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The MRP1-Mediated Effluxes of Arsenic and Antimony do not Require Arsenic–Glutathione and Antimony–Glutathione Complex Formation

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Arsenic trioxide is an effective treatment for acute promyelocytic leukemia, but resistance to metalloïd salts is found in humans. Using atomic absorption spectroscopy, we have measured the rate of uptake of arsenic trioxide and of antimony tartrate in GLC4 and GLC4/ADR cells overexpressing MRP1 and the rate of their MRP1-mediated effluxes as a function of the intracellular GSH concentration. In sensitive cells, after 1 h, a pseudosteady state is reached where intra- and extracellular concentrations of metalloid are the same. This precludes the formation, at short term, of complexes between arsenic or antimony with GSH. In resistant cells reduced intracellular accumulation of arsenic (or antimony), reflecting an increased rate of arsenic (or antimony) efflux from the cells, is observed. No efflux of the metalloid is observed in GSH depleted cells. The two metalloïds and GSH are pumped out by MRP1 with the same efficiency. Moreover for the three compounds 50% of the efflux is inhibited by 2 μM MK571. This led us to suggest that As- and Sb-containing species could be cotransported with GSH.

KEY WORDS: arsenic trioxide; MRP1 transporter; glutathione.

INTRODUCTION

Organic arsenicals were the first antimicrobials agents specifically synthesized for the treatment of infectious diseases such as syphilis and sleeping sickness. For the treatment of diseases caused by trypanosomatid parasites, organic derivatives of arsenic and the related metalloid antimony are still the drugs of choice (Borst and Ouellette, 1995). Arsenic trioxide, As_2O_3, has been used for a long time in traditional Chinese medicines for treatment of various diseases, and it has recently been shown to be clinically active in acute promyelocytic leukemias (Shen *et al.*, 1997). Based on its selective toxicity to specific leukemic cells, arsenic trioxide is now used in the treatment of patients with acute promyelocytic leukemia with curative result (Chen *et al.*, 1997a,b; Soignet *et al.*, 1998).

Resistance to metalloid salts is found in bacteria, fungi, parasites, and animals. In some organisms, resistance involves overproduction of intracellular thiols (Mukhopadhyay *et al.*, 1996). In many cases, resistance to arsenic salts is the result of removal of the metalloid from the cytosol usually by extrusion from the cell (Dey and Rosen, 1995). In bacteria resistance to arsenite and antimony is common and well characterized; it is conferred by the ArsB, a 45-kDa membrane transport protein that catalyzes extrusion from the cells (Dey and Rosen, 1995). Up to now no eukariotic ArsB orthologs have been identified. However, other two families of membrane proteins have eukaryotic representatives that have been shown to produce arsenite resistance: the ACR family and the MRP family. This last group includes drug pumps, such as human MRP1, which is frequently amplified in cancer cells (Borst and Schinkel, 1997). MRP1-overexpressing tumor

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Key to abbreviations: MRP1, Multidrug resistance associated protein; GSH, glutathione; MK571, 3-([{3-(2-[7–chloro-2-quinolinyl]ethenyl) phenyl}-{(3-dimethylamin-3-oxopropyl)-thio}-methyl]thio)propanoic acid; BSO, L-buthionine-(S, *R*) sulphoximine; AAS, atomic absorption spectroscopy; CD, circular dichroism.

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cells have been shown to be cross-resistant to sodium arsenite and potassium antimony tartrate, suggesting that MRP1 overexpression may limit efficiency of arsenic-derived anticancer drugs. Also, from the observation that MRP1-catalyzed export of glutathione from cells is increased by arsenite, it has been suggested that MRP1 functions as an As(III)–triglutathione [$As(GS)_3$] carrier (Zaman *et al.*, 1995).

In this study, using GLC4/ADR cells overexpressing MRP1, we have examined the rate of uptake of arsenic trioxide and of antimony potassium tartrate, and the rate of their MRP1-mediated efflux as a function of the intracellular GSH concentration. Our data show that resistance is correlated with reduced intracellular accumulation of arsenic (or antimony) reflecting an increase rate of arsenic (or antimony) efflux from the cells. In sensitive cells after 1 h a pseudosteady state is reached where intra- and extracellular concentration of metalloid are the same. This precludes the formation, at least at short term, of complexes between arsenic or antimony with GSH. In addition spectroscopic studies of the GSH-$As(OH)_3$ system shows that at pH 7 the complexation of GSH to arsenic is slow. These results led us to suggest that As(III)- and Sb(III)-containing species could be cotransported with GSH.

MATERIALS AND METHODS

Drugs and Chemicals

Arsenic trioxide (As_2O_3), antimony potassium tartrate, or bis[μ-[2,3-dihydroxybutanedioato(4-)-01,02: 03,04]]-diantimonate dipotassium trihydrate and L-buthionine-(*S*,*R*) sulphoximine (BSO) were from Sigma. 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio) propanoic acid (MK571) was provided by Dr R. N. Young (Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments. The HEPES Na^+ buffer solutions contained 20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM glucose at pH 7.3.

Materials

Cellular arsenic and antimony contents were quantified using a Zeeman atomic absorption spectrometer (Varian SpectrAA 220). Circular dichroism measurements Jobin Yvon Model Mark CD-6 dichrograph and NMR with a 500 MHz Varian spectrometer.

Cell Lines and Cultures

GLC4 and the MRP1-expressing GLC4/ADR cells (Zijlstra *et al.*, 1987) as well as K562 leukemia cells and the P-gp expressing K562/ADR cells (Mankhetkorn *et al.*, 1996) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum in a humidified incubator with 5% CO_2. The resistant K562/ADR and GLC4/ADR cells were cultured with 400 nM or 1.2 μM doxorubicin, respectively until 1–4 weeks before experiments. Cell cultures used for experiments were split 1:2 one day before use to ensure logarithmic growth. They were counted, with a Coulter counter, immediately before use in the assay. Cell viability was assessed by trypan blue exclusion and was higher than 95% under the various experimental conditions used.

The cytotoxicity of the compounds was determined by incubating cells (10^5) with different concentrations of the compound for 72 h in standard 6-well plates. Then the IC_{50} values (50% inhibitory drug concentrations) were determined by counting the cells using a Coulter counter. The resistance factor (RF) was defined as the IC_{50} for the resistant cells divided by the IC_{50} for the corresponding sensitive cells.

Cellular Drug Accumulation

For arsenite or antimony accumulation assays, the cells were treated with various concentration of arsenic trioxide or antimony potassium tartrate. In most of cases the concentration used was 50 μM and the incubation was performed in the culture medium. After specified time intervals, aliquots containing 10^6 cells were taken and washed two times with phosphate saline buffer. The pellet obtained after centrifugation was digested with nitric acid, and the arsenic or antimony content was measured by atomic absorption spectrometry (AAS).

Drug Efflux

Cells (10^6/mL) were incubated with arsenic trioxide (or antimony potassium tartrate) in the culture medium for 1 h. Cells were then centrifuged, washed two times with PBS, and the pellet was resuspended in arsenic (or antimony) free buffer. After specified time intervals ranging from 5 min to 2 h, aliquots containing 10^6 cells were

taken centrifuged and the pellet and supernatant analyzed for their arsenic (or antimony) content.

Mathematical Calculations

The diffusion of the arsenic- and antimony-based compounds into the cells is passive, and so it obeys Fick's law. In sensitive cells the release of arsenic- or antimony-containing complex is passive. However, in resistant GLC4/ADR cells the release of the drugs is the sum of a passive diffusion and of an active transporter-mediated efflux. The following equations are easy to establish

$$(\mathscr{V}_{\text{cell}})\ \mathrm{d}\,\mathscr{C}_{\text{i}}/\mathrm{dt} = k_+\ C_{\text{e}} - k_{\text{efflux}}\ \mathscr{C}_{\text{i}} \qquad (1)$$

where $\mathscr{V}_{\text{cell}}$ is the volume of one cell, $\mathscr{C}_{\text{i}}$ is the intracellular arsenic (or antimony) concentration and d $\mathscr{C}_{\text{i}}$/dt its variation as a function of time. It follows that ($\mathscr{V}_{\text{cell}}$) d $\mathscr{C}_{\text{i}}$/dt $= V_+$ is the number of moles that enter into one cell per second. C_{e} is the extracellular metalloïd concentration and k_+ its passive influx coefficient. In sensitive cells, k_{efflux} is equal to k_- the passive efflux coefficient; in resistant cells, k_{efflux} is equal to $k_- + k_{\text{a}}$ where k_{a} is the active efflux coefficient characteristic of the transporter and of the substrate (Marbeuf-Gueye *et al.*, 1998).

The integration of Eq. (1) yields the variation of $\mathscr{C}_{\text{i}}$ as a function of time

$$\mathscr{C}_{\text{i}} = (k_+/k_{\text{efflux}})\ C_{\text{e}}[1 - \exp(-k_{\text{efflux}}/\mathscr{V}_{\text{cell}})t] \qquad (2)$$

C_{e} can be considered as a constant, the total arsenic, or antimony, taken by the cells being about 1 millieme of C_{e}.

The three parameters k_+, k_-, and k_{a} were computed by nonlinear regression analysis of $\mathscr{C}_{\text{i}}$ versus t data and assuming that the accumulation follows Eq. (2).

For the efflux of the metalloid-based compounds, when the cells are loaded with the metalloid and then resuspended in drug free medium, the decrease of the intracellular metalloid concentration can be written as

$$\mathscr{C}_{\text{i}} = \mathscr{C}_{\text{i}}(t = 0)\ [\exp(-k_{\text{efflux}}/\mathscr{V}_{\text{cell}})t] \qquad (3)$$

GSH Measurements

To quantify free GSH either inside the cells or the amount released in the extracellular medium, an enzymatic technic was used (Salerno and Garnier-Suillerot, 2001). Monochlorobimane, itself nonfluorescent, is conjugated to GSH by glutathione S-transferase to yield a fluorescent adduct (Fernandez-Checa and Kaplowitz, 1990). We have used this property to develop a very rapid and sensitive fluorometric method for GSH measurement (Salerno and Garnier-Suillerot, 2001). Briefly, a 10 mM stock solution of monochlorobimane was prepared in ethanol, and aliquots were stored at -80°C in the dark. The nonenzymatic reaction which occurred between GSH and monochlorobimane was very slow. However, when glutathione S-transferase was added, the increase of the fluorescent signal characteristic of monochlorobimane-GSH derivative formation was very fast. The initial rate of monochlorobimane-GSH formation was determined as the increase of the fluorescent signal between 100 and 150 s after the addition of glutathione S-transferase to monochlorobimane plus GSH. The monochlorobimane and glutathione S-transferase concentrations were kept constant, equal to 100 μM and 0.5 u/mL, respectively. The fluorescence signal recorded over a short time (50 s), which was used as a measure of the inital rate of monochlorobimane–GSH formation, is directly proportional to the concentration of GSH at least within the range from 0 to 20 μM (this corresponding to the concentrations expected when 10^6 cells/mL were lysed, the intracellular GSH concentrations being within the 0–20 mM range). We have checked that GSSG did not give rise to any modification of the fluorescence signal.

For the intracellular GSH determination, cells, 2×10^6 suspended in 2 mL of buffer, were disrupted by sonication on ice (3×10 s, power 2). The rate of monochlorobimane-GSH formation was followed after addition of monochlorobimane 100 μM and GSH_{T} 0.5 u/mL, as described earlier.

For the determination of GSH released by the cells, they were resuspended in HEPES buffer (10^6/mL). After specified time intervals, 2-mL aliquots containing 2×10^6 cells were centrifuged, the GSH concentration present in the extracellular medium, and therefore released from the cells, and the GSH present in the pellet were determined. Extracellular concentration of GSH was not affected by 250 μM acivicin, indicating negligible activity of γ-glutamyltransferase in membrane of GLC4/ADR cells.

Syntheses of As(GS)$_3$

The complex of GSH with As_2O_3 was synthesized and isolated. The general procedure used was that reported by Cullen *et al.* (1984). Solutions of GSH, 2 mM, and As_2O_3, 0.7 mM, were mixed for 6 h at room temperature under an atmosphere of nitrogen, the pH of the solution was $\sim$3. Methanol was added to precipitate the product of the reaction and the mixture was filtered. NMR spectra

were obtained in 0.15M NaCl, D_2O solution. The concentration of the arsenic solutions used for the ^1H NMR spectra was 1 mM as arsenic. All experiments were carried out at room temperature. CD spectra were recorded under the same experimental conditions.

RESULTS

Cell-Growth Inhibition

Cell lines that we used were made resistant by coculture with doxorubicin. The IC_{50} values obtained with arsenic trioxide and antimony potassium tartrate for sensitive and resistant GLC4 and K562 (for comparison) cells are shown in Table I, together with the RF factor. For comparison, the values obtained for doxorubicin have been added.

Uptake of Arsenic by Sensitive and Resistant GLC4 Cells

In typical experiment, cells (10^6/mL) were incubated with 50 μM As_2O_3. Figure 1 shows the plot of the intracellular concentration of arsenic recorded inside the cells as a function of the time of incubation of the cells with the drug (the mean volume of a cell was 10^{-12} L). Four independent experiments, at least, were performed on 4 different days. In sensitive cells the uptake occurred in two steps: The first increase of an intracellular arsenic concentration (Step 1) was observed within the first 60 min and the intracellular arsenic concentration was then equal to the extracellular concentration, i.e., $\sim 50 \pm 5$ μM (Fig. 1). This was followed by a slower uptake (Step 2), and after 5 h and 24 h the intracellular arsenic concentration in sensitive cells was equal to 80 ± 8 μM and $\sim 250 \pm 25$ μM, respectively. In resistant cells a fast uptake was also observed within the first hour, and the intracellular arsenic concentration was then equal to $\sim 21 \pm 2$ μM (Fig. 1). As time elapsed, one observed a very slow increase of the

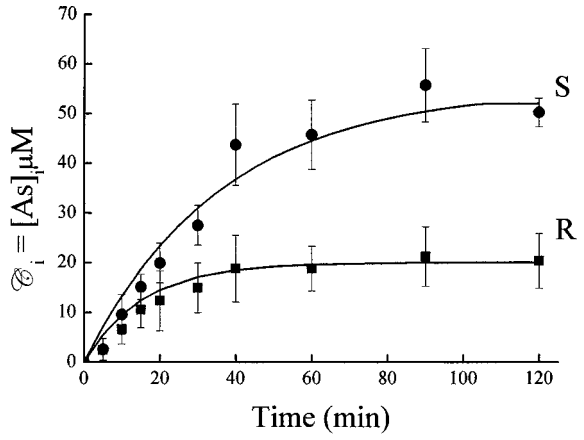

Fig. 1. Time dependency of the uptake of As_2O_3 by sensitive and resistant GLC4 cells. Cells (10^6/mL) were incubated with 50 μM As_2O_3 and after various intervals of time the intracellular concentration of As was determined (the cell volume was taken equal to 10^{-12} L). The values represent mean $\pm$ SD of 3–5 independent experiments performed on 5 different days. The line is the fitting of the experimental data using Eq. (2).

arsenic intracellular concentration: After 5 h and 24 h $[As]_i$ was equal to $\sim 24 \pm 2$ μM and 40 ± 4 μM, respectively. These data clearly show that after a 1–2-h incubation with 50 μM As_2O_3 the resistant cells contained 2.5-fold lower arsenic concentration than did parental cells.

The three parameters k_+, k_-, and k_a were then calculated, as described, from the curves of $\mathscr{C}_i$ versus t and are reported in Table II. Experiments were also performed with 200 and 500 μM As_2O_3. The values of the parameters calculated in two latter cases were very similar to those obtained with 50 μM.

In principle V_a should be determined for various intracellular arsenic concentrations $\mathscr{C}_i$, and assuming that the transport follows the Hill equation (Hill, 1985).

$$V_a = V_M \cdot \mathscr{C}_i^{n_H} / (K_m^{n_H} + \mathscr{C}_i^{n_H}) \qquad (4)$$

the maximal efflux rate (V_M), the apparent Michaelis-Menten constant (K_m) and the cooperativity constant for

Table I. Cross-Resistance of Doxorubicin-Resistant K562 and GLC4 Cells[a]

Drug	K562 (IC_{50} μM)	K562/ADR (IC_{50} μM)	K562 (RF)	GLC4 (IC_{50} μM)	GLC4/ADR (IC_{50} μM)	GLC4 (RF)
Arsenic trioxide	1.2 ± 0.3	0.7 ± 0.2	1	1.0 ± 0.2	10 ± 2	10
Tartrate antimony	30	30	1	7 ± 2	160 ± 20	23
Doxorubicin	10 ± 2	340 ± 30	34	9 ± 2	670 ± 70	74

[a] IC_{50} is the drug concentration required to inhibit 50% of cell growth. Resistance factor value was calculated as resistant cell IC_{50}/sensitive cell IC_{50}. The values represent mean $\pm$ SD of triplicate determinations.

Table II. Uptake and Efflux Parameters for As- and Sb-Containing Species by GLC4, GLC4/ADR, K562, and K562/ADR

Cell line	Kinetics parameter × 10^{-16} L · cell^{-1} · s^{-1} As[a]	Kinetics parameter × 10^{-16} L · cell^{-1} · s^{-1} Sb[a]
GLC4, GLC4/ADR	$k_+ = 4.3 \pm 1.0$	$k_+ = 2.7 \pm 0.7$
GLC4, GLC4/ADR	$k_- = 4.9 \pm 1.2$	$k_- = 2.8 \pm 0.7$
GLC4/ADR	$k_a = 6.4 \pm 1.4$	$k_a = 8.4 \pm 1.8$
K562, K562/ADR	$k_+ = 6.1 \pm 1.2$	nd
K562, K562/ADR	$k_- = 5.7 \pm 1.2$	nd
K562/ADR	$k_a = 0$	$k_a = 0$

Note. Data are mean ± SD of four independent experiments on different days. The parameters were calculated using Eq. (2).
[a] As and Sb stand for arsenic- and antimony-containing compounds.

the transport of arsenic (n_H) should be computed by nonlinear regression analysis of transport velocity (V_a) versus free cytosolic $\mathscr{C}_i$ substrate concentration. However, in the case of arsenic the complete curve $V_a = f(\mathscr{C}_i)$ cannot be obtained, and therefore it is not possible to obtain these three parameters characteristic for the transporters. In such a case, the efficiency of the efflux can be characterized by calculating a mean active efflux coefficient (k_a) according to the equation:

$$V_a = k_a \cdot \mathscr{C}_i \qquad (5)$$

The relation between k_a and the parameters V_M, K_m, and n_H (12) is as follows:

$$k_a = (V_M/n_H \cdot K_m) \quad (n_H - 1)(1 - 1/n_H) \qquad (6)$$

When $n_H = 1$, $k_a = V_M/K_m$.

When the complete curve $V_a = f(\mathscr{C}_i)$ cannot be obtained, as it is the case here, a good estimation of the active coefficient k_a can be obtained according to the procedure described earlier.

Arsenic Uptake in GSH-Depleted Sensitive and Resistant GLC4 Cells

Cells were incubated in the culture medium for 24 h in the presence of 25 μM BSO. Under these conditions the concentration of GSH remaining inside the cells was lower than 0.2 mM. Figure 2 shows the increase of the intracellular arsenic concentration when the cells, either sensitive or resistant, were incubated with 50 μM As_2O_3. For both cell lines the uptake is comparable to that observed for sensitive cells (Fig. 1). These results clearly indicate that GSH is involved in the decrease arsenic retention in resistant cells.

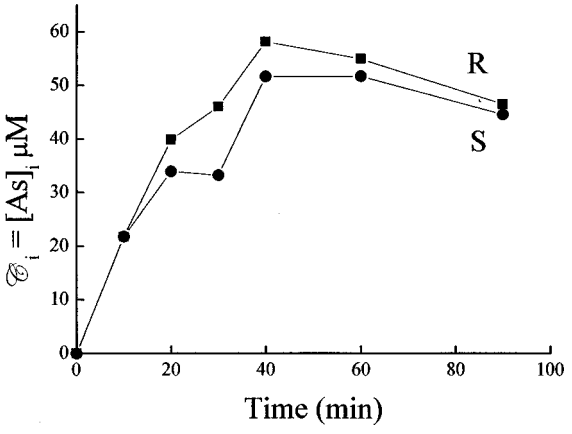

Fig. 2. Time dependency of the uptake of As_2O_3 by sensitive and resistant GLC4 cells GSH-depleted. Cells, 10^6/mL, were GSH depleted by incubation in the presence of 25 μM BSO for 20 h. They were they incubated with 50 μM As_2O_3 and the concentration of As determined as a function of time. The values represent the result of one typical experiment. The cell volume was taken equal to 10^{-12} L.

Arsenic Uptake in Energy-Depleted GLC4 Cells

The cells were energy-depleted by coincubation for 30 min with N_3^- in the absence of glucose. The uptake of arsenic was then determined after 1 h of incubation with 50 μM As_2O_3. The amount of arsenic incorporated in these cells was similar to that obtained with sensitive cells. These data clearly show that the decrease of arsenic retention in resistant cells is an energy-dependent phenomenon.

Arsenic Uptake in Resistant GLC4 Cells in the Presence of MK571

A dose-dependent increase of arsenic uptake in resistant cells was observed in the presence of MK571. Figure 3 shows the uptake in the presence of 2 μM and 5 μM MK571 respectively. A half maximum effect is observed at ~2 μM. MK571 is an inhibitor of MRP1 (Leier *et al.*, 1996), of MRP2 (Büchler *et al.*, 1997) and also, probably, of most of the transporters of the MRP class. However, Kool *et al.* (1997) have shown that, even if GLC4 cells have a low expression not only of MRP1 but also of MRP4 and MRP5, MRP1 only is overexpressed in GLC4/ADR cells. In addition, MK571 had no effect on As accumulation in GLC4 cells. From these data we can infer that the defect of arsenic incorporation in resistant cells is due to the presence of MRP1.

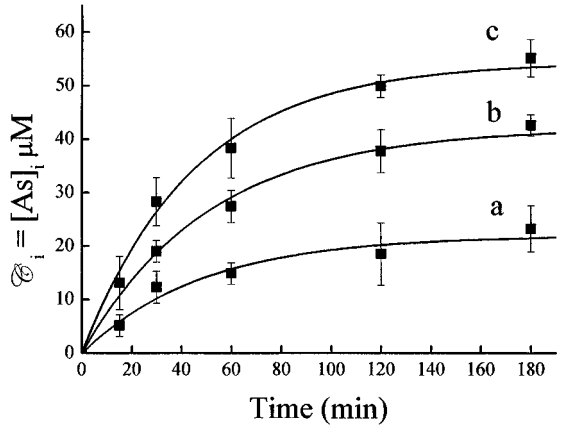

Fig. 3. Time dependency of the uptake of As_2O_3 by sensitive and resistant GLC4 cells in the presence of MK571. Resistant cells (10^6/mL) were incubated with 50 μM As_2O_3 in the absence (a) or in the presence of 2 μM (b) or 5 μM (c) of MK571. After various intervals of time the intracellular concentration of As was determined (the cell volume was taken equal to 10^{-12} L). The values represent mean $\pm$ SD of three independent experiments performed on 3 different days). The line is the fitting of the experimental data using Eq. (2).

Arsenic Uptake in Sensitive and Resistant K562 Cells

To ensure that the incorporation of As observed in GLC4 cells, i.e., after 1 h the presence of a plateau with the same intra- and extracellular concentration of As, was not dependent on the cell line we have also measured the uptake of arsenic in sensitive and resistant K562 cells (Fig. 4).

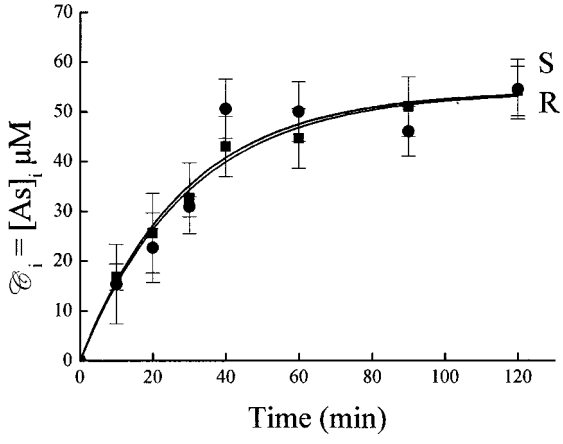

Fig. 4. Time dependency of the uptake of arsenic by sensitive and resistant K562 cells. Cells (10^6/mL) were incubated with 50 μM As_2O_3 and after various intervals of time the intracellular concentration of arsenic was determined (the cell volume was taken equal to 10^{-12} L). The values represent mean $\pm$ SD of four independent experiments performed on 4 different days). The line is the fitting of the experimental data using Eq. (2).

No difference in the uptake by both types of cell could be detected. Here also, a fast uptake of arsenic within the 1st h was observed and the extra- and intracellular concentrations of arsenic were the same. The three parameters k_+, k_-, and k_a were then calculated, as described earlier, from the curves of $\mathscr{C}_i$ versus t and are reported in Table II.

Release of Arsenic by Sensitive and Resistant GLC4 Cells

The reduced retention of arsenic in GLC4/ADR cells is likely to be due to a MRP1-mediated export of the arsenic-based drug. Thus, we have performed a direct determination of the kinetics of arsenic efflux in both cell lines. GLC4 cells (10^6/mL), either sensitive or resistant, were incubated with 50 μM of As_2O_3 for 1 h; according to Fig. 1, under these conditions a pseudosteady state is reached. Cells were then centrifuged and resuspended in free As_2O_3 HEPES buffer. After various times aliquots were taken, centrifuged, and the arsenic concentrations were determined in the supernatant and in the pellet. Figure 5 shows the percentage of arsenic recovered in the cell pellet as a function of time. An efflux is observed in sensitive and resistant cells; however, the percentage of efflux is about 2.5-fold higher in resistant versus sensitive cells.

Intra- and Extracellular GSH Concentration as a Function of the Incubation Time of Resistant GLC4 Cells With As_2O_3

Cells were incubated either in the absence or in the presence of 50 μM As_2O_3 and after 1 h they were centrifuged. The pellet was suspended in arsenic free buffer and the extra- and intracellular GSH concentrations determined versus time. The intracellular GSH concentration did not vary with time whether cells were or were not incubated with As_2O_3. However, as it has previously been observed, an increase of the GSH efflux was observed in the presence of As_2O_3. We determined that the rate was about 1.7-fold higher in the presence of As_2O_3.

NMR and CD Monitoring of the Interaction of Arsenic Trioxide With GSH

The implication of GSH in arsenic excretion prompted us to look for arsenic–GSH complexes as

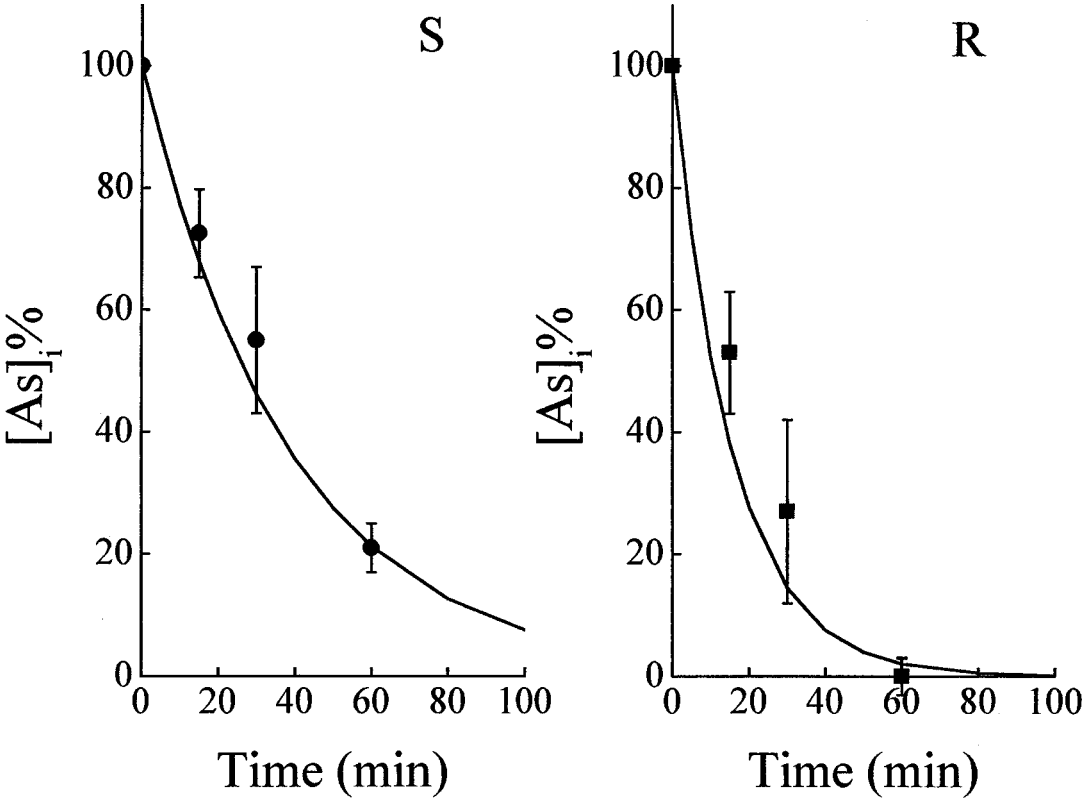

Fig. 5. Kinetics of arsenic efflux in sensitive and resistant GLC4 cells. Cells (10^6/mL) were incubated with either 50 μM or 10 μM As_2O_3. After 1 h they were centrifuged and resuspended in Hepes-free As_2O_3 buffer. After various intervals of time aliquots were taken, centrifuged, and the concentrations of arsenic in the supernatant and the cell pellet were determined. The line is the fitting of the experimental data using Eq. (3).

intermediates in arsenic transport. Although such complexes have not been identified in vivo to date, their existence has been postulated and it has been reasoned that failure to detect these complexes may result from their inherent instability (Cullen *et al.*, 1984; Dey *et al.*, 1996; Scott *et al.*, 1993; Wang *et al.*, 1966).

To provide additional informations on the stability and lability of the arsenic–GSH complexes, we synthesized the $As(GS)_3$ complex and measured its NMR and CD spectra under the conditions described in Materials and Methods. ^{1}H NMR spectrum exhibited mainly bound form of Cys βCH_2 and αCH at 3.36 p.p.m. and 4.70 p.p.m. respectively. The CD spectrum exhibited a positive band at 230 nm ($\Delta\varepsilon$/GSH $= +0.9$). The CD spectrum of this complex was also recorded after 10-fold dilution, and no modification was observed even after 1 h. We thus inferred that the dissociation of this complex is very low.

In a second set of experiments, the complexation of GSH to As(III) was monitored as a function of time via NMR and CD spectra. At pH$\sim$3, after $\sim$5 min, only free form of Cys βCH_2 at 2.95 and αCH at 4.57 was observed. As time elapsed, both free Cys βCH_2 and αCH resonances decreased in intensity, while there was an appearance and an increase in intensity of the bound forms of Cys βCH_2 at 3.36 and αCH at 4.70. After 6 h only the bound forms of Cys βCH_2 and αCH were detected. The CD spectrum of free GSH exhibited a negative band at 220 nm ($\Delta\varepsilon = -1.5$). The CD spectrum of GSH and As_2O_3 at 3:1 molar ratio at pH 3.0 and Δt $\sim$5 min exhibited also a negative band at 220 nm ($\Delta\varepsilon = -1.5$). As time elapsed, the amplitude of this band decreased and after $\sim$6 h the positive band at 230 nm ($\Delta\varepsilon = +0.9$) characteristic of GSH complexed to As(III) was observed. In the same experiment performed at pH 7.3, after $\sim$5 min about 10% of the bound form of Cys βCH_2 and αCH was detected, and within 3 h no modification of the spectra was observed. The CD spectrum revealed also that about 10% of GSH was complexed and no further modification of the signal was observed as time elapsed.

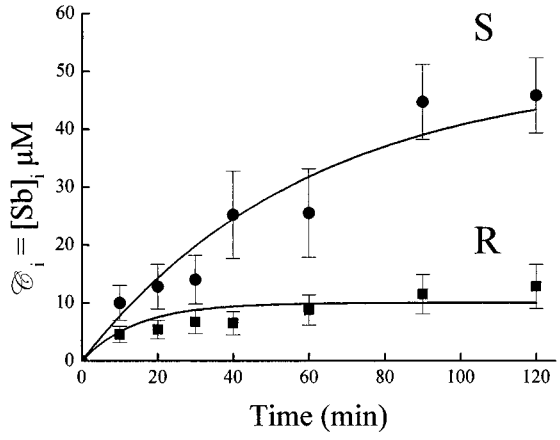

Fig. 6. Time dependency of the uptake of tartrate antimony by sensitive and resistant GLC4 cells. Cells (10^6/mL) were incubated with 50 μM tartrate antimony and after various intervals of time the intracellular concentration of antimony was determined (the cell volume was taken equal to 10^{-12} L). The values represent mean $\pm$ SD of four independent experiments.

Experiments Performed With Potassium Antimony Tartrate

Experiments strictly analogous to those described for arsenic trioxide were performed with potassium antimony tartrate, and similar data were obtained. (1) A lower accumulation of antimony in resistant GLC4 cells versus sensitive one was observed. Figure 6 shows the plot of the intracellular concentration of antimony recovered inside GLC4 cells as a function of the time of incubation of the cells with the drug. These data clearly show that after a 1–2-h incubation with 50 μM tartrate antimony resistant GLC4 cells contained fourfold lower antimony concentration than did parental cells. The three parameters k_+, k_-, and k_a were then calculated, as described earlier, from the curves of $\mathscr{C}_i$ versus t and are reported in Table II. They are slightly different from those determined for arsenic. (2) Antimony accumulation in GSH-depleted or ATP-depleted resistant GLC4 cells was the same as in sensitive cells. (3) 2 μM MK571 inhibited 50% of the MRP1-mediated efflux of antimony-based compound. (4) The efflux of antimony-based compound from GLC4 resistant cells was fourfold higher than in parental cells. (5) Sensitive and resistant K562 cells incorporated the same amount of antimony.

DISCUSSION

Arsenite in the form of arsenic trioxide has been shown to be an effective treatment for acute promyelocytic leukemia (Chen *et al.*, 1997a). In a recent study, arsenite treatment produced complete remission of acute promyelocytic leukemia in 11 of 12 patients (Soignet *et al.*, 1998). However, 3 of the 11 patients became unresponsive to treatment with arsenite, suggesting that resistance may have arisen in those individuals. Thus, it is important to determine the routes of arsenic detoxification in higher eukaryotes including humans.

It is now well-documented that MRP1-overexpressing cells poorly accumulate arsenic and antimony because of enhanced cellular efflux which depends on the presence of GSH (Chen *et al.*, 1997a; Vernhet *et al.*, 1999, 2000). It has also been shown that this resistance was reversed in the presence of MK571 (Vernhet *et al.*, 1999).

To develop a better understanding of arsenic and antimony excretion by MRP1-overpressing cells, we measured the kinetic parameters for the uptake and released of these metalloids in MDR and parental cancer cells. Our results show that the doxorubicin-selected cells, which overexpress MRP1, are cross-resistant to As(III) and Sb(III) and displayed enhanced efflux of both metalloids. Our data indicate also that GSH is involved in the transport of both metalloids although, under our experimental conditions, the formation of a metalloid–glutathione complex for MRP1-mediated efflux was not supported.

Although the presence of glutathione is required for the MRP1-mediated efflux of arsenic and antimony, different hypothesis can be made: (1) the metalloid forms a complex with GSH and this complex is pumped out by MRP1 and (2) the metalloids are cotransported with GSH, as this is the case for daunorubicin (Renes *et al.*, 1999; Salerno and Garnier-Suillerot, 2001) and vincristine (Loe *et al.*, 1998). It follows that one of the questions that have to be solved here is, what is the chemical nature of the arsenic and antimony species which are pumped out via MRP1 protein?

Actually, a central issue in arsenic biochemistry is the role of sulfhydryl-containing molecules. GSH is known to bind a variety of essential and nonessential metals, playing critical roles in the cellular and systemic metabolism of these metals (Rabenstein *et al.*, 1979). The formation of the $As(GS)_3$ complex has been thoroughly studied (Delnomdedieu *et al.*, 1994; Scott *et al.*, 1993). It has been shown that three mol of glutathione are consumed in the formation of the glutathione–arsenic complex and that the binding site is the cysteinyl sulfydryl. It is important to point out that these experiments have been performed without adjustment of the pH of the solution and that under the experimental conditions used the pH is acidic ($\sim$3). In addition, 6 h are required for the complete formation of the complex. Our NMR and CD results are in agreement with these data. So, it appears that the kinetics of

formation of $As(GS)_3$ is very slow at pH 3 and even slower at pH 7.3. According to the data of Delnomdedieu *et al.* (1994), the $As(GS)_3$ complex, which has been prepared at acidic pH value, is stable over the pH range from 1.5 to 7.0–7.5 and rather unstable above pH 7.5. Thus, there is little chance to observe its major formation when As(III) and GSH are mixed at pH higher than 7. This conclusion agrees with our observations.

Actually, with one exception that will be discussed later, the formation of arsenic–glutathione complex has not been demonstrated in vivo. Thus, the significance of the As(III)-glutathione complexes in transport and metabolism in vivo is still unknown and require further studies (Scott *et al.*, 1993). However, based on the following observations, the formation and the transporter-mediated efflux of As-GSH complex has been often postulated. It has been shown that either As(V) or As(III) treatment induced increased biliary excretion of nonprotein thiols in the rat (Gregus and Gyurasics, 2000). An increased biliary excretion of glutathione is also generated by the glutathione-dependent hepatobiliary transport of antimony and bismuth (Gyurasics *et al.*, 1992). Because this enhancement was not observed when the thiol groups were blocked by a firmly bound metal ion (e.g. Hg(II)), and based on the close chemical similarity of the trivalent antimony, bismuth, and arsenic, the authors hypothesized that generation of biliary GSH by arsenic, antimony, and bismuth is due to hepatic formation, biliary excretion, and subsequent decomposition of unstable arsenic–GSH, antimony–GSH, and bismuth–GSH complexes. Because the pH of bile is neutral or slightly alkaline, this proposition is in agreement with the possible dissociation of the GSH–As complexes observed at pH > 7.5 (Delnomdedieu *et al.*, 1994).

Accordingly to our knowledge only Kala *et al.* (2000) have demonstrated the presence of As(III)–glutathione complex in the bile of rats injected with arsenic and proposed that the MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic. They have observed that when rats are intravenously treated with sodium arsenite at high dose (5 mg/kg, i.e., $\sim$300 μM arsenic) arsenic is recovered in the bile complexed to GSH. However, when lower doses that are relevant to human exposure (0.1–0.5 mg/kg, i.e., $\sim$5–30 μM) are used, arsenic complexed to GSH was almost undetectable. According to these authors, a likely explanation for this finding is that $As(GS)_3$ is found only at high doses (5 mg/kg) because the ability of cells to methylate arsenic is saturated, whereas at lower doses all arsenic can be methylated (Kala *et al.*, 2000). It must be kept in mind that the major human metabolic pathway for inorganic arsenic is methylation (Aposhian, 1997; Kala *et al.*, 2000). Under experimental conditions similar to those used by Kala *et al.* (2000), Gregus *et al.* (2000) have not detected As–GSH species and have found that monomethylarsenous acid is the major biliary metabolite in rats.

In MRP1-overexpressing cells, Zaman *et al.* (1995) have observed that MRP1 increases the export of glutathione from the cell and this increase is further elevated in the presence of arsenite. They subsequently proposed that $As(GS)_3$ complex may be the form in which arsenite is excreted. We also observed increased extracellular concentration of GSH when cells are incubated with As_2O_3, the efflux of GSH being about 1×10^{-18} and 1.7×10^{-18} mole per cell and per second in the absence and presence of As_2O_3, respectively. However, we have calculated that under our experimental conditions 1.3×10^{-20} mole As are pumped out per cell and per second yielding 3.9×10^{-20} mole GSH per cell and per second with the hypothesis that As is efflux as an $As(GS)_3$ complex. This value is about 20 times lower than that observed (3.9×10^{-20} versus 7×10^{-19}). It follows that the hypothesis of an efflux of As as an $As(GS)_3$ complex cannot take into account the increase of the export of GSH in the presence of As_2O_3.

As the chemical nature of the arsenic (and antimony) species which is pumped out by MRP1 is still unknown, let us consider now the uptake of arsenic and antimony by the cells. One question is, what are the arsenic and antimony species which diffuse passively through the plasma membrane? As(III) and Sb(III) are semimetals that can form either oxyanions or soft metal covalent bonds with the thiolate groups. The chemistry of arsenic in water is not simple, but there is little doubt that at pH 7, aqueous solutions of inorganic arsenites contain $As(OH)_3$ $pK_1 = 9.2$ (Cullen *et al.*, 1984). It follows that the uptake of arsenic in the cells occurs by passive diffusion of the neutral trihydroxide. Now, once inside the cells, what is the "devenir" of $As(OH)_3$. The data obtained with sensitive cells are very important to answer this question. Actually, the observation that when cells are incubated with As_2O_3, at the pseudosteady state (Step 1, obtained after 1 h) the extra- and intracellular concentration of arsenic are the same indicates that there is a transmembrane equilibrium of the species that diffuses passively through the membrane; this means that $As(OH)_3$ is still present inside the cells, without undergoing a chemical transformation and is passively effluxed from the cells. In addition, the mathematical analysis of the kinetic data as well as the direct measurement of the efflux rate clearly shows that the passive influx coefficient k_+ and efflux coefficient k_- are the same. These data are corroborated by the observations that (i) at pH $\sim$7.3, the rate of complexation of GSH to

arsenic is very slow; (ii) the rate of passive uptake and efflux of arsenic species in sensitive cells does not depend on GSH level (if arsenic–GSH complex was formed, one could have expected different rates of arsenic accumulation inside the GSH-depleted or GSH-rich sensitive cells). So we can conclude that within the first 1–2-h the amount of arsenic-gluathione complex, if any, is very low. Now as time elapsed, one observes a slow increase of the arsenic intracellular concentration which becomes higher than the extracellular arsenic concentration. One very likely interpretation is that the arsenic accumulation is due to the transformation of the permeant $As(OH)_3$ species into another one which is unable to diffuse freely through the membrane such as an As–glutathione complex or arsenic binding to thiol-containing proteins. All this reasoning holds for antimony.

These data are also corroborated by the experiments with sensitive and resistant K562 cells. In both cell lines, after 1–2 h, at the pseudosteady state, the intra- and extracellular arsenic or antimony concentration are the same. This shows that this phenomenon does not depend on the nature of the sensitive cell line. Also, these experiments confirm that arsenic- and antimony-containing species are not P-gp substrates.

The important point is that negligible amount, if any, of As–GSH complex is formed during the first 1–2 h. The arsenic concentration is lower in resistant cells than in sensitive cells; thus, the arsenic species is pumped out by MRP1. This shows that $As(OH)_3$ species is the substrate for MRP1. In addition from the observation that there is no active efflux in GSH-depleted cells, we suggest that $As(OH)_3$ could be cotransported with GSH. In addition, 2 μM MK571 are able to inhibit the 50% of MRP1-mediated efflux of $As(OH)_3$, and this is exactly the concentration required to inhibit 50% of MRP1-mediated efflux of GSH (Salerno and Garnier-Suillerot, 2001).

At this step it is interesting to compare the kinetic parameters for the MRP1-mediated efflux of GSH with those for the MRP1-mediated efflux of As- and Sb-containing species. The parameter k_a, which is proportional to V_M/K_m allows an easy comparison of the transport efficiency of the various substrates. We have previously shown the low efficiency of GSH as MRP1 substrate with $k_a = 4.4 \times 10^{-16}$ L. cell^{-1}. s^{-1}, and shown that 50% of the ATP-dependent transport of GSH was inhibited by 2 μM MK571. Here we have measured $k_a = 6.4 \pm 1.4$ L. cell^{-1}. s^{-1} and 8.4 ± 1.8 L. cell^{-1}. s^{-1} for As and Sb, respectively, and it is shown that here also, 50% of the ATP-dependent transport of these species was inhibited by 2 μM MK571. The similarities of the kinetic parameters for the transport of these three species, with k_a lying within the range 4–9 $\times$ 10^{-6} L. cell^{-1}. s^{-1}, and the observation that a metalloïd–GSH complex cannot be formed at short term within the cells, led us to suggest that As- and Sb-containing species could be cotransported with GSH.

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