRP1	*Sf*9–MRP2
Control	0	100	100
CDDP	1	131 ± 4*	112 ± 11
	10	124 ± 6	146 ± 8*
As_2O_3	1	99 ± 3	73 ± 6*
	10	95 ± 5	80 ± 1*
$HgCl_2$	1	170 ± 8*	130 ± 5*
	10	128 ± 6*	129 ± 3*
LTC_4	0.05	235 ± 12*	204 ± 6*

Data are expressed as % of the control (means ± SD, $n = 3$). *$P < 0.05$ significantly different from corresponding vehicle-treated *Sf*9 vesicles.

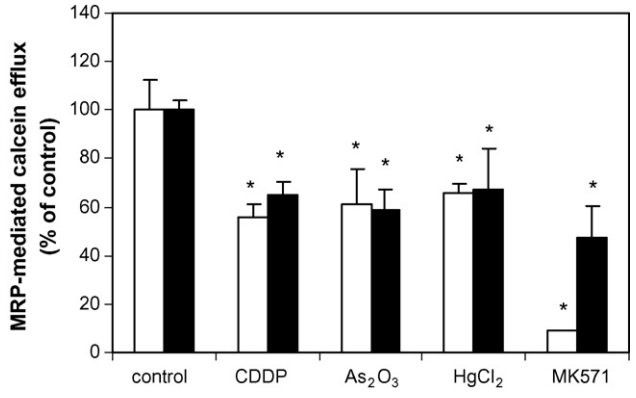

Fig. 2. The effect of CDDP (10 μM), As_2O_3 (5 μM) or $HgCl_2$ (5 μM) on the calcein efflux from MDCKII-MRP1 (basolateral side; white bars) and MDCKII-MRP2 cells (apical side; black bars). The known MRP1 and MRP2 inhibitor, MK571 (25 μM), was included as positive control. At first, cells were loaded with calcein-AM for 2 h at 7 °C, after which calcein efflux was measured in fresh medium containing the metal compound at 37 °C. Each point represents mean ± S.D. of a typical experiment performed in triplicate. *$P < 0.05$ significant different (Student's *t*-test) from non-treated cells.

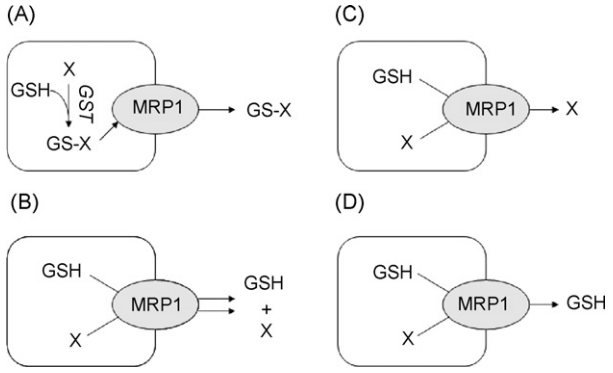

Fig. 3. Some models of involvement of glutathione in MRP1-mediated transport. (A) Transport of hydrophobic compounds that are conjugated to glutathione. (B) Co-transport or cross-stimulated transport of compound X with glutathione. (C) Transport of compound X is stimulated by glutathione, but cross-stimulated transport of glutathione has not been observed (allosteric regulation). (D) Transport of glutathione is stimulated by compound X, but compound X does not appear to be a transport substrate (adapted from Cnubben et al., 2005).

associated ATPase activity, indicating that it acts as an inhibitor of MRP2. $HgCl_2$ stimulated both MRP1- and MRP2-associated ATPase activity by 70 and 28%, and 30 and 29% at 1 and 10 μM, respectively. These data indicate that both CDDP and $HgCl_2$ can be transported as the parent compound by MRP1 and MRP2. In contrast, As_2O_3 as the parent compound is not a substrate for MRP1, and an inhibitor for MRP2. GSH (2 mM) by itself stimulated the ATPase activity by 30% which has been reported by others (Chang et al., 1997; Hooijberg et al., 1999), but addition of the heavy metal-containing compounds did not show any additional effects (results not shown).

4. Discussion

Heavy metal-containing compounds have a high affinity for GSH and as such their effects on MRP1 and MRP2 transport processes can be expected to involve various mechanisms. It is known or has been suggested that glutathione is involved in the MRP-mediated efflux of heavy metal-containing compounds, and different hypotheses of its MRP-mediated transport have been postulated. Among these hypotheses are (1) the metalloid forms a complex with GSH and this complex is pumped out by MRP1 and/or MRP2 (Fig. 3A), and/or the transport of the metalloid (or the metalloid–GSH complex) by MRP1 and/or MRP2 might be co-transported with GSH or cross-stimulated by GSH (Fig. 3B), and/or the metalloid is transported by MRP1 and/or MRP2 without GSH involved (Fig. 3C), and/or transport of GSH is stimulated by the metalloid without the latter being transported itself (Fig. 3D). Using intact monolayers of MRP1 and MRP2 transfected MDCKII cells as well as vesicles of isolated membranes of MRP-overexpressing *Sf*9 cells, the interaction of CDDP, As_2O_3, and $HgCl_2$ as heavy metal-containing model compounds with intracellular GSH levels and MRP1 and MRP2 were studied, to a better understanding of possible processes involved. Literature data indicated that the affinity for GSH and the stability of the GSH adducts appears to be CDDP ≪ As_2O_3 ≪ $HgCl_2$ (Dabrowiak et al., 2002; Hagrman et al., 2004; Csanaky and Gregus, 2005; Ballatori, 2002). The present study point at a different interaction with MRP1 and MRP2 for all three heavy metal-containing compounds which appears to be dependent on the affinity for GSH. The results of the study are summarized in Table 4 and discussed in more detail below.

4.1. Cisplatin

CDDP is used in the treatment of ovarian, testicular and bladder cancers (Giaccone, 2000), but its dose administered is limited by its nephrotoxicity due to active uptake of CDDP by the renal hOCT2 transporter (Ciarimboli et al., 2005). An overexpression of the active efflux transporter MRP2, but not of MRP1, has been associated with resistance of cultured cells to CDDP (Sharp et al., 1998; Cui et al., 1999; Itoh et al., 2002). An induction of both γ-GCS and MRP2 (and not MRP1) has been shown in cells treated with CDDP (Ishikawa et al., 1996; Kauffmann et al., 2002; Vernhet et al., 2000), whereas it is still unclear which mechanism is involved in the transport of CDDP by MRP2. Although formation of complexes of CDDP–GSH was described before (Ishikawa et al., 1996; Bernareggi et al., 1995), a process which could be enhanced by GSTP1-1 (Goto et al., 1999), at low and more physiological concentrations of CDDP, i.e. 0.25–30 μM and a ratio of cisplatin:GSH of 1:500, it appeared that this reaction was very slow with $t_{1/2}$ of about 360 h (Dabrowiak et al., 2002; Hagrman et al., 2004). The fact that no cisplatin–GSH complexes could be detected *in vivo* can be explained by this slow formation of GSH complexes (Bernareggi et al., 1995). This observation is also consistent with data showing poor correlation with intracellular GSH levels and cytotoxicity of CDDP (Pendyala et al., 1995). Also in the present study no significant effect of CDDP on the intracellular GSH levels or efflux of free unconjugated GSH was observed in any of the three tested MDCKII cell lines. CDDP has been shown to inhibit MRP2-mediated transport of LTC_4, indicating that CDDP itself can interact with MRP2 (Watabe et al., 1999). This was confirmed in the present study by the observation that CDDP inhibited the MRP2-mediated calcein efflux. Moreover, CDDP stimulated MRP2-ATPase activity, indicating that CDDP itself is a substrate for MRP2. MRP1 and MRP2 are both ATP binding cassette proteins, and the mode of action of these ABC proteins is that stimulation of the transporter-associated ATPase is needed for transport of substrates. Stimulation of the transporter-associated ATPase activity is therefore a good indication if a compound is a substrate. It has to be noted, however, that false negatives of this assay has been reported (Polli et al., 2001).

In the present study, similar effects of CDDP were observed on MRP1-mediated calcein efflux and MRP1-ATPase activity, indicating that CDDP itself might also be a substrate for MRP1. These data together indicate that at low physiological concentrations, CDDP itself can be transported out of the cell by both MRP1 and MRP2. The role of GSH in the transport of CDDP by MRP1 and MRP2 is probably limited as co-incubation of GSH in the ATPase assay did not affect MRP1- and MRP2-associated ATPase activity.

4.2. Arsenic trioxide

As_2O_3 is efficiently used in the treatment of acute promyelocytic leukemias (Shen et al., 1997). Enhanced expression of MRP1 is associated with resistance to As_2O_3 (Ishikawa et al., 1996; Vernhet et al., 2000), but knowledge on the exact mechanism of interaction with transporters is unknown. It was suggested from *in vitro* studies that arsenic species are either co-transported with GSH (Salerno et al., 2002) or as a tri-GSH conjugate (Kala et al., 2000; Leslie et al., 2004) by MRP1. Formation of As^{3+}-GSH complexes at physiological levels and clinical relevant concentrations (0.2–5 μM As_2O_3) have been described *in vivo* but these complexes appeared to be readily reversible (Delnomdedieu et al., 1994; Shen et al., 1997; Gailer and Lindner, 1998; Csanaky and Gregus, 2005). In the present study, MRP2 expression and to a lesser extent MRP1 expression in the MDCKII cells decreased cytotoxicity of As_2O_3. Exposure to As_2O_3 for 20 h, enhanced intracellular GSH levels and the MRP1-and MRP2-mediated efflux of free unconjugated GSH, indicating that GSH is involved in the MRP-mediated transport process of As_2O_3. As_2O_3 inhibited MRP1- and MRP2-mediated transport of calcein in the MDCKII cell system. The parent compound As_2O_3 itself, however, did not stimulate MRP1-associated ATPase activity, and addition of 2 mM GSH did not change these results. An inhibition of MRP2-

Table 4
Effects of heavy metal-containing compounds on intracellular GSH, efflux of unconjugated GSH, and MRP1- and MRP-mediated transport

Compound	MDCKII-MRP1 cells					*Sf*9-MRP1 vesicles ATPase activity	
	Total GSH levels (1 h exposure)	Intracellular GSH levels (20 h exposure)	Efflux of unconjugated GSH		MRP1-mediated calcein transport	−GSH	+GSH
			Apical	Basolateral			
CDDP	−/↓	−	−	−	↓	↑	↑
As_2O_3	−/↓	↑	↑↑↑	↑↑↑	↓	−	−
$HgCl_2$	↓↓	↑	↓	↓↓	↓	↑↑	↑↑
Compound	MDCKII-MRP2 cells					*Sf*9-MRP2 vesicles ATPase activity	
	Total GSH levels (1 h exposure)	Intracellular GSH levels (20 h exposure)	Efflux of unconjugated GSH		MRP2-mediated calcein transport	−GSH	+GSH
			Apical	Basolateral			
CDDP	−	−	−	−	↓	↑[a]	↑
As_2O_3	↓	↑	↑↑↑	−	↓	↓	↓
$HgCl_2$	↓	↑	↓↓	−	↓	↑↑	↑↑

For experimental details see Section 2. The effects are summarized according to the percentage of enhancement (↑) or decrease (↓) in comparison to its corresponding control −: no effect, −/↓: >40% effect but not significantly different; one arrow: significantly different and 20–40% effect, two arrows: significantly different and 40–60% effect, three arrows: >75%.

[a] No effect on MRP2-mediated ATPase was observed with 1 μM CDDP.

mediated ATPase by As_2O_3 was detected pointing at an interaction of As_2O_3 with MRP2.

These data together indicate that at low physiological concentrations As_2O_3 itself is no substrate for MRP1 and MRP2. Besides, GSH did not have any effect on the stimulation of MRP1- and MRP2-associated ATPase activity, pointing at no cross-stimulated transport by GSH. However, the MRP-mediated efflux of unconjugated GSH after exposure to As_2O_3 in the intact MRP1 and MRP2 cells can be explained by the fact that either there is a co-transport of GSH or, more likely the labile As_2O_3–GSH complexes fall apart outside the cell, after which free GSH is released. These observations are consistent with *in vivo* data (Csanaky and Gregus, 2005) showing no effect of acivicin, the inhibitor of γ-glutamyl transferase, on $As(SG)_3$ levels in the urine, whereas in a similar experiment with methylmercury treatment with acivicin clearly enhanced methylmercury–SG complexes in the urine. In conclusion, the data together point at – at low physiological concentrations – As_2O_3 is presumably transported not as the parent compound itself, but as a labile complex with GSH whose dissociation probably occurs especially at low levels of GSH outside the cell, releasing unconjugated free GSH.

4.3. Mercurychloride

In contrast to CDDP and As_2O_3, the metal Hg^{2+} is known to have a very high affinity for reduced sulfhydryl groups, among them GSH (Ballatori and Clarkson, 1985; Ballatori, 2002). There is evidence for formation of Hg–GSH complexes and these are postulated to be good substrates for MRP1 and/or MRP2 (Zalups, 2000). In the present study, exposure to $HgCl_2$ enhanced intracellular GSH in both MRP1 and MRP2 cells, but no MRP1- and MRP2-mediated efflux of unconjugated GSH could be detected. In fact, exposure to $HgCl_2$ diminished efflux of unconjugated GSH completely. $HgCl_2$ inhibited the MRP1- and MRP2-mediated transport of calcein. $HgCl_2$ increased both MRP1- and MRP2-ATPase activity, but addition of 2 mM GSH had no additional effect, indicating that $HgCl_2$ itself may also interacts with both MRP1 and MRP2. All these data together indicate that $HgCl_2$ and its glutathionyl conjugates can interact with both MRP1 and MRP2, and although we do not have direct evidence for the transport of Hg–GSH complexes in this study, the data strongly indicate that GSH plays an important role in this process.

The data of the present study indicate a direct interaction of all three heavy metal-containing compounds with both MRP1 and MRP2. Yet, for CDDP, acquired resistance with only MRP2 has been described, whereas for As_2O_3 an acquired resistance with MRP1 has been described. In this respect it should be noted that in normal healthy tissue MRP1 and MRP2 are differentially expressed in the various cell types, and the mechanisms behind acquired resistance may be various and related to the basal level of expression. In normal tissue, both MRP1 and MRP2 may be involved in the cellular response (de novo resistance) to heavy metal-containing compounds, but whether either MRP1 or MRP2 is involved in acquired resistance is dependent of many factors, as was for example shown for cisplatin resistance and MRP2 expression levels in ovarian tumors (Guminski et al., 2006).

In conclusion, the present study show that all three heavy metal compounds, CDDP, As_2O_3, and $HgCl_2$ interact with both MRP1 and MRP2 transporter proteins and GSH, although through different mechanisms. At low, for cisplatin and arsenic trioxide more physiological concentrations, the affinity of the heavy metal for GSH and the stability of its possible formed metal–SG complexes depend on the intracellular concentrations of GSH and heavy metal-containing compound, and appear to determine the mechanism of transport via MRP1 and MRP2.

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