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Short communication

Resistance of human multidrug resistance-associated protein 1-overexpressing lung tumor cells to the anticancer drug arsenic trioxide

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Abstract

The human multidrug-resistance protein (MRP1) confers resistance to some heavy metals such as arsenic and antimony, mainly through mediating an increased cellular efflux of metal. However, it was recently suggested that arsenic, used under its trioxide derivative form as anticancer drug, is not handled by MRP1. The aim of the present study was to test this hypothesis in MRP1-overexpressing human lung tumor GLC4/Sb30 cells. Using the cytotoxicity MTT assay, GLC4/Sb30 cells were found to be 10.8-fold more resistant to arsenic trioxide (As_2O_3) than parental GLC4 cells. MK571, a potent inhibitor of MRP1 activity, almost totally reversed resistance of GLC4/Sb30 cells, but did not alter the sensitivity of GLC4 cells. Moreover, As_2O_3-loaded GLC4/Sb30 cells poorly accumulated arsenic through an increased MK571-sensitive efflux of metal. Finally, depletion of cellular glutathione levels in buthionine sulfoximine-treated GLC4/Sb30 cells was found to result in increased accumulation and reduced efflux of arsenic in cells exposed to As_2O_3, outlining the glutathione-dependence of MRP1-mediated transport of the metal. These results indicate that MRP1 overexpression in human tumor cells can confer resistance to As_2O_3, which may limit the clinical use of this anticancer drug for treatment of MRP1-positive tumors.

Keywords: Arsenic trioxide; Multidrug resistance-associated protein 1; Metal resistance; Lung tumor cells

1. Introduction

As_2O_3, a trivalent arsenical salt, has been used for many decades in traditional Chinese medicines for treatment of various human diseases including tumors, and was recently confirmed to be clinically active in acute promyelocytic leukemias [1]. At low concentrations, As_2O_3 has been shown to induce a high rate of clinical remission in patients suffering from such leukemias without severe toxicity [1,2]. Moreover, experimental studies have provided further evidence of the antiproliferative properties of As_2O_3 towards malignant lymphoid cells [3] and solid tumor cells [4–6]. Taken together, these observations are in favor of a promising use of As_2O_3 as a potent anticancer drug. However, as observed with various other anticancer drugs, treatment with As_2O_3 has also been associated with a rapid development of clinical resistance in some patients [2].

Clinical efficiency of anticancer drugs is frequently limited by emergence of various mechanisms of resistance in tumor cells. One of these mechanisms is related to increased expression of efflux pumps, including that of MRP1, an ATP-binding cassette transporter [7]. MRP1 is thought to mediate outwardly-directed transport of drugs, including GSH conjugates, thus preventing intracellular accumulation of various anticancer drugs and conferring a multidrug resistance phenotype [7]. MRP1 can also confer resistance to heavy metals such as arsenic and antimony [8–10]. In particular, MRP1-overexpressing tumor cells have been shown to be cross-resistant to arsenical salts such as sodium arsenite and sodium arsenate, suggesting that MRP1 overexpression may limit the efficiency of arsenic-derived anticancer drugs. However, a recent study has reported that increased MRP1 levels in human leukemia HL60 cells did

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Abbreviations: MRP1, multidrug resistance-associated protein 1; As_2O_3, arsenic trioxide; MTT, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and BSO, buthionine sulfoximine.

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not prevent the cytotoxic effects of As_2O_3 [11], indicating that the use of arsenic under its trioxide derivative form may make it possible to bypass MRP1-mediated resistance. In the present study, to test such a hypothesis we evaluated the sensitivity of MRP1-overexpressing human lung tumor GLC4/Sb30 cells to As_2O_3.

2. Materials and methods

2.1. Chemicals

As_2O_3 and BSO, an inhibitor of γ-glutamylcysteine synthetase [12], were purchased from Sigma Chemical Co. MK571, a leukotriene D4 receptor antagonist [13] that is known to inhibit MRP1 activity [14], was provided by Dr. Ford-Hutchinson (Merck-Front Inc., Quebec, Canada).

2.2. Cell culture

Human lung tumor GLC4 cells and their MRP1-overexpressing variants GLC4/ADR and GLC4/Sb30 were cultured in RPMI supplemented with 10% fetal bovine serum. GLC4 cells and doxorubicin-selected GLC4/ADR cells [15] were provided by Dr. E.G.E. de Vries (University Hospital Groningen, Groningen, The Netherlands), whereas GLC4/Sb30 cells were generated in our laboratory by a step-wise selection with antimony [9]. GLC4/Sb30 and GLC4/ADR cells were routinely maintained in the presence of 92 μM potassium antimony tartrate and 1 μM doxorubicin, respectively, until 3 days before experiments.

2.3. Western blotting

MRP1 expression was analyzed on crude membranes by Western blot as previously described [9], using the monoclonal antibody MRP1m6 (Monosan, dilution 1/250) and the enhanced ECL chemoluminescent system (Amersham) for the detection of immunoreactive proteins.

2.4. Drug sensitivity

Sensitivity of cells to As_2O_3 was tested using the MTT colorimetric assay, as previously described [9]. IC_{50} values were defined as the mean concentrations reducing absorbance by 50%.

2.5. Measurement of cellular arsenic contents

Cellular arsenic contents were quantified using a Zeeman atomic absorption spectrometer (Spectra A300, Varian) and normalized to cellular protein content, using the Bio-Rad protein assay [16].

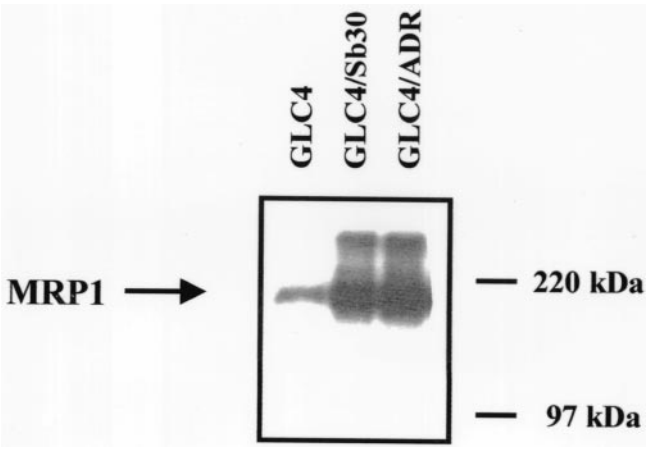

Fig. 1. MRP1 expression in GLC4, GLC4/Sb30, and GLC4/ADR cells. Crude membrane proteins (25 μg) were analyzed by Western blotting using the specific monoclonal antibody MRP1m6 and with horseradish peroxidase-conjugated goat anti-mouse antibody. Immunoreactive proteins were detected using a chemoluminescent system.

2.6. Measurement of GSH levels

Intracellular GSH levels were determined using the Tietze's recycling method [17] and normalized to cellular protein contents.

2.7. Statistical analysis

The results were analyzed by the Duncan multirange *t*-test and the criterion of significance of differences between means (±SEM) was $P < 0.05$.

3. Results and discussion

In a first set of experiments, we compared As_2O_3 toxicity towards GLC4, GLC4/Sb30, and GLC4/ADR cells. As shown by immunoblotting in Fig. 1, both GLC4/Sb30 and GLC4/ADR cells overexpressed MRP1 when compared to

Table 1
Sensitivity of GLC4, GLC4/Sb30, and GLC4/ADR cells to As_2O_3

MK571 (μM)	IC_{50} (μM)[a]		
	GLC4	GLC4/Sb30	GLC4/ADR
0	0.77 ± 0.11	8.35 ± 1.16 (10.8)[b]	6.37 ± 0.3 (8.3)[b]
20	0.64 ± 0.05 (1.2)[c]	1.24 ± 0.29* (6.7)[c]	1.31 ± 0.26* (5.1)[c]

[a] IC_{50} values were determined using an MTT assay. Data are the means ± SEM of at least three independent experiments performed in duplicate.

[b] The resistance factor, calculated as the ratio of the IC_{50} of GLC4/Sb30 or GLC4/ADR cells to the IC_{50} of GLC4 cells, is shown in parentheses.

[c] The fold sensitization, calculated as the ratio of the IC_{50} in the absence of MK571 to the IC_{50} in the presence of MK571, is shown in parentheses.

* $P < 0.05$, when compared to MK571-untreated cells.

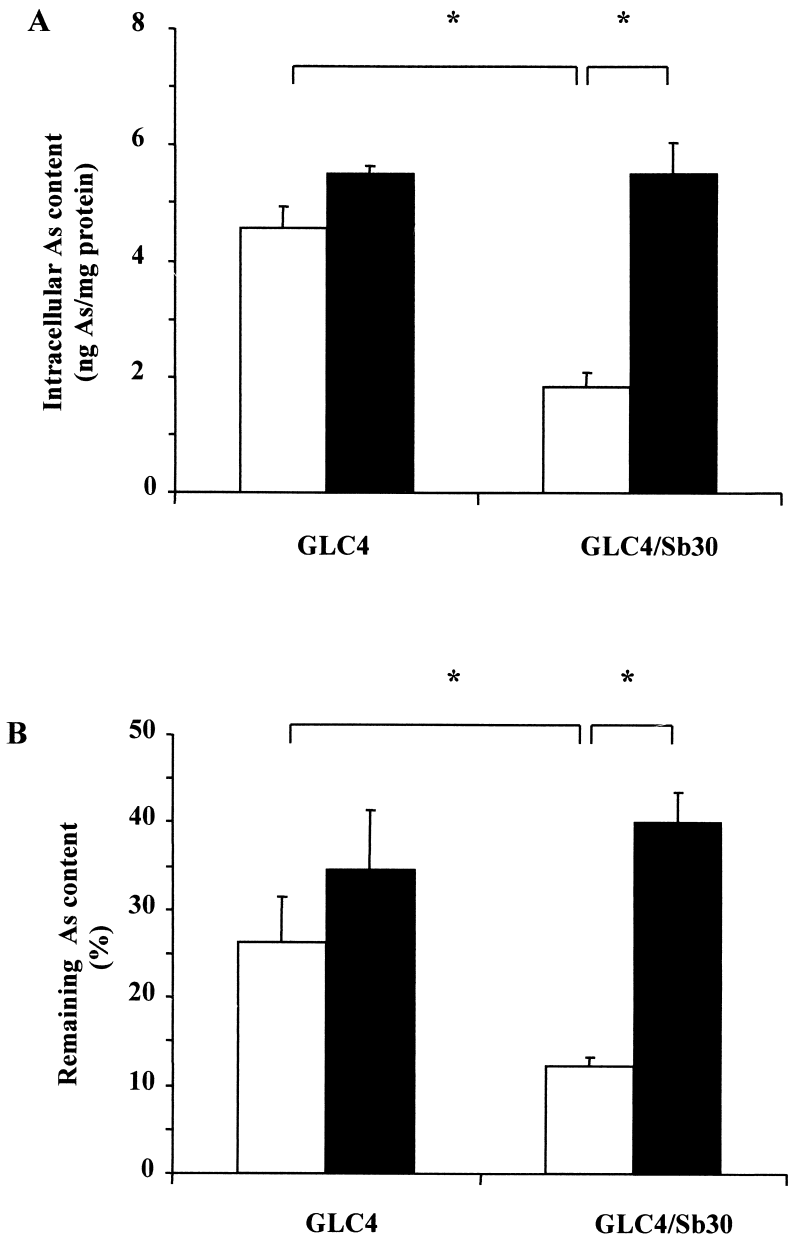

Fig. 2. Accumulation (A) and efflux (B) of arsenic in GLC4 and GLC4/Sb30 cells. (A) Cells were incubated with 10 μM As_2O_3 in RPMI medium for 2 hr in the absence or presence of 50 μM MK571. Cells were washed and lysed with distilled water. Intracellular arsenic contents were quantified by atomic absorption spectrometry and normalized to cellular protein contents. (B) As_2O_3-loaded cells were incubated in As_2O_3-free RPMI medium for 2 hr in the absence or presence of 50 μM MK571. Remaining intracellular arsenic contents were quantified as described above. Results are expressed as percentage of initial intracellular arsenic accumulation. Each bar is the mean ± SEM of four independent experiments. Control cells (□), MK571-treated cells (■). *$P < 0.05$; As, arsenic.

GLC4 cells. Cytotoxic MTT assays indicated that GLC4 cells were highly sensitive to As_2O_3, with IC_{50} values being in the range of therapeutic concentrations shown to be effective in treatment of acute promyelocytic leukemias [1] (Table 1). In contrast, GLC4/Sb30 cells were 10-fold more resistant to the cytotoxic effects of this metal than the parental cells. Moreover, the potent MRP1 inhibitor MK571 [14], at the non-cytotoxic concentration of 20 μM, was shown to strongly increase sensitivity of these resistant cells to As_2O_3. Indeed, MK571-treated GLC4/Sb30 cells were found to be 6.7-fold more sensitive than their untreated counterparts, whereas MK571 had no significant effect in parental GLC4 cells. Similarly, GLC4/ADR cells exhibited a marked level of resistance to As_2O_3 (8.3-fold), one almost totally reversible by MK571 (Table 1); this demonstrates that resistance to this arsenical salt may occur in different MRP1-overexpressing cell lines.

In further experiments, MRP1, which had been previ-

ously reported to be fully active in GLC4/Sb30 cells [9,10], was demonstrated to reduce intracellular arsenic levels in GLC4/Sb30 cells exposed to As_2O_3. Indeed, Fig. 2A clearly indicates that after a 2-hr incubation with 10 μM As_2O_3, GLC4/Sb30 cells contained 60% less arsenic than did parental cells. Interestingly, MK571 specifically increased arsenic accumulation in resistant cells, allowing the metal content to reach the levels observed in GLC4 cells (Fig. 2A). The reduced retention of arsenic in GLC4/Sb30 cells is likely due to an increased MRP1-dependent export of the metal since: (1) after a 2-hr incubation of As_2O_3-loaded cells in As_2O_3-free medium, the percentage of remaining intracellular metal was 2.1-fold lower in GLC4/Sb30 cells than in parental cells; and (2) MK571 was found to specifically block arsenic efflux in resistant cells (Fig. 2B). Moreover, the involvement of several other drug transporters can be excluded. Indeed, GLC4 and GLC4/Sb30 cells do not express detectable levels of P-glycoprotein and MRP2 [9], and display similar levels of both *MRP3* and *MRP5* gene expression.[1] It could be noted that the levels of resistance to As_2O_3 did not correlate with the decrease in cellular arsenic retention observed in GLC4/Sb30 cells. However, it is noteworthy that such a discrepancy, also reported for other compounds in different human MRP1-overexpressing cell lines [18,19], may result from MRP1-mediated alterations in drug distribution among intracellular compartments [19]. Indeed, MRP1 mediates drug sequestration into some compartments, which further prevents drug interaction with its intracellular targets.

GSH has been shown to play a major role in the MRP1-mediated export of various compounds [7]. We therefore studied the effects of BSO, a potent GSH-depleting agent, on arsenic transport in As_2O_3-treated cells. After a 24-hr treatment of GLC4/Sb30 cells with 25 μM BSO, intracellular GSH levels were 80% lower than those measured in untreated cells (7.9 $\pm$ 1.0 vs 1.5 $\pm$ 0.1 nmol/mg protein in untreated and BSO-treated GLC4/Sb30 cells, respectively). In such GSH-depleted cells, accumulation of As_2O_3 was found to be significantly increased by 53% when compared with untreated cells (25.7 $\pm$ 1.9 ng vs 16.8 $\pm$ 2.8 ng As/mg protein in BSO-treated GLC4/Sb30 and GLC4/Sb30 cells). In addition, efflux studies indicate that outward transport of arsenic from GLC4/Sb30 cells was strongly impaired in response to BSO, since 41.2 $\pm$ 4% and 13.2 $\pm$ 0.4% of initial metal content were recovered in BSO-treated and untreated GLC4/Sb30 cells, respectively, after a 2-hr incubation in metal-free culture medium (Fig. 3). By contrast, BSO treatment of parental GLC4 cells, which similarly depleted intracellular GSH levels, did not significantly modify arsenic retention in these cells (Fig. 3). Down-modulation of arsenic efflux by GSH depletion is therefore likely restricted to MRP1-overexpressing cells; this argues against

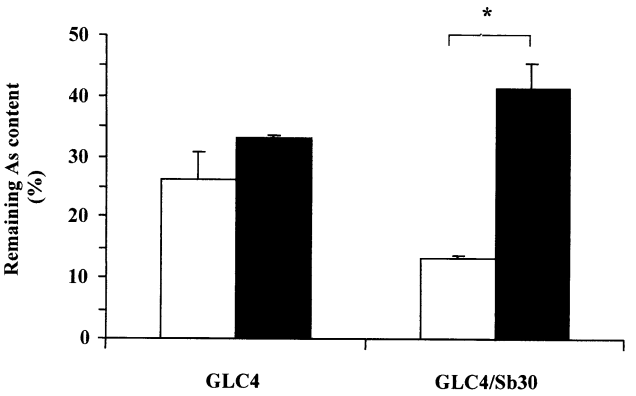

Fig. 3. Effect of BSO on arsenic cellular efflux. Untreated and BSO-treated cells were cultured with 10 μM As_2O_3 in RPMI medium for 2 hr, washed, and then incubated in As_2O_3-free RPMI medium for 2 hr. Remaining intracellular arsenic contents were quantified by atomic absorption spectrometry and normalized to cellular protein contents. Results are expressed as percentage of initial intracellular arsenic accumulation. Each bar is the mean $\pm$ SEM of three independent experiments. Control cells (□), BSO-treated cells (■). *$P < 0.05$; As, arsenic.

an MRP1-unrelated mechanism such as increased binding of arsenic to intracellular proteins, and supports the conclusion that GSH is required for MRP1-mediated transport of arsenic used under its trioxide derivative. How GSH precisely interacts with the MRP1-related efflux of arsenic deserves further study. It should be kept in mind that a coordinated increase in both intracellular GSH levels and glutathione *S*-transferase activities has been reported to enhance the MRP1-independent efflux of arsenic, thereby allowing cells to escape the cytotoxic effects of the metal [20,21]. Involvement of such mechanisms in the resistance of GLC4/Sb30 cells to As_2O_3 should, however, be ruled out. Indeed, neither intracellular GSH levels measured in cells cultured in metal-free medium for at least three days [22] nor GSH *S*-transferase activities[1] were found to be significantly different in GLC4 and GLC4/Sb30 cells.

In contrast to the data shown in the present study, Perkins *et al*. failed to detect increased resistance to As_2O_3 in MRP1-overexpressing human promyelocytic leukemia HL-60 cells [11]. The reason for such a discrepancy is unclear. It may, however, be hypothesized that the cell type, the level of MRP1 overexpression, or putative posttranslational alterations could influence the handling of arsenic under its trioxide derivative in MRP1-overexpressing cells.

In summary, our study has demonstrated that MRP1 overexpression in human tumor cells confers resistance to the anticancer drug As_2O_3 mainly through an enhanced MRP1-mediated export of metal. These results have important potential implications; indeed, since high MRP1 levels have been previously detected in various solid tumors and leukemias [7], the clinical efficiency of As_2O_3 as an anticancer drug may be consequently limited in such malignancies.

[1]Vernhet L, Allain N, Fardel O. Unpublished observations.

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