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# Kinetics of glutathione and daunorubicin efflux from multidrug resistance protein overexpressing small-cell lung cancer cells

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**Abstract**

The present study examined how the multidrug resistance protein (MRP1), which is an ATP-dependent anionic conjugate transporter, also mediates the transport of reduced glutathione (GSH) and the co-transport of the cationic drug, daunorubicin, with GSH in living GLC4/Adr cells. To obtain information on the affinity of GSH for the multidrug resistance protein in GLC4/Adr cells, we investigated the GSH concentration dependence of the ATP-dependent GSH efflux. The intracellular GSH concentration was modulated by preincubation of the cells with 25 μM buthionine sulfoximine, an inhibitor of GSH synthetase, for 0–24 h. The transport of GSH was related to the intracellular GSH concentration up to ~5 mM and then plateaued. Fitting of the obtained data according to the Michaelis–Menten equation revealed a $K_m$ of 3.4 ± 1.4 mM and a $V_{max}$ of 1.5 ± 0.2 × $10^{-18}$ mol/cell/s. The ATP-dependent transport of GSH was inhibited by 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio)propanoic acid (MK571), with 50% inhibition being obtained with 1.4 μM MK571. We investigated the GSH concentration dependence of the MRP1-mediated ATP-dependent transport of daunorubicin under conditions where the transport of daunorubicin became saturated. The daunorubicin transport was related to the intracellular GSH concentration up to ~5 mM and then plateaued. We were therefore in the situation where GSH acted as an activator: its presence was necessary for the binding and transport of daunorubicin by MRP1. However, GSH was also transported by the multidrug resistance protein. The concentration of GSH that gave half the maximal rate of daunorubicin efflux was 2.1 ± 0.8 mM, very similar to the $K_m$ value obtained for GSH. In conclusion, the rate of daunorubicin efflux, under conditions where the transport of daunorubicin became saturated, and the rate of GSH efflux determined at any intracellular concentration of GSH were very similar, yielding a 1:1 stoichiometry with respect to GSH and daunorubicin transport. These results support a model in which daunorubicin is co-transported with GSH. 

*Keywords:* Multidrug resistance; MRP1 (multidrug resistance protein); Efflux; Anthracycline; Glutathione

## 1. Introduction

The MRP1-encoded multidrug resistance protein (MRP1) and the multidrug resistance-encoded resistance protein (P-glycoprotein) are both plasma membrane transporters thought to be responsible in part for the resistance of tumor cells to multiple chemically unrelated drugs (Cole et al., 1992; Bradley et al., 1998). Both proteins cause resistance to a broad spectrum of anticancer drugs such as anthracyclines, vinca alkaloids and epipodophyllotoxins (Grant et al., 1994; Zaman et al., 1994), which suggests that these drugs belong to classes of overlapping substrates. However, cMOAT/MRP2, which is the major organic anion transporter in the canalicular membrane of hepatocytes, has been shown to be overexpressed in cancer cell lines resistant to cisplatin, doxorubicin, vincristine and etoposide (Cui et al., 1999; Belinsky and Kruh, 1999; Kawabe et al., 1999). The three proteins belong to the ATP-binding cassette (ABC) superfamily, which are known to be dependent on ATP hydrolysis for the translocation of substrates across membranes (Higgins, 1992).

The ABC family of drug transporters can be divided into two major clusters: the P-glycoprotein cluster and the MRP cluster. The important difference between the two classes of proteins is the role of glutathione: the depletion of intracellular glutathione (GSH) has no effect on the P-glycoprotein-mediated transport of drugs (Versantvoort et al., 1995), but inhibits the MRP1-mediated transport of drugs (Versantvoort et al., 1995). MRP1 is an organic transporter able to transport glutathione conjugates, such as dinitrophenyl glutathione. However, it was found at an early stage that MRP1 was able to transport non-anionic

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drugs, such as anthracyclines, vinca alkaloids, epipodophyllotoxins (Zaman et al., 1995; Marbeuf-Gueye et al., 1998). Attempts to detect derivatives of these drugs conjugated to an anionic ligand (glutathione, glucuronic acid, sulphate) have remained unsuccessful and the consensus now is that these drugs are transported as such. Thus, it has been demonstrated (i) that etoposide is co-transported with GSH (Rappa et al., 1997), (ii) that unmodified vincristine is transported by MRP1 but only in the presence of physiological concentrations of GSH (Loe et al., 1996), and (iii) that MRP1-mediated daunorubicin transport is dependent on the concentration of GSH (Renes et al., 1999). However, these experiments did not answer the question of whether GSH is an activator or a co-transported substrate.

In this study, using GLC4/Adr cells overexpressing MRP1, we examined the MRP1-mediated efflux of daunorubicin as a function of the intracellular GSH concentration, as well as the MRP1-mediated export of GSH in the presence and absence of daunorubicin. Our data show an ATP-dependent efflux of GSH which does not depend on the presence of the drug ($< 10$ μM) and an ATP-dependent and GSH-dependent efflux of daunorubicin with a 1:1 stoichiometry with respect to GSH and daunorubicin transport. These results support a model in which daunorubicin is co-transported with GSH.

## 2. Materials and methods

### 2.1. Cell culture

GLC4 and MRP1-expressing GLC4/ADR cells (Zijlstra et al., 1987), as well as K562 leukemia cells and P-glycoprotein expressing K562/ADR cells (Lozzio and Lozzio, 1975) were cultured in RPMI 1640 (Sigma, St. Louis, MO) medium supplemented with 10% fetal calf serum (Biomedia, Boussens, France) at 37°C 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 the experiments. Cell cultures used for experiments were split 1:2 a day before use in order to assure logarithmic growth.

### 2.2. Drugs and chemicals

Purified doxorubicin and daunorubicin were kindly provided by Laboratoire Pharmacia–Upjohn. Concentrations were determined by diluting stock solutions to approximately $10^{-5}$ M with $\varepsilon_{480} = 11{,}500\ M^{-1}\ cm^{-1}$. Stock solutions were prepared just before use. 2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(*trans*-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide (PAK-104P) was a gift from Dr. Shudo, Dr. Iwasaki and Dr. Akiyama (Nissan Chemical Industries, Japan). 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). Triton X-100 was from Sigma and was dissolved in water. Reduced GSH, glutathione disulfide (GSSG), glutathione transferase from equine liver ($GSH_T$), L-buthionine-(*S*,*R*) sulphoximine (BSO), and avicin were from Sigma. Monochlorobimane was from Molecular Probes (Eugene, OR). Before the experiments, the cells were counted, centrifuged and resuspended in HEPES buffer solutions containing 20 mM HEPES plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ at pH = 7.3, with or without 5 mM glucose. All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments.

### 2.3. Free GSH measurement

In order to quantify free GSH, either inside the cells or the amount released in the extracellular medium, an enzymatic method was used. Several fluorescent probes have been used for GSH measurements and especially for flow cytometric GSH measurements, but in recent years, monochlorobimane has become the reagent of choice. Monochlorobimane, itself non-fluorescent, 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. A 10-mM stock solution of monochlorobimane was prepared in ethanol, and aliquots were stored at −80°C in the dark. Fig. 1(A) shows the variation of the fluorescence signal ($\lambda_{ex} = 380$, $\lambda_{em} = 480$ nm) as a function of time when 5 μM GSH was mixed with 100 μM monochlorobimane in HEPES buffer at pH 7.3 and 37°C. The non-enzymatic reaction which occurred between GSH and monochlorobimane was very slow. However, when glutathione S-transferase was added, the increase in the fluorescent signal characteristic of monochlorobimane-GSH derivative formation was very fast. According to the manufacturer, the enzymatic activity is optimum in phosphate buffer. However, as most of our experiments were performed in HEPES buffer, we used this buffer after having checked that the activity was only slightly lower than that observed in phosphate buffer. The initial rate of monochlorobimane-GSH formation was determined as the increase in the fluorescence signal between 100 and 150 s ($\Delta F$) after the addition of glutathione S-transferase to monochlorobimane plus GSH (Fig. 1(A)). The variation in the initial rate of monochlorobimane-GSH formation as a function of the glutathione S-transferase concentration was linear up to ~1 u/ml (data not shown). In the following experiments, the monochlorobimane and glutathione S-transferase concentrations were kept constant, equal to 100 μM and 0.5

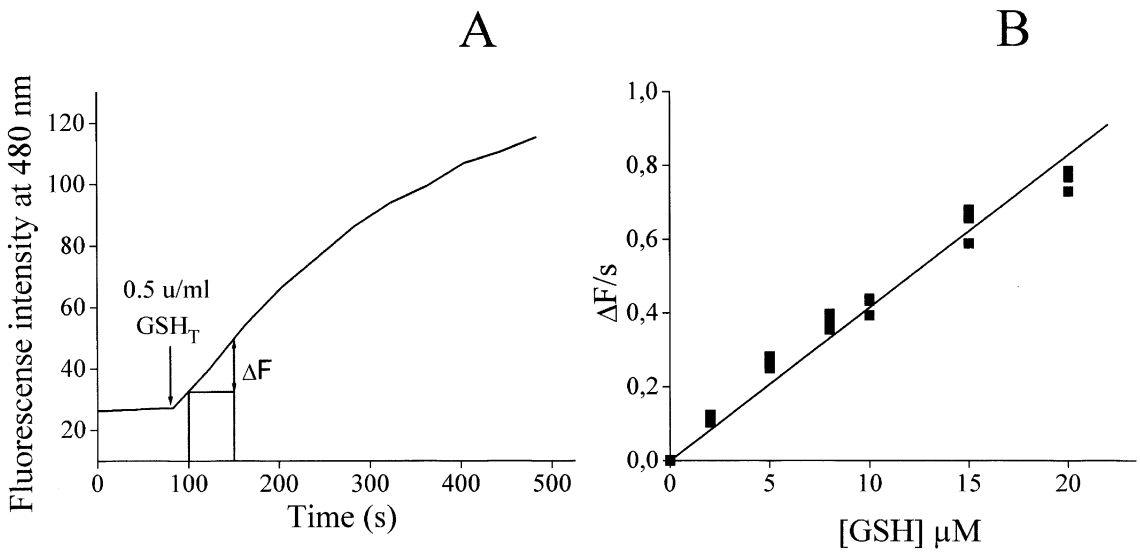


Fig. 1. Determination of the GSH-monochlorobimane adduct. (A) The change in fluorescence intensity at 480 nm was followed as a function of time when 100 μM monochlorobimane was added to 5 μM GSH, before and after the addition (arrow) of 0.5 u/ml glutathione S-transferase. $\Delta F$ is the change in fluorescence intensity over 50 s. (B) The rate of change in the fluorescence intensity has been plotted as a function of the GSH concentration added to 100 μM monochlorobimane and 0.5 u/ml glutathione S-transferase.

u/ml, respectively. Then, we checked (Fig. 1(B)) that the fluorescence signal recorded over a short time (50 s), which was used as a measure of the initial rate of monochlorobimane-GSH formation, was directly proportional to the concentration of GSH at least within the range 0–20 μM (corresponding to the concentrations expected when $10^6$ cells/ml were lysed, the intracellular GSH concentrations being within the 0–20 mM range). The curve shown in Fig. 1(B) was used as the calibration curve.

### 2.4. Intracellular GSH content

Cells, $2 \times 10^6$ suspended in 2 ml of buffer, were disrupted by sonication on ice ($3 \times 10$ s, power 2). The rate of monochlorobimane-GSH formation was monitored after the addition of monochlorobimane, 100 μM and GSH$_T$ 0.5 u/ml, as described above. In the absence of glutathione S-transferase, the rate of formation of the fluorescent derivative was very slow. We checked that GSSG did not give rise to any modification of the fluorescence signal.

### 2.5. GSH depletion

In order to examine the effect of glutathione depletion by L-buthionine sulphoximine on daunorubicin efflux, and the relation between GSH efflux and GSH content, cells were cultured in the presence of 25 μM L-buthionine-sulphoximine for various times ranging from 0 to 24.

### 2.6. GSH released by the cells

In order to distinguish between GSH released into the medium and that retained within the cells, the cells were resuspended in HEPES buffer ($10^6$/ml). At various times, 2-ml aliquots containing $2 \times 10^6$ cells were centrifuged, and the GSH concentration present in the extracellular medium, and therefore released from the cells, and the GSH concentration present in the pellet were determined. The extracellular concentration of GSH was not affected by 250 μM acivicin, indicating negligible activity of γ-glutamyltransferase in the membrane of GLC4/Adr cells.

### 2.7. Cellular anthracycline accumulation

The rationale and validation of our experimental set-up for measuring the kinetics of the active transport of anthracyclines by tumor cells has been extensively described and discussed before (Mankhetkorn et al., 1996; Frézard and Garnier-Suillerot, 1991a,b; Borrel et al., 1994a,b). It is based on the continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the decrease in the fluorescence signal of anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) after incubation with the cells in a 1-cm quartz cuvette. The decrease in fluorescence occurring during incubation with cells is due to the quenching of fluorescence after intercalation of anthracycline between the base pairs of DNA. We have previously shown that this methodology allows the accurate measurement of the free cytosolic concentration of anthracyclines under steady state conditions, their initial rates of uptake and kinetics of active efflux (Mankhetkorn et al., 1996; Frézard and Gar-

nier-Suillerot, 1991a,b; Borrel et al., 1994a,b; Pereira et al., 1994).

### 2.8. Determination of the MRP1-mediated efflux of anthracycline derivatives

Cells ($1 \times 10^6$/ml; 2 ml per cuvette) were preincubated for 30 min in HEPES buffer with sodium azide, but without glucose (energy-deprived cells). Depletion of ATP in these cells was 90%, as checked with the luciferin–luciferase test (Kimmich et al., 1975). The cells remained viable throughout the experiment, as checked with Trypan blue and calcein vital stain (not shown). After addition of anthracycline, the decrease in the signal was monitored until steady state was reached. Since the pH of the medium was chosen to equal the intracellular pH, at steady state the extracellular free drug concentration ($C_e$) was equal to the cytosolic free drug concentration ($\mathscr{C}_i$). Then glucose was added, which led to the restoration of control ATP levels within 2 min. and to an increase in the fluorescence signal due to the efflux of anthracycline. ATP-dependent anthracycline efflux was determined from the slope of the tangent of the curve $F = f(t)$, where $F$ is the fluorescence intensity at the time of addition of glucose. Since under these conditions at the moment of addition of glucose $\mathscr{C}_i = C_e$, the passive influx and efflux were equal, the net initial efflux represents the MRP1-mediated active efflux only (Mankhetkorn et al., 1996; Marbeuf-Gueye et al., 1999).

## 3. Results

### 3.1. Intracellular and extracellular GSH concentrations as a function of time

The amount of GSH released into the extracellular medium was determined as a function of the time of incubation of cells in HEPES buffer at pH 7.3 and 37°C. Intracellular GSH was determined simultaneously. Such determinations were performed for GLC4, GLC4/ADR and, as a control, for K562 and K562/ADR (data not shown) as well as in energy-deprived cells (Fig. 2). Clearly, there was an energy-dependent efflux of GSH from GLC4/ADR cells, because the small amount of GSH recovered in the extracellular medium of energy-deprived GLC4/Adr cells was the same as that observed for GLC4, K562 and K562/ADR cells and did not depend on time. The level of GSH in ATP-depleted cells was similar to that in ATP-rich cells (at least for 2 h, which is the time required to make the incubation with $N_3^-$, leading to ATP-depletion, and then GSH or daunorubicin efflux measurements). The amount of GSH measured in the extracellular medium, even at $t = 0$, was due to the release of GSH by some cells that were permeabilised during centrifugation. Using Trypan blue, we found that about 5% of the cells were permeabilised, which could account for the $0.05 \pm 0.01$ μM of GSH recovered in the extracellular medium. In all efflux experiments, net ATP-dependent transport was calculated by subtracting values at $t = 0$ from those at $t$.

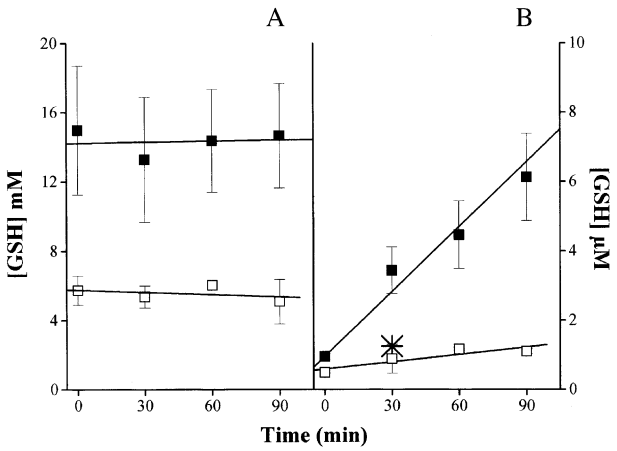


Fig. 2. Intracellular (A) and extracellular (B) GSH concentrations as a function of the time of incubation of cells in HEPES buffer. GLC4 cells (□), GLC4/ADR cells (■), energy-deprived GLC4/ADR cells (*).

### 3.2. Rate of GSH efflux from GLC4/ADR cells as a function of the intracellular GSH concentration

To obtain information on the affinity of GSH for the MRP1 protein in GLC4/ADR cells, we investigated the GSH concentration dependence of the ATP-dependent GSH efflux. The intracellular GSH concentration was modulated by preincubation of the cells with 25 μM L-buthionine-sulphoximine, an inhibitor of GSH synthetase, for 0–24 h (Table 1). Every 2 h, an aliquot of the cell preparation was taken and suspended in a GSH-free buffer for 30 min. The GSH released in the extracellular medium and those present in the cells were then determined. The relationship between intracellular GSH levels and GSH efflux is depicted in Fig. 3 (the volume of the cell was estimated as $10^{-12}$ l). The transport of GSH was related to the intracellular GSH concentration up to ~5 mM and then plateaued. Fitting of the obtained data according to the Michaelis–Menten equation revealed a $K_m$ of $3.4 \pm 1.4$ mM and a $V_{max}$ of $1.5 \pm 0.2 \times 10^{-18}$ mol/cell/s.

### 3.3. Rate of GSH efflux from GLC4/ADR cells in the presence of MK571, PAK-104P, vinblastine or daunorubicin

The amounts of GSH released into the extracellular medium in the presence of various MK571 concentrations (1–5 μM), or various PAK-104P concentrations (10–100 μM), 5 μM vinblastine or 10 μM daunorubicin were determined.

Table 1
Comparison of the rates of MRP1-mediated efflux of GSH and DNR at different intracellular GSH concentrations in GLC4/Adr cells

| $[GSH]_i^a$ | 2.5 mM | 5 mM | 6.1 mM | 7.7 mM | 10 mM |
|---|---|---|---|---|---|
| $\Delta t(h)$ | 7 | 4 | 3 | 2 | 0 |
| $V_{GSH}$ ($10^{-18}$ mol/cell/s) | 0.61 ± 0.06 | 0.88 ± 0.08 | 0.96 ± 0.09 | 1.05 ± 0.10 | 1.12 ± 0.11 |
| $V_{DNR}^b$ ($10^{-18}$ mol/cell/s) | 0.65 ± 0.06 | 0.80 ± 0.08 | 0.96 ± 0.10 | 1.03 ± 0.1 | 1.06 ± 0.1 |
| $V_{GSH}/V_{DNR}$ | 0.94 ± 0.19 | 1.10 ± 0.2 | 1.00 ± 0.2 | 1.02 ± 0.2 | 1.06 ± 0.21 |

[a]The intracellular GSH concentration was modulated by incubation of the cells with 25 μM L-buthionine-sulphoximine, an inhibitor of GSH synthetase, for 0–24 h.

[b]Cells, $10^6$/ml were incubated with DNR 8 μM and the rate of daunorubicin efflux was determined as described under Section 2. The values represent means ± SD of triplicate determinations.

A dose-dependent inhibition of GSH efflux was observed with MK571, with a half-maximum inhibition at 1.4 ± 0.3 μM (data not shown). Because MK571 is a specific inhibitor of MRP1, this showed that the kinetic data obtained were MRP1-specific. It has recently been shown that PAK-104P is able to inhibit the MRP1-mediated efflux of several compounds (Sumizawa et al., 1997; Marbeuf-Gueye et al., 2000). A dose-dependent inhibition of GSH efflux was observed with PAK-104P, with a half-maximum inhibition at 25 ± 5 μM (data not shown). Actually, we observed that PAK-104P also modulated the GSH intracellular level: at low PAK-104P concentrations, the GSH level was not affected, but at high PAK-104P concentrations (> 50 μM) a slight decrease in the intracellular GSH concentration was observed after a 30-min incubation. However, under our experimental conditions, this could not account for the decrease in GSH efflux when the intracellular GSH concentration was higher than 6 mM.

The rate of GSH efflux (at ~12 mM intracellular concentration) was not affected by either daunorubicin or vinblastine at the concentrations used.

### 3.4. Rate of daunorubicin efflux from GLC4 / ADR cells as a function of the GSH intracellular concentration

We investigated the GSH concentration dependence of MRP1-mediated ATP-dependent daunorubicin transport. Cells were split into two batches. L-buthionine-sulphoximine, 25 μM, was added to batch 1, whereas batch 2 was not treated. After specified time intervals, ranging from 2 to 24 h, aliquots from both batches were withdrawn, and the ability of cells to efflux daunorubicin and their GSH content were determined. We checked that, after short incubations, 25 μM buthionine sulphoximine was unable to inhibit daunorubicin transport. A typical example of an experiment aimed at determining the rate of daunorubicin efflux is shown in Fig. 4. Two sets of experiments were performed with 1 and 8 μM daunorubicin, respectively. Previously, we have shown that, under the experimental conditions used for daunorubicin transport measurement in GLC4/ADR cells, daunorubicin transport is saturated at concentrations (in the medium) higher than 7 μM (Marbeuf-Gueye et al., 1998). The relationship between intracellular GSH levels and daunorubicin efflux is depicted in Fig. 5. For both drug concentrations, daunoru-

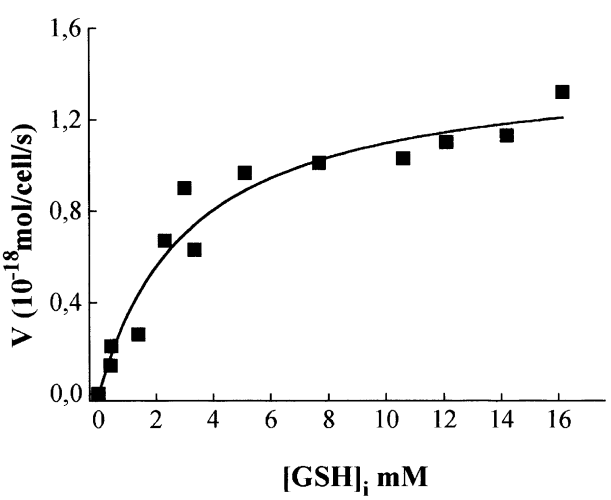


Fig. 3. Kinetics of the MRP1-mediated efflux of GSH, plotted as a function of the intracellular GSH concentration. $V$ and $[GSH]_i$ were determined as described. Data points are from a representative experiment. The data were fitted using the equation $V = V_{max}[GSH]_i/([GSH]_i + K_m)$.

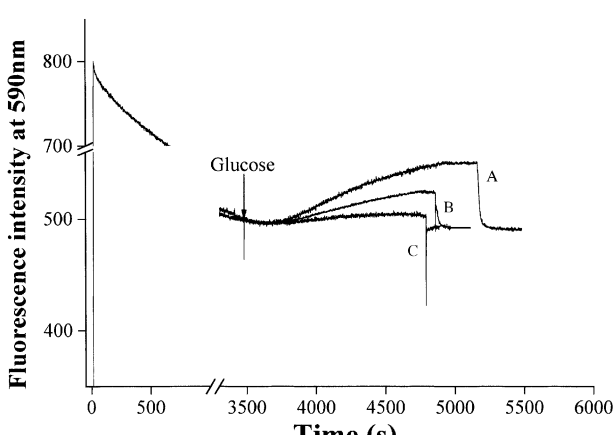


Fig. 4. Incorporation of daunorubicin (1 μM) in GLC4/ADR cells after ATP depletion with azide and determination of the active efflux rate ($V_a$) after restoration of ATP synthesis by the addition of glucose. The cells were without L-buthionine sulphoximine (A), or incubated with 25 μM L-buthionine sulphoximine for 6 h (B), 24 h (C). The intracellular GSH concentration was 12 mM (A), 2 mM (B), 0.2 mM (C).

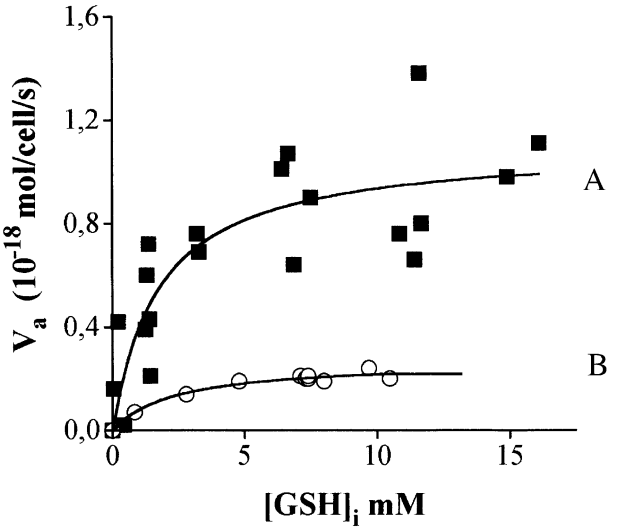


Fig. 5. Kinetics of the MRP1-mediated efflux of daunorubicin plotted as a function of the intracellular GSH concentration. $V_a$ and $[GSH]_i$ were determined as described. The daunorubicin concentration was 8 μM (A, ■) or 1 μM (B, ○) Data points are from three to five independent experiments performed on different days. The data were fitted using Eq. (1).

bicin transport was related to the intracellular GSH concentration and then plateaued.

The MRP1-mediated efflux of non-negatively charged drugs requires the presence of GSH. We are therefore in a situation where GSH acts as an activator: its presence is necessary for the binding and transport of daunorubicin by MRP1. However, GSH is also transported by MRP1.

The rate $V$ of daunorubicin transport should follow the equation

$$V = (V_M)_A[A]/(K_A + [A]) \quad (1)$$

where $[A]$ is the intracellular GSH concentration, $V_M$ is the maximum rate of daunorubicin efflux in the presence of an excess of intracellular GSH, $K_A$ is the concentration of GSH that gives half the maximal rate of daunorubicin efflux and should be equal to the affinity constant of GSH for the transporter. Fitting of the obtained data for 1 μM daunorubicin, according to Eq. (1), yielded $K_A = 2.3 \pm 0.6$ mM and $(V_M)_A = 0.27 \pm 0.2 \times 10^{-18}$ mol/cell/s. When the daunorubicin concentration was 8 μM, $K_A = 1.6 \pm 0.8$ mM and $(V_M)_A = 1.2 \pm 0.2 \times 10^{-18}$ mol/cell/s. For the two daunorubicin concentrations used, the $K_A$ and $K_m$ values determined for GSH transport were comparable.

Table 1 shows the rate of daunorubicin efflux, under conditions in which the transport of the drug was saturated, and the rate of GSH efflux. These rates were determined at various intracellular GSH concentrations obtained by incubation of the cells with L-buthionine sulphoximine, as explained in Section 2. As can be seen, at any intracellular GSH concentration, the rates of GSH and daunorubicin efflux were very similar, which strongly suggests that the stoichiometry between GSH and daunorubicin transport is 1:1. It must be noticed that in the case of a subsaturating concentration of daunorubicin, the ratio of the rate of GSH efflux to the rate of daunorubicin efflux was higher than 1 (for instance, it was about 5 in the case of Fig. 5, curve b, when 1 μM daunorubicin was used).

## 4. Discussion

The purpose of this study was to get some insight into the mechanism by which MRP1 mediates the ATP-dependent transport of the cationic drug daunorubicin. For this purpose, using living GLC4/ADR cells, we studied the MRP1-mediated efflux of GSH, measured the efflux of daunorubicin as a function of the intracellular GSH concentration and determined the stoichiometry of the GSH/daunorubicin efflux. According to previous reports, our data show that MRP1 co-transports GSH and daunorubicin. In addition, we demonstrate that the stoichiometry of this transport is 1:1.

It has been said that GSH is an MRP1 substrate. However, reports are contradictory. Several attempts failed to demonstrate MRP1-mediated, as well as cMOAT-mediated [$^3$H]GSH transport in membrane vesicles. This was most likely due of the low affinity of GSH for the transporter, which led to relatively low transport rates at the relatively low concentrations (μM) used (Paulusma et al., 1999; Leier et al., 1996). Based on these data, it was concluded that GSH is not transported by MRP1 (Leier et al., 1996). However, using living cells, in which the intracellular GSH concentration is high (mM), an increase in GSH excretion from non-small-cell lung carcinoma cells transfected with MRP1 cDNA (Zaman et al., 1995), from GLC4/ADR cells (Versantvoort et al., 1995; this study), and from polarized MDCKII cells stably expressing the human cMOAT or human MRP1 (Paulusma et al., 1999) has been observed. These data strongly suggest that MRP1, as well as cMOAT, mediate the low-affinity transport of GSH and that this transport is ATP-dependent. Our results, showing an ATP-dependent transport of GSH by MRP1 with low affinity ($K_m \sim 4$ mM), are in line with these data.

Several attempts have been made to connect intracellular GSH concentration and GSH efflux. Actually, in some cell lines, overexpression of MRP1 is associated with a significant (two- to six-fold) decrease in intracellular GSH levels (Lautier et al., 1996; Schneider et al., 1995). However, in other cell lines, such as the GLC4 cell line used in this study, just the opposite is observed (Versantvoort et al., 1995; this study). Therefore, comparison of the intracellular GSH concentration of resistant cells with that of sensitive cells cannot provide additional support for either the MRP1-mediated transport of GSH alone or the MRP1-mediated co-transport of GSH with drug or with some currently unknown cellular metabolite.

Let us now consider the MRP1-mediated transport of other molecules and especially that of drugs such as anthracyclines, vinca alkaloids and etoposide. MRP1 has been demonstrated to be the main active transporter of structurally diverse conjugated organic anions. In contrast, it has not been possible to detect the direct active transport of chemotherapeutic agents by MRP1-enriched membrane vesicles under similar conditions. (Loe et al., 1996, 1998; Leier et al., 1996). However, the MRP1-dependent active transport of certain unconjugated xenobiotics such as vincristine, daunorubicin and aflatoxin $B_1$ is observed if vesicle preparations are supplemented with physiological concentrations of GSH, which strongly suggests that MRP1 actively co-transports some unmodified substrate with GSH (Renes et al., 1999; Loe et al., 1998). Rappa et al. (1997) have provided evidence that MRP1 exports GSH physiologically, presumably in association with an endogenous compound(s), that baseline MRP1 expression protects cells from the toxic effects of xenobiotics by effluxing the xenobiotics and GSH by a co-transport mechanism, and that disruption of the gene encoding MRP prevents the co-transport of xenobiotics and GSH. In addition, Renes et al. (1999) have shown that MRP1-mediated daunorubicin transport rates are dependent on the concentration of GSH, being maximal at concentrations equal or higher than 10 mM; the apparent $K_m$ value for GSH was 2.7 mM. Previous studies have already established that drug transport in MRP1-overexpressing tumor cells can be regulated by the intracellular GSH level: depletion of cellular GSH levels, in MRP1-overexpressing cell lines, following exposure of the cells to L-buthionine-sulphoximine, results in an increase in drug accumulation (Versantvoort et al., 1995; Zaman et al., 1995; Marbeuf-Gueye et al., 1998). However, up to now, the role of GSH on the MRP1-mediated transport of drugs in living cells has not been quantitatively addressed. Our data clearly corroborates the dependence on the intracellular GSH concentration of the MRP1-mediated efflux of daunorubicin, showing in addition (i) that the efflux of daunorubicin parallels that of GSH, and that (ii) under conditions where the drug transport is saturated, there is a 1:1 stoichiometry with respect to GSH and daunorubicin transport.

Let us now consider the modulation of GSH transport by drugs. It has been shown that vincristine stimulates the ATP-dependent transport of GSH in a concentration-dependent fashion. In the absence of vincristine, no ATP-dependent GSH transport was detected (Leier et al., 1996; Loe et al., 1998). These authors suggest that there is a 1:1 stoichiometry with respect to GSH and vincristine transport; however, as they emphasised, this was based on determinations at only one concentration of vincristine and GSH. Nevertheless, their data indicate that the transport rates of both compounds are not vastly different, and that a true co-transport mechanism may be involved. Surprisingly, the stimulation was observed at high vincristine concentrations only (10–100 μM), whereas the Michaelis constant for vincristine transport by MRP1 is close to 1 μM. Our data and a previous report (Versantvoort et al., 1995) show that MRP1 transports GSH independently of daunorubicin and of vinblastine as well (at least at the low concentrations that can be used when working with living cells). It should be noticed that a similar result was obtained for cMOAT by Van Aubel et al. (1999), they observed that the initial uptake rate of GSH at 0.1 or 5 mM into Sf9-MRP2 membranes vesicles was not affected by either 0.2, 10 μM or 0.1 mM vinblastine and concluded that MRP2 transported GSH independently of vinblastine. However, we observed that the transport of GSH was inhibited by the LTD4 receptor antagonist MK571, with 50% inhibition being obtained with 1.4 μM. This value is comparable to that obtained for the inhibition of the MRP1-mediated transport of S-(2,4-dinitrophenyl)glutathione by MK572 ($K_i = 1$ μM) (Leier et al., 1996). Also PAK-104P, which can be used at higher concentrations than daunorubicin, is able to inhibit the MRP1-mediated efflux of GSH. Recently, we have shown that PAK-104P is very potent in inhibiting the efflux of non-negatively charged molecules such as daunorubicin, pirarubicin, and calcein acetoxymethyl ester, with a $K_i = 0.05$ μM (Marbeuf-Gueye et al., 2000). However, its ability to inhibit the efflux of negatively charged molecules such as calcein (Marbeuf-Gueye et al., 2000), leukotriene $C_4$ (Sumizawa et al., 1997) and GSH (this work) is about 30 times lower. These data suggest the existence of two different mechanisms for the inhibition by PAK-104P of the MRP1-mediated efflux of molecules: a first mechanism, involving a low-affinity site for PAK-104P, and which concerns negatively charged molecules such as calcein, leukotriene $C_4$, and GSH, and a second mechanism involving a high-affinity site for PAK-104P and which concerns non-negatively charged molecules such as anthracyclines, calcein acetoxymethyl ester.

Recent data concerning the ATPase activity of MRP1 and its relation with the transport of molecules are also conflicting. Mao et al. (1999) have shown that the ATPase activity of purified MRP1 and MRP1 reconstituted into proteoliposomes is stimulated by the organic anion substrates, leukotriene $C_4$ and 17β-estradiol, by glutathione disulfide but not by reduced glutathione or unconjugated chemotherapeutic agents. However, Hooijberg et al. (2000), using MRP1 in natural membranes, have demonstrated a dose-dependent (up to 5 mM) stimulation of its ATPase activity by GSH. Vincristine and daunorubicin do not induce MRP1 ATPase activity, and the effect of GSH on MRP1 ATPase activity is not increased by daunorubicin or by vincristine. These data are in agreement with the observation that daunorubicin does not perturb GSH efflux (Versantvoort et al., 1995; this work).

We conclude from our data that the MRP1-mediated transport of daunorubicin requires the presence of GSH, with daunorubicin/GSH 1:1 co-transportation. However, the MRP1-mediated transport of GSH does not seem to be

dependent on the presence of daunorubicin. It should also be emphasised that research into molecule transport in intact cells is bound to provide many valuable insights into mechanisms and kinetics in vivo, and that methods using intact cells and vesicles are complementary.

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## References

Belinsky, M.G., Kruh, G.G., 1999. MOAT-E (ARA) is a full-length MRP cMOAT subfamily transporter expressed in kidney and liver. Br. J. Cancer 80, 1342–1349.

Borrel, M.-N., Pereira, E., Fiallo, M., Garnier-Suillerot, A., 1994a. Mobile ionophores are a novel class of P-glycoprotein inhibitors. The effects of ionophores on 4′−O−tetrahydropyranyl-adriamycin incorporation in K562 drug-resistant cells. Eur. J. Biochem. 223, 125–133.

Borrel, M.-N., Fiallo, M., Priebe, W., Garnier-Suillerot, A., 1994b. P-glycoprotein mediated efflux of hydroxyrubicin, a neutral anthracycline derivative, in resistant K562 cells. FEBS Lett. 356, 287–290.

Bradley, G., Juranka, P.F., Ling, V., 1998. Mechanism of multidrug resistance. Biochim. Biophys. Acta 948, 87–128.

Cole, S.P.C., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M.W., Deeley, R.G., 1992. Overexpression of a novel transporter gene in a multidrug resistant human lung cancer cell line. Science 258, 1650–1654.

Cui, Y.H., Konig, J., Buchholz, U., Spring, H., Leier, I., Keppler, D., 1999. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol. Pharmacol. 55, 929–937.

Fernandez-Checa, J.C., Kaplowitz, N., 1990. The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal. Biochem. 190, 212–219.

Frézard, F., Garnier-Suillerot, A., 1991a. Determination of the osmotic active drug concentration in the cytoplasm of anthracycline-resistant and-sensitive K562 cells. Biochim. Biophys. Acta 1091, 29–35.

Frézard, F., Garnier-Suillerot, A., 1991b. Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. Eur. J. Biochem. 196, 483–491.

Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K.C., Cole, S.P.C., Deeley, R.G., 1994. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res. 54, 357–361.

Higgins, C.F., 1992. ABC transporters—from microorganisms to man. Annu. Rev. Cell Biol. 8, 67–113.

Hooijberg, J.H., Pinedo, H.M., Vrasdonk, C., Priebe, W., Lankelma, J., Broxterman, H.J., 2000. The effect of glutathione on the ATPase activity of MRP1 in its natural membranes. FEBS Lett. 469, 47–51.

Kawabe, T., Chen, Z.C., Wada, M., Uchiumi, T., Ono, M., Akiyama, S., Kuwano, M., 1999. Enhanced transport of anticancer agents and leukotriene C-4 by the human canalicular multispecific organic anion transporter (CMOAT/MRP2). FEBS Lett. 456, 327–331.

Kimmich, G., Randles, J., Brand, J., 1975. Assay of picomole amounts of ATP, ADP and AMP using the luciferase enzyme system. Anal. Biochem. 69, 187–192.

Lautier, D., Canitrot, Y., Deeley, R.G., Cole, S.P.C., 1996. Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. Biochem. Pharmacol. 52, 967–977.

Leier, I., Jedlitschky, G., Buchholtz, U., Center, M., Cole, S.P.C., Deeley, R.G., Keppler, D., 1996. ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. Biochem. J. 314, 433–437.

Loe, D.W., Almquist, K.C., Deeley, R.G., Cole, S.P.C., 1996. Multidrug resistance protein (MRP)-mediated transport of leukotriene $C_4$ and chemotherapeutic agents in membrane vesicles—demonstration of glutathione-dependent vincristine transport. J. Biol. Chem. 271, 9675–9682.

Loe, D.W., Deeley, R.G., Cole, S.P.C., 1998. Characterization of vincristine transport by the M-r 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res. 58, 5130–5136.

Lozzio, C.B., Lozzio, B.B., 1975. Human chronic myelogeneous leukemia cell line positive Philadelphia chromosome. Blood 45, 321–334.

Mankhetkorn, S., Dubru, F., Hesschenbrouck, J., Fiallo, M., Garnier-Suillerot, A., 1996. Relation among the resistance factor, kinetics of uptake, and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin in multidrug-resistant K562 cells. Mol. Pharmacol. 49, 532–539.

Mao, Q., Leslie, E.M., Deeley, R.G., Cole, S.P.C., 1999. ATPase activity of purified and reconstituted multidrug resistance protein MRP1 from drug-selected H69AR cells. Biochim. Biophys. Acta 1461, 69–82.

Marbeuf-Gueye, C., Broxterman, H.J., Dubru, F., Priebe, W., Garnier-Suillerot, A., 1998. Kinetics of anthracycline efflux from multidrug resistance protein-expressing cancer cells compared with P-glycoprotein-expressing cancer cells. Mol. Pharamacol. 53, 141–147.

Marbeuf-Gueye, C., Ettori, E., Priebe, W., Kozlowski, H., Garnier-Suillerot, A., 1999. Correlation between the kinetics of anthracycline uptake aand the resistance factor in cancer cells expressing the multidrug resistance protein or the P-glycoprotein. Biochim. Biophys. Acta 1450, 374–384.

Marbeuf-Gueye, C., Salerno, M., Quidu, P., Garnier-Suillerot, A., 2000. Inhibition of the P-glycoprotein- and multidrug resistance protein-mediated efflux of anthracyclines and calceinacetoxymethyl ester by PAK-104P. Eur. J. Pharmacol. 391, 207–216.

Paulusma, C.C., van Geer, M.A., Evers, R., Heijn, M., Ottenhoff, R., Borst, P., Oude Elferink, R.P.J., 1999. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. Biochem. J. 338, 393–401.

Pereira, E., Borrel, M.-N., Fiallo, M., Garnier-Suillerot, A., 1994. Noncompetitive inhibition of P-glycoprotein-associated efflux of THP-adriamycin by verapamil in living K562 leukemia cells. Biochim. Biophys. Acta 1225, 209–216.

Rappa, G., Lorico, A., Flavell, R.A., Sartorelli, A.C., 1997. Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural produce toxins. Cancer Res. 57, 15232–15237.

Renes, J., De Vries, E.G.E., Nuenhuis, E.F., Jansen, P.L.M., Müller, M., 1999. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. Br. J. Pharmacol. 126, 681–688.

Schneider, E., Yamazaki, H., Sinha, B.K., Cowan, K.H., 1995. Buthionine sulphoximine-mediated sensitisation of etopoxide-resistant human breast cancer MCF7 cells overexpressing the multidrug resistance-associated protein involves increased drug accumulation. Br. J. Cancer 71, 738–743.

Sumizawa, T., Chen, Z.S., Chuman, Y., Seto, K., Furukawa, T., Haraguchi, M., Tani, A., Shudo, N., Akiyama, A.I., 1997. Reversal of multidrug-associated protein-mediated drug resistance by the pyridine analog PAK-104P. Mol. Pharmacol. 51, 399–405.

Van Aubel, R.A.M.H., Koenderink, J.B., Peters, J.G.P., Van Os, C.H., Russel, F.G.M., 1999. Mechanisms and interaction of vinblastine and

reduced glutathione transport in membrane vesicles by the rabbit multidrug resistance protein MRP2 expressed in insect cells. Mol. Pharmacol. 56, 714–719.

Versantvoort, C.H.M., Broxterman, H.J., Bagrij, T., Scheper, R.J., Twentyman, P., 1995. Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. Br. J. Cancer 72, 82–89.

Zaman, G.J.R., Flens, M.J., Van Leusden, M.R., De Haas, M., Mülder, H.S., Lankelma, J., Pinedo, H.M., Scheper, R.J., Baas, F., Broxterman, H.J., Borst, P., 1994. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc. Natl. Acad. Sci. U. S. A. 91, 8822–8826.

Zaman, G.J.R., Lankelma, J., Van Tellingen, O., Beijnen, J., Dekker, H., Paulusma, C., Oude Elferink, R.J.P., Baas, F., Borst, P., 1995. Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. Proc. Natl. Acad. Sci. U. S. A. 92, 7690–7694.

Zijlstra, J.G., De Vries, E.G.E., Mulder, N.H., 1987. Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res. 47, 1780–1784.