Overexpression of the multidrug resistance-associated protein (MRP1) in human heavy metal-selected tumor cells

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Received 11 December 1998

Abstract Cellular and molecular mechanisms involved in the resistance to cytotoxic heavy metals remain largely to be characterized in mammalian cells. To this end, we have analyzed a metal-resistant variant of the human lung cancer GLC4 cell line that we have selected by a step-wise procedure in potassium antimony tartrate. Antimony-selected cells, termed GLC4/Sb30 cells, poorly accumulated antimony through an enhanced cellular efflux of metal, thus suggesting up-regulation of a membrane export system in these cells. Indeed, GLC4/Sb30 cells were found to display a functional overexpression of the multidrug resistance-associated protein MRP1, a drug export pump, as demonstrated by Western blotting, reverse transcriptase-polymerase chain reaction and calcein accumulation assays. Moreover, MK571, a potent inhibitor of MRP1 activity, was found to markedly down-modulate resistance of GLC4/Sb30 cells to antimony and to decrease cellular export of the metal. Taken together, our data support the conclusion that overexpression of functional MRP1 likely represents one major mechanism by which human cells can escape the cytotoxic effects of heavy metals.

Key words: Multidrug resistance-associated protein; Heavy metal; Drug resistance; Membrane transport; Antimony

1. Introduction

Most heavy metals are devoid of physiological functions and are potentially toxic for prokaryotes and eukaryotes. Thus, arsenic, cadmium and antimony have been shown to be highly toxic toward cultured cells and are presumed to be genotoxic and/or carcinogenic agents in humans [1]. Their cytotoxic effects can, however, be reduced in heavy metal-selected cells but the cellular mechanisms involved are not fully understood. A major one is related to overexpression of low molecular weight cysteine-rich proteins known as metallothioneins (MT), which bind heavy metals such as cadmium, thereby preventing them from reaching their intracellular targets [1]. Another mechanism is linked to decreased metal intracellular accumulation and reduced levels of arsenic or antimony have been demonstrated in different resistant cell lines [2–4]. Low accumulation of metal could result from either a decreased cellular influx or an increased cellular efflux. It may be linked to cellular glutathione content [4] and is most likely related to an altered expression of membrane systems of metal transport; however, the transporters involved remain largely unknown in mammalian cell lines. For efflux processes, it can been hypothesized that they may be members of the ATP-binding cassette (ABC) transporter superfamily that contains several detoxifying pumps such as P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and canalicular multispecific organic anion transporter (cMOAT) [5]. Indeed, ABC membrane proteins such as *Leishmania* LtpgpA and yeast cadmium resistance facor 1 (YCF1) have already been incriminated in heavy metal resistance in unicellular eukaryotic organisms [6,7]. Moreover, transfection experiments of the cDNA encoding human MRP1 into human tumor cells have recently demonstrated that this efflux pump, in addition to mediating outwardly directed transport of anticancer drugs [8,9], may also interact with genotoxic heavy metals such as arsenite and antimony [8]. In order to improve knowledge of the detoxifying pathways displayed by heavy metal-selected human cells, in particular with respect to carrier-mediated membrane transport of metals, we have generated and analyzed a human antimony-selected lung cancer cell line. Our results demonstrate that these antimony-selected cells, which were also cross-resistant to arsenic, displayed enhanced efflux of antimony and overexpressed MRP1.

2. Materials and methods

2.1. Cell culture

An antimony-resistant variant of the human small cell lung cancer cell line GCL4, kindly provided by Dr. E.G.E. de Vries (University Hospital Groningen, Groningen, The Netherlands), was generated by chronic exposure of GLC4 cells to potassium antimony tartrate (Sb(III)) during which the salt metal concentration was increased step-wise from 2 to 92 μM over an 8-month period. Antimony-selected GLC4 cells, named GLC4/Sb30, were then routinely maintained in the presence of 92 μM Sb(III) until 3 days before experiments. The culture medium used for both GLC4 cells and GLC4/Sb30 cells was RPMI medium supplemented with 10% fetal calf serum and 2 mM glutamine.

2.2. Drug sensitivity

Cytotoxic effects of metals and anticancer drugs were analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as previously described [10]. IC_{50} values were defined as the mean concentrations reducing absorbance by 50%.

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Abbreviations: MT, metallothioneins; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1; cMOAT, canalicular multispecific organic anion transporter; YCF1, yeast cadmium resistance factor 1; Sb(III), potassium antimony tartrate; RT-PCR, reverse transcriptase-polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PII: S0014-5793(98)01716-5

2.3. Antimony accumulation and efflux

Cellular antimony contents were determined after both incubation of cells with 100 μM Sb(III) for 2–6 h (accumulation studies) and re-incubation of Sb(III)-loaded cells in Sb(III)-free RPMI medium for up to 3 h (efflux studies) in the absence or presence of 50 μM MK571, a leukotriene D4 receptor antagonist [11], kindly provided by Dr. Ford-Hutchinson (Merck-Front Inc., Quebec, Que., Canada), which is known to inhibit MRP1 activity [12]. Cellular antimony contents were quantified using a Zeeman atomic absorption spectrometer (Spectra A300, Varian, Victoria, Australia) and normalized to cellular protein content, using the Bio-Rad protein assay [13].

2.4. Western blotting

Proteins were analyzed either on whole cell lysates (for MT) or on crude membrane protein extracts prepared according to Germann et al. [14] (for P-gp and MRP1). Proteins were separated on a 7.5% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane (Amersham, Les Ulis, France) for 24 h at 4°C. Nitrocellulose membranes were blocked with 1% skim milk, 1% bovine serum albumin and 0.1% Tween-20 Tris buffer, and sequentially incubated with specific monoclonal antibodies raised against MRP1 (MRPm6, Monosan, Uden, The Netherlands; dilution 1/250), P-gp (C219, Centocor, Malvern, USA, dilution 1/200) or MT (Dako-MT, E9, Dako, Carpinteria, USA, dilution 1/200) and then with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1/2500 dilution). Immunoreactive proteins were detected using a chemiluminescent system (ECL, Amersham, UK).

2.5. RT-PCR experiments

Total cellular RNA was extracted from cells by the guanidinium thiocyanate/cesium chloride method [15] and mRNA levels were analyzed by RT-PCR as previously reported [10]. The primers used for MRP1 and β-actin detection were exactly as previously described [10,16]. The primers used for cMOAT analysis were: cMOAT sense primer, 5′-CTAGCAGCCATAGAGCTGGC-3′; cMOAT antisense primer, 5′-TGGCTCCAGAGTTCTGCTGG-3′.

2.6. Southern blotting

Genomic DNA (20 μg) was digested with *Eco*RI, electrophoresed through a 0.8% agarose gel and transferred onto Hybond-N^+ membrane (Amersham). Membranes were prehybridized for at least 3 h at 65°C, hybridized with a ^{32}P-labelled 0.4 kb MRP1 cDNA probe for 18 h at 65°C and then autoradiographed at −80°C for 24 h. The MRP1 gene probe was generated by RT-PCR and was identical to MRP1 gene sequences previously described [17,18].

2.7. MRP1 activity

MRP1 activity was assessed by determination of intracellular accumulation of the fluorescent anionic dye calcein, as previously described [19]. Cells were loaded with 0.5 μM calcein AM for 1 h at 37°C without or with probenecid, a modulator of MRP1 [19]. Cellular calcein contents were then quantified by fluorimetry, using a Titertek Fluoroscan spectrofluorometer (Flow Laboratories, Puteaux, France); excitation and emission wavelengths were 488 nm and 538 nm, respectively.

2.8. Statistical analysis

Results were analyzed by Student's *t*-test and the criterion of significance of the differences between means (± S.E.M.) was $P < 0.05$.

3. Results

3.1. Resistance pattern of GLC4Sb30 cells

GLC4/Sb30 cells, generated by a step-wise selection in Sb(III), were first examined for their sensitivity to cytotoxic agents using the MTT assay. Antimony IC_{50} values were 3.2 ± 0.3 μM and 112.6 ± 11.0 μM for GLC4 cells and GLC4/Sb30 cells, respectively, demonstrating that GLC4/Sb30 cells were 35-fold more resistant to Sb(III) when compared to their parental counterparts. Table 1 indicates that GLC4/Sb30 cells were highly cross-resistant to the pentavalent antimonial salt meglumine and to the trivalent arsenical salt arsenite. By contrast, these cells were only slightly resistant, if at all, to cadmium chloride and zinc sulfate. Antimony-selected cells were also found to be cross-resistant to the two chemotherapeutic drugs doxorubicin and vincristine.

3.2. Cellular accumulation and efflux of antimony

Cellular accumulation of antimony was determined in GLC4 and GLC4/Sb30 cells exposed to 100 μM Sb(III) for various lengths of time by atomic absorption spectrometry. Antimony contents of GLC4/Sb30 cells, which reached a steady state within the first 2 h of incubation, were lower than those measured in GLC4 cells whatever the duration of exposure to antimony (Fig. 1A). They represented about 60% of the values measured in GLC4 cells after a 6 h incubation with the metal. Efflux studies were then performed in order to determine whether impairment of antimony accumulation in GLC4/Sb30 cells was due to enhanced metal cellular export. Results indicated that the antimony-selected cells poorly retained antimony after either a 1 h or a 3 h efflux period when compared to parental sensitive cells (Fig. 1B).

3.3. Expression of detoxifying proteins

Western blot analysis failed to detect either MT or P-gp in both GLC4 and GLC4/Sb30 cells whereas high levels of MT and P-gp were evidenced in cadmium-treated GLC4 cells and P-gp-overexpressing drug-resistant K562R/7 cells, respectively (data not shown). In contrast, immunoblotting with the monoclonal antibody MRPm6 revealed overexpression of a MRP1-related 190 kDa protein in GLC4/Sb30 cells when compared to the parental cells (Fig. 2A). RT-PCR experiments also revealed enhanced levels of MRP1 mRNAs in GLC4/Sb30 cells compared to GLC4 cells whereas similar amounts of β-actin mRNAs were evidenced in both sensitive and resistant cells (Fig. 2B). In contrast, mRNAs of cMOAT, an ABC membrane transporter sharing numerous substrates with MRP1 [5], were not detected in either GLC4 cells or

Table 1
Cross-resistance of GLC4/Sb30 cells to heavy metals and chemotherapeutic drugs

	IC_{50}[a]		Resistance factor[b]	*P*
	GLC4	GLC4/Sb30		
Meglumine antimonate	1170 ± 0.3	32 170 ± 8.5	27.5	0.001
Sodium arsenite	1.6 ± 0.2	16.1 ± 3.5	10.0	0.006
Cadmium chloride	11.5 ± 2.2	25.9 ± 4.2	2.2	0.013
Zinc sulfate	128.7 ± 21.3	172.8 ± 24.2	1.3	0.22
Vincristine	0.43 ± 0.1[c]	3.34 ± 0.6[c]	7.7	0.004
Doxorubicin	10.1 ± 1.2[c]	51.8 ± 5.6[c]	5.1	0.03

[a]The IC_{50}s were determined using a MTT assay and are expressed as μM concentrations, unless otherwise indicated. Data are the mean ± S.E.M. of at least three independent experiments. Statistical significance (*P*) was determined by Student's *t*-test.
[b]The resistance factors were calculated by dividing the IC_{50} values of GLC4/Sb30 cells by those of GLC4 cells.
[c]nM.

GLC4/Sb30 cells, but were markedly visualized in human hepatocytes used as positive control cells (data not shown). Genomic DNAs extracted from GLC4 and GLC4/Sb30 cells were further subjected to Southern blotting and hybridized with a MRP1 cDNA probe (Fig. 2C). Results showed increased levels of MRP1-related DNA in GLC4/Sb30 cells. Finally, MRP1 activity was examined using a calcein accumulation assay (Fig. 3). Cellular levels of the fluorescent dye were found to be strongly decreased in GLC4/Sb30 cells in comparison to those observed in GLC4 cells. However, probenecid, a well-known inhibitor of MRP1-mediated transport [19], was able to markedly enhance calcein accumulation in the anti-

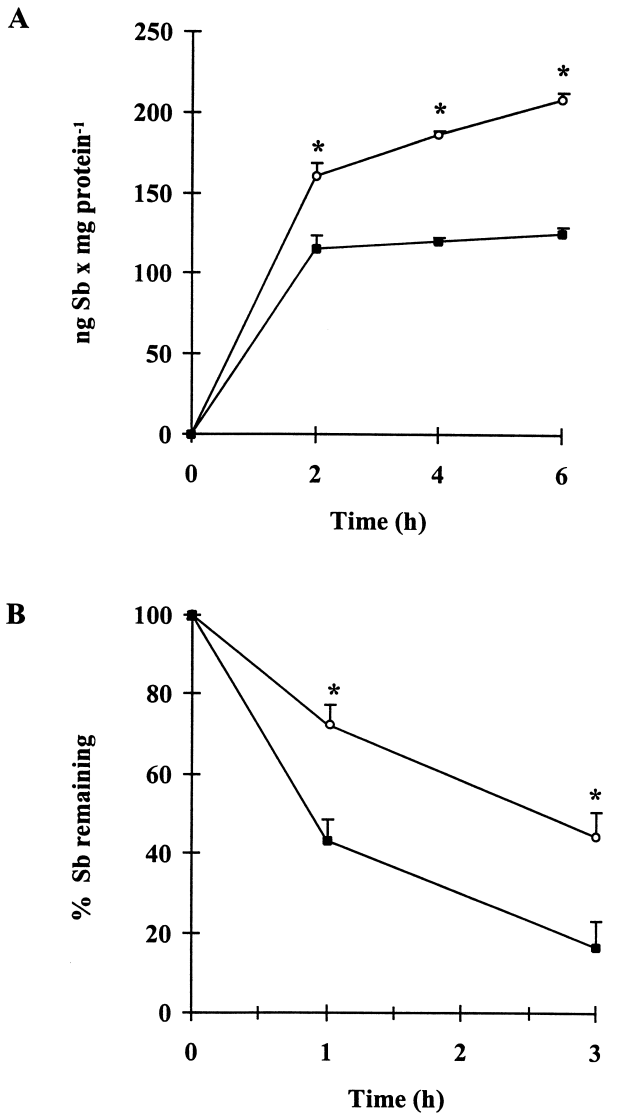

Fig. 1. Accumulation (A) and efflux (B) of antimony in GLC4/Sb30 cells. A: GLC4 cells and GLC4/Sb30 cells were incubated with 100 μM Sb(III) and intracellular antimony content was measured at different time intervals and normalized to cellular protein content. B: GLC4 cells and GLC4/Sb30 cells were first pre-incubated with 200 μM Sb(III) for 1 h, washed and then incubated in Sb(III)-free medium. Intracellular antimony content was measured at different time intervals and expressed as the percentage of initial intracellular antimony accumulation. Each point is the mean ± S.E.M. of at least three independent experiments performed in duplicate. GLC4 cells (○), GLC4/Sb30 cells (■). *$P < 0.05$.

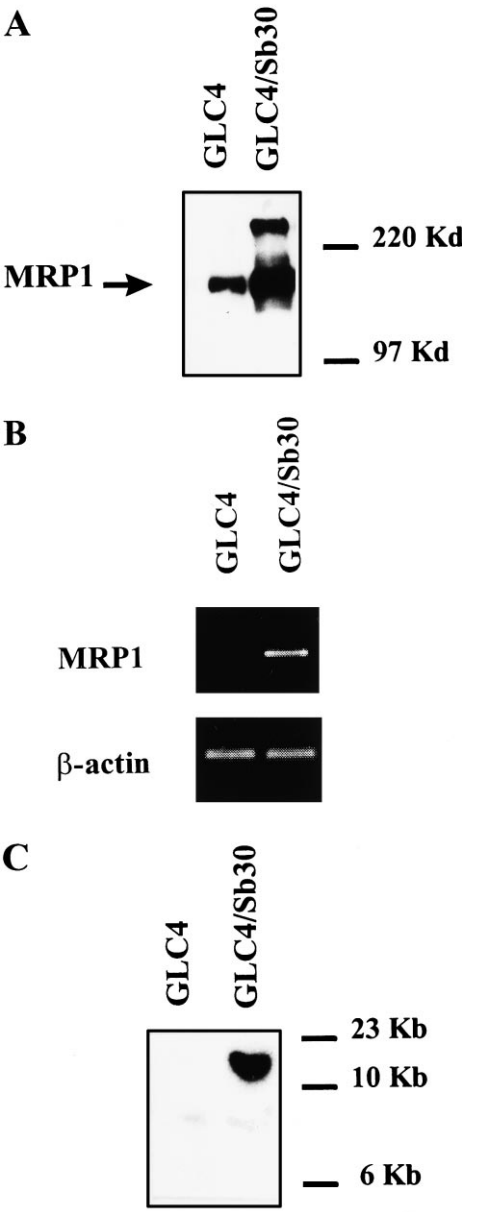

Fig. 2. Analysis of MRP1 expression in GLC4/Sb30 cells. A: MRP1 protein levels. Crude membrane proteins (25 μg) were prepared and analyzed by Western blotting using the specific MRP1 monoclonal antibody MRPm6. Position of molecular weight standard is indicated on the right. B: MRP1 mRNA levels. Total RNAs were prepared and analyzed by RT-PCR using MRP1- and β-actin-specific primers. C: MRP1 DNA analysis. Genomic DNA (20 μg) was digested with *Eco*RI and electrophoresed through a 0.8% agarose gel. The blot was hybridized with a cDNA-specific fragment of MRP1 and autoradiographed for 24 h at −80°C. Position of DNA molecular fragment standards is indicated on the right.

mony-resistant cells without altering dye levels in the parental GLC4 cells (Fig. 3).

3.4. Effect of MK571

In order to determine whether MRP1 activity was involved in the resistance of GLC4/Sb30 cells to Sb(III) cytotoxicity, the effects of the MRP1 inhibitor MK571 on both metal sensitivity and metal efflux were studied using MTT assays and atomic absorption spectrometry measurements, respectively. As indicated in Table 2, MK571, used at the non-cytotoxic

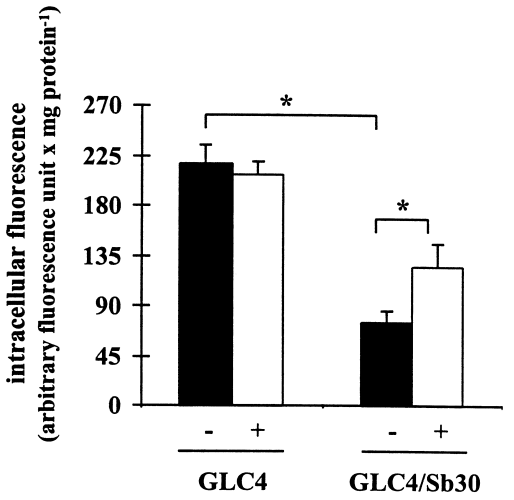

Fig. 3. MRP1 activity in GLC4/Sb30 cells. Cells were incubated with 0.5 μM calcein AM for 1 h at 37°C in the absence (−) or presence (+) of 1 mM probenecid. Cellular calcein contents were then analyzed by spectrofluorimetry as described in Section 2. Each column is the mean ± S.E.M. of three independent experiments performed in triplicate. $*P < 0.05$.

concentration of 50 μM, almost fully reversed the resistance of GLC4/Sb30 cells to Sb(III) while it only marginally increased metal sensitivity of the parental cells. In addition, efflux experiments showed that MK571 greatly enhanced cellular retention of antimony in GLC4/Sb30 cells, allowing it to reach the levels observed in sensitive GLC4 cells. Indeed, after a 3 h efflux period, the amounts of antimony retained in GLC4/Sb30 cells, MK571-treated GLC4/Sb30 cells and parental GLC4 cells corresponded to 25.3 ± 6.2, 65.1 ± 13.4 and 53.3 ± 4.5% of the initial cell-associated antimony value, respectively.

4. Discussion

Detoxifying pathways displayed by human heavy metal-selected cells remain largely to be characterized. In this study, we have established a human lung cancer cell line highly resistant to both trivalent and pentavalent antimonial and trivalent arsenical salts by an 8 month exposure to Sb(III). Analysis of these antimony-selected cells demonstrates that they did not express metal-detoxifying MT and showed low resistance, if at all, to known MT-interacting metals such as cadmium and zinc; in contrast, they displayed reduced levels of cellular antimony due to enhanced cellular efflux of the metal. Taken together, these data indicate that decreased metal retention is likely a mechanism contributing to the resistance of GLC4/Sb30 cells. Impairment of metal cellular accumulation has already been reported in some protozoa [2] and mammalian heavy metal-selected cells [3,4] and therefore appears to correspond to a common detoxifying pathway displayed by metal-resistant cells. It should, however, be kept in mind that such a mechanism of resistance may involve, besides increased cellular efflux, decreased cellular uptake of metals. Indeed, in contrast to our GLC4/Sb30 cells, antimony-selected 2008/H ovarian cells have been shown to display metal accumulation defect without change in metal efflux rates [3]. This suggests that alterations of different membrane transport systems of metals can occur in resistant cells according to their origin or the protocol used for their selection.

Overexpression of the efflux pump MRP1, initially demonstrated in tumor cells selected by anticancer drugs [20,21], was also observed in GLC4/Sb30 cells at both protein and mRNA levels. In contrast, antimony-selected cells failed to express other detoxifying ABC membrane transporters such as P-gp or cMOAT. Direct and indirect arguments are in favor of a major role for MRP1 in resistance to heavy metals in GLC4/Sb30 cells: (i) these antimony-selected cells displayed a pattern of cross-resistance to heavy metals similar to that found in human cells transfected with a MRP1 cDNA [8], thereby suggesting that selected cells and transfected cells shared the same mechanism of resistance; (ii) MRP1 appeared to be fully functional in GLC4/Sb30 cells since these cells showed decreased probenecid-sensitive accumulation of the MRP1 substrate calcein and were cross-resistant to doxorubicin and vincristine, two anticancer drugs well-known to be transported by human MRP1 [8]; (iii) the MRP1 gene copy number was augmented in GLC4/Sb30 cells as assessed by Southern blot analysis and such a gene amplification, which certainly accounts for at least part of the increased MRP1 levels in GLC4/Sb30 cells, is usually observed for genes encoding resistance markers toward the agent used for selection [20,22]; and (iv) MK571, a potent inhibitor of MRP1 known to specifically reverse MRP1-mediated anticancer drug resistance [12], was also found to markedly down-modulate resistance of GLC4/Sb30 cells to antimony and to inhibit cellular export of the metal. In contrast, MK571 only weakly increased the sensitivity to antimony of parental GLC4 cells and this effect can likely be attributed to inhibition of the MRP1 constitutively present at low, but detectable, levels in these cells.

It is noteworthy that the levels of resistance to antimony (35-fold) displayed by GLC4/Sb30 cells do not correlate with the decrease (1.7-fold) in metal accumulation occurring in these resistant cells when compared to their parental counterparts. A possible explanation for this discrepancy is that additional cellular factors unrelated to MRP1, which remain to be determined, may also participate in a major way in the resistance of GLC4/Sb30 cells. However, it should be kept in mind that inhibition of MRP1 activity in GLC4/Sb30 cells by MK571 allowed them to almost fully reverse their resistance. In addition, similar discordances between the levels of resist-

Table 2
Effect of MK571 on the Sb(III) sensitivity of GLC4/Sb30

	IC_{50} (μM)[a]		
	Sb(III)	Sb(III)+MK571 (50 μM)	*P*
GLC4	3.2 ± 0.3	1.2 ± 0.3 (2.6)[b]	0.004
GLC4/Sb30	112.6 ± 11.0	7.1 ± 0.9 (15.8)	< 0.001

[a]The IC_{50}s were determined using a MTT assay. Data are the mean ± S.E.M. of four independent experiments. Statistical significance (*P*) was determined by Student's *t*-test.
[b]The fold sensitization, calculated as the ratio of IC_{50} in the absence of MK571 to IC_{50} in the presence of MK571, is shown in parentheses.

ance and those of drug accumulation and efflux have already been reported in various MRP1-overexpressing cell lines [8,9]; more strikingly, multidrug-resistant H69AR cells, from which MRP1 cDNA was originally cloned, did not show any change in drug accumulation [23]. In these cell lines, alterations of drug distribution among intracellular compartments have, however, been reported. MRP1 is therefore thought not only to act as a plasma drug efflux pump, but also to contribute to the sequestration of cytotoxic compounds in some intracellular compartments, thus resulting in protection of cellular targets of drugs [9]. Such a mechanism may likely be operating in GLC4/Sb30 cells and, in addition to MRP1-mediated cellular metal efflux, MRP1-related alteration of cellular metal localization may account for the high levels of resistance displayed by these cells. Interestingly, the ABC protein YCF1, which confers heavy metal resistance to yeast and shares strong structural similarities with MRP1, has recently been demonstrated to function as a vacuolar pump [24].

In summary, the present study supports the conclusion that overexpression of the MRP1 export pump constitutes one major molecular basis of resistance in human heavy metal-selected cells and furthermore demonstrates that MRP1 overexpression can occur in response to chronic exposure not only to anticancer drugs but also to cytotoxic heavy metals.

Acknowledgements: This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Association pour la Recherche sur le Cancer and by the Ligue Nationale contre le Cancer (Comité d'Ille et Vilaine). A.C. and L.P. are recipients of fellowships from the Association pour la Recherche sur le Cancer and from the Ligue Nationale contre le Cancer (Comité des Côtes d'Armor), respectively. We gratefully acknowledge Dr. Ford-Hutchinson for the gift of MK571.

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