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Kinetics of Anthracycline Efflux from Multidrug Resistance Protein-Expressing Cancer Cells Compared with P-Glycoprotein-Expressing Cancer Cells

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ABSTRACT

The multidrug resistance protein (MRP) has been shown to mediate ATP-dependent efflux of anticancer agents of diverse structure, such as daunorubicin (DNR), vincristine and etoposide. Thus, this protein does confer a multidrug resistant phenotype to cancer cells, similar to P-glycoprotein (Pgp). The substrate specificity of both transporter proteins is partly overlapping but is otherwise very distinct; because MRP is a multiple organic anion transporter, it transports certain glutathione conjugates and may be partly dependent on intracellular glutathione levels for the transport of anthracyclines. We have studied the transport kinetics of a series of anthracyclines in MRP and Pgp that overexpress tumor cell lines to obtain information on the substrate specificity of these proteins. The anthracyclines have modifications in the sugar moiety. The mean active efflux coefficient k_a, used to characterize the efficiency of the active efflux, was very similar for DNR and one of its 4′-deoxy- derivatives (eso-DNR) for MRP and Pgp [10–20 $\times$ 10^{-10}/sec/(cells/ml)]. The permanently neutral derivatives 3′-deamino-3′-hydroxy-doxorubicin (OH-DOX) and 3′-deamino-3′-hydroxy-daunorubicin (OH-DNR) were effluxed by both proteins but had a lower k_a [2 $\times$ 10^{-10} and 6 $\times$ 10^{-10}/sec/(cells/ml) (OH-DOX) and 2 $\times$ 10^{-10} and 5 $\times$ 10^{-10}/sec/(cells/ml) (OH-DNR)] for MRP and Pgp. Two anthracyclines, the doxorubicin derivative pirarubicin and 2′-bromo-4′-epi-DNR seemed to have a slightly higher k_a value for Pgp than for MRP. The apparent Michaelis-Menten constants (K_m) and maximal efflux rates (V_M) for the active transport were within a narrow range for both transporters, except for OH-DOX and OH-DNR, which had a lower V_M in the case of MRP-mediated transport, suggesting a role of the amino group in the interaction with glutathione. Determination of the Hill coefficient (n_H) of the MRP-mediated efflux gave most values close to 2, which suggests cooperativity of the transport of anthracyclines as reported before for Pgp. In conclusion, the transport kinetics of anthracyclines by MRP and Pgp are very similar.

MDR is a form of resistance to natural product derived anticancer agents, such as the anthracyclines, vinca alkaloids, and epipodophyllotoxins, characterized by an increased ATP-dependent efflux of the cytotoxic agent over the cellular plasma membrane. Two plasma membrane drug transporter proteins, Pgp and MRP, have been cloned and shown by transfection to induce the MDR phenotype to tumor cells (Bradley *et al.*, 1988; Broxterman *et al.*, 1995a; Loe *et al.*, 1996a). Both proteins create a concentration gradient of the anthracycline DNR over the cellular plasma membrane (Zaman *et al.*, 1994; Frézard and Garnier-Suillerot, 1991a, 1991b), resulting in a lowered intracellular drug concentration. Thus MDR produced by overexpression of MRP seems to be phenotypically similar to that caused by Pgp. However, major differences exist with regard to the substrates handled by these proteins. Although the chemical requirements for a compound to be a substrate for Pgp are not clear (Pearce *et al.*, 1989), its substrate specificity seems to be restricted to neutral or cationic molecules. In contrast, MRP has been identified as a transporter of organic anions, such as the glutathione conjugates leukotriene C_4 (Loe *et al.*, 1996a; Leier *et al.*, 1994), dinitrophenylglutathione (Heijn *et al.*, 1997), and the fluorescent dye calcein (Feller *et al.*, 1995). In addition, the MRP-mediated efflux of these anionic species, as well as the classical MDR drugs, but not the Pgp-mediated drug efflux, is inhibited by drugs known to affect organic anion transport, like probenecid (Feller *et al.*, 1995) or the anionic quinoline derivative MK571 (Gekeler *et al.*, 1995). A further distinction between the transport properties of both

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ABBREVIATIONS: MDR, multidrug resistance; Pgp, P-glycoprotein; MRP, multidrug resistance protein; DNR, daunorubicin; DOX, doxorubicin; Br-DNR, 2′-bromo-4′-epi-daunorubicin; PIRA, pirarubicin; eso-DNR, 4′-deoxy-daunorubicin; OH-DOX, 3′-deamino-3′-hydroxy-doxorubicin; OH-DNR, 3′-deamino-3′-hydroxydaunorubicin; C_e, extracellular free drug concentration; C_i, cytosolic free drug concentration; V_a, transport velocity.

proteins is that the efflux of the typical MDR drugs, such as DNR by MRP, seems to be dependent on the intracellular glutathione levels (Versantvoort *et al.*, 1995). This was concluded from the demonstration that MRP-mediated cellular DNR (Versantvoort *et al.*, 1995) or epirubicin (Davey *et al.*, 1995) efflux is inhibited by lowering the cellular glutathione levels with DL-buthionine-(*S,R*)-sulfoximine, which can be reversed by repletion of cellular glutathione levels by treatment of the cells with glutathione ethyl ester (Versantvoort *et al.*, 1995). These and other data (Loe *et al.*, 1996b) have led to the speculation that MRP might be identical to the glutathione-conjugate (or GS-X) pump and would export drugs such as the anthracyclines only after they were chemically transformed to negatively charged glutathione conjugates (Ishikawa *et al.*, 1995). The rapid efflux of drugs such as DNR (Versantvoort *et al.*, 1994) with no detectable concomitant increase in glutathione efflux (Versantvoort *et al.*, 1995), as well as the demonstration of ATP-dependent uptake of DNR or etoposide in MRP-expressing inside-out vesicles (Paul *et al.*, 1996), and the inhibition of MRP-mediated dinitrophenyl-glutathione transport by anthracyclines in such a system (Heijn *et al.*, 1997) have, however, challenged the hypothesis that MRP would exclusively transport the involved cytostatic drugs after metabolic conversion to conjugates (Broxterman *et al.*, 1996).

Previous studies by our group have been directed at the question of the substrate specificity of Pgp-mediated drug efflux to contribute to a rational drug design for the treatment of drug resistant tumors (Mankhetkorn *et al.*, 1996; Garnier-Suillerot, 1995; Priebe, 1995). With that purpose, we studied the ability of various chemical classes of molecules to be recognized by Pgp (Borrel *et al.*, 1995; Borrel *et al.*, 1994a) and the kinetics of Pgp-mediated efflux of a series of anthracyclines (Frézard and Garnier-Suillerot, 1991b; Mankhetkorn *et al.*, 1996; Pereira *et al.*, 1994; Borrel *et al.*, 1994b). In the present work, we have systematically measured efflux parameters of DNR and three of its sugar-modified analogs and two DOX analogs with different pK_a values and lipophilicity in MRP-overexpressing small cell lung cancer cells and compared those with Pgp-mediated efflux parameters. Our data show that despite the different substrate profile of Pgp and MRP, the efflux properties of these anthracyclines are very similar.

Materials and Methods

Cell culture

K562 leukemia cells and the Pgp-expressing K562/ADR cells (Mankhetkorn *et al.*, 1996), as well as GLC_4 and the MRP-expressing GLC_4/ADR cells (Zijlstra *et al.*, 1987), were cultured in RPMI 1640 (Sigma Chemical, St. Louis, MO) medium supplemented with 10% fetal calf serum (Biomedia, Boussens, France) at 37° in a humidified incubator with 5% CO_2. The resistant K562/ADR and GLC_4/ADR cells were cultured with 400 nM or 1.2 μM DOX, respectively, until 1–4 wk before experiments. Cell cultures used for experiments were split 1:2 1 day before use to assure logarithmic growth.

Drugs and chemicals. DNR and PIRA were kindly provided by Roger Bellon laboratory. OH-DOX, OH-DNR, eso-DNR, and Br-DNR were provided by author W.P. (Priebe, 1995). Stock solutions were prepared in water just before use. Concentrations were determined by diluting stock solutions in water to approximately 10 μM and using ϵ_{480} = 11,500/M/cm. Experiments were performed in HEPES/Na buffer solutions containing 20 mM HEPES plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.25, in the presence of 10 mM sodium azide. To synthesize ATP via the glycolysis, 5 mM glucose was added at the start of efflux experiments (see below).

Cellular drug accumulation. The rationale and validation of our experimental set-up for measuring the kinetics of active transport of anthracyclines from tumor cells has been extensively described and discussed before (Frézard and Garnier-Suillerot, 1991a, 1991b; Mankhetkorn *et al.*, 1996; Borrel *et al.*, 1995; Borrel *et al.*, 1994a, 1994b; Pereira *et al.*, 1994). It is based on a continuous spectrofluorometric monitoring (Perkin Elmer LS50B spectrofluorometer) of the decrease of the fluorescence signal of the anthracycline at 590 nm (λ_{ex} = 480 nm) after incubation with cells in a 1-cm quartz cuvette. The decrease of fluorescence that occurred during incubation with cells is caused by quenching of the fluorescence after intercalation of anthracycline between the base-pairs of DNA. We have previously shown that this method allows us to measure accurately the free cytosolic concentration of anthracyclines in steady state, their initial rates of uptake, and kinetics of active efflux (Frézard and Garnier-Suillerot, 1991a, 1991b; Mankhetkorn *et al.*, 1996; Borrel *et al.*, 1995; Borrel *et al.*, 1994a, 1994b; Pereira *et al.*, 1994).

Determination of the MRP or Pgp-mediated efflux of anthracycline derivatives. Cells (1×10^6/ml; 2 ml per cuvette) are preincubated for 30 min in HEPES buffer with sodium azide but without glucose. 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. After addition of anthracyclines, the decrease of the signal is followed until steady state is reached. Because the pH of the medium is chosen to equal the intracellular pH, at steady state the C_e is equal to the C_i. Then glucose is added, which leads to restoration of control ATP levels within 2 min and increase of the fluorescence signal because of the efflux of anthracycline. The ATP-dependent anthracycline efflux is 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. Under these conditions, at the moment glucose is added, $C_i = C_e$ and the passive influx and efflux are equal; therefore, the net initial efflux represents the MRP or Pgp-mediated active efflux only. Similar experiments were performed with the drug-sensitive cell lines and no efflux was measured (Fig. 2).

Mathematical calculations. The maximal efflux rate (V_M), apparent Michaelis-Menten constant (K_m) and cooperativity constant (n_H) for the transport of anthracyclines were computed by nonlinear regression analysis of V_a versus C_i data using the MacCurveFit program and assuming that the transport follows the Hill equation (Hill, 1985):

$$V_a = V_M \cdot C_i^{nH}/(K_m^{nH} + C_i^{nH}) \quad (1)$$

where n_H, the Hill coefficient, represents the cooperativity constant.

To characterize the efflux, earlier, when we did not yet know that the transport of anthracyclines was cooperative (Frézard and Garnier-Suillerot, 1991b; Mankhetkorn *et al.*, 1996; Spoelstra *et al.*, 1992) we have defined a mean active efflux coefficient (k_a) according to the equation:

$$V_a = k_a \cdot n \cdot C_i \quad (2)$$

where n is the number of cells per ml.

A relation between k_a and the parameters V_M, K_m, and n_H can be obtained from the derivation of eq. 1:

$$dV_a/dC_i = (V_a \cdot {}^{nH} \cdot K_m^{nH})/C_i(C_i^{nH} + K_m^{nH}) \quad (3)$$

When $n_H \cdot K_m^{nH}/(C_i^{nH} + K_m^{nH}) = 1$ or alternatively when

$$C_i = K_m \cdot (n_H - 1)^{1/n_H} \quad (4)$$

eq. 1 [which can be written as $V_a/C_i = V_m \cdot C_i^{nH-1}/(K_m^{nH} + C_i^{nH})$] becomes

$$V_a/C_i = (V_M/n_H \cdot K_m) \cdot (n_H - 1)^{(1-1/n_H)}$$

$$\text{or } k_a \cdot n = (V_M/n_H \cdot K_m) \cdot (n_H - 1)^{(1-1/n_H)} \quad (5)$$

When $n_H = 2$, it follows that $k_a \cdot n = V_M/2\ K_m$; in other words, $k_a \cdot n$ is equal to the slope of the tangent to the curve $V_a = f(C_i)$ when $C_i = K_m$. Thus k_a was calculated using eq. 5 and the computed values V_M, K_m, and n_H.

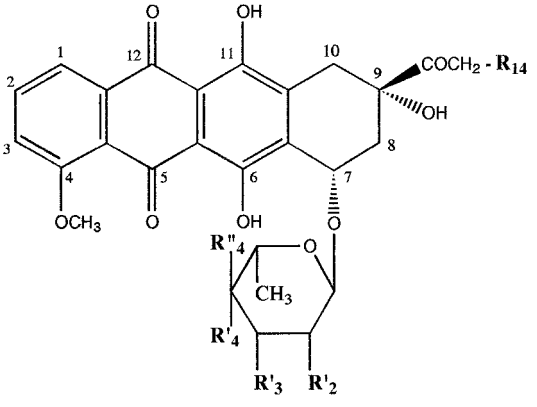
Hydrophobicity of the anthracycline derivatives. An estimation of the hydrophobicity of a compound is given by log*P*, where P is the partition coefficient in an *n*-octanol/water system. In a first approximation, the log*P* value of a compound can be estimated by adding the *fi* values of its fragments (Rekker, 1977). We have determined the variation of this calculated log*P* (c log*P*) of tested compounds using DNR as a reference compound and the equation c log*P* = log P_0 +Σ*fi*, where P_0 is the partition coefficient for DNR in *n*-octanol/water and *fi* is the *n*-octanol/water fragmental constant of the fragments of a compound that differentiates it from DNR. The values are reported in Fig. 1.

Results

The structures of the anthracycline derivatives used are shown in Fig. 1, together with the pK_a values of deprotonation of their amino group and their hydrophobicity estimated from their c log*P* values. Because the concentration of neutral form of the anthracyclines is in equilibrium between extracellular medium and cytosol when steady state is reached (the condition used to calculate C_i) the extracellular pH has to be equal to cytosolic pH to allow these calculations. We have chosen here an extracellular pH of 7.25, because the cytosolic pH in both the sensitive and the resistant K562 and GLC_4 cells was shown to be within the range of 7.2–7.3 (Frézard and Garnier-Suillerot, 1991a; Versantvoort *et al.*, 1992).

Determination of the K_m, V_M, and n_H coefficients of anthracycline efflux

Typical examples of these experiments are shown in Fig. 2. The time required to reach the steady state of anthracycline accumulation in the energy-depleted cells varied between 20 min (for Br-DNR and PIRA) and 90 min (for DNR). At this time-point, $C_i = C_e$ was calculated from the (nonquenched) fluorescence (Frézard and Garnier-Suillerot, 1991a, 1991b; Mankhetkorn *et al.*, 1996) and V_a was calculated as $C_T/F_0 \cdot (dF/dt)$, where F_0 is the fluorescence of a C_T μM anthracycline solution and dF/dt the slope of the tangent to the curve F(t) after addition of glucose which initiated the active efflux component. The same experiment was performed using the parent cell lines, in which no active efflux could be detected (shown for DNR and PIRA in Fig. 2).



Anthracycline derivatives	R_{14}	R'_2	R'_3	R'_4	R''_4	pKa	relative hydrophobicity Σ f_i
Eso-DNR	H	H	NH_2	H	H	8.7	+1.67
DNR	H	H	NH_2	OH	H	8.4	0
PIRA	OH	H	NH_2		H	7.7	-1.06
Br-DNR	H	Br	NH_2	H	OH	6.5	+0.09
OH-DNR	H	H	OH	OH	H	---	-0.06
OH-DOX	OH	H	OH	OH	H	---	-1.73

Fig. 1. Structures, pK_a and relative hydrophobicity values of the anthracyclines used. Variation of hydrophobicity evaluated as sum of fragmental values *fi*, referred to DNR (the calculated log *P*, c log *p* = log P_0 + Σ*fi* where log P_0 is the partition coefficient for DNR in *n*-octanol/water).

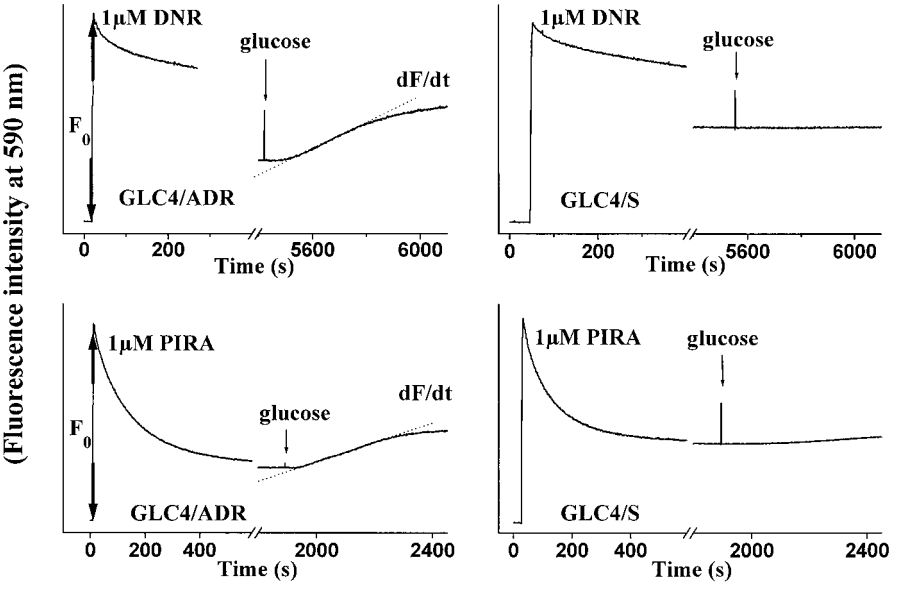


Fig. 2. Incorporation of PIRA and DNR in GLC4/S and GLC4/ADR cells after ATP depletion by incubation with azide and determination of the active efflux rate (V_a) after restoration of ATP synthesis by the addition of glucose (normalized fluorescence intensity).

The K_m, V_M, and n_H coefficients of the active transport were then calculated as described from the curves of V_a versus C_i. Fig. 3 shows two examples of the curves obtained for DNR and Br-DNR. The values of the three parameters for all anthracyclines are reported in Tables 1 and 2 for GLC4/ADR and K562/ADR cells, respectively. It seems that the apparent K_m values were within the rather narrow range of 0.4–2.4 µM for the GLC_4/ADR as well as K562/ADR cells, indicating a similar affinity of the anthracyclines for MRP and Pgp. However, the V_M values seemed to vary in a systematic way, dependent on the anthracycline and on the cell line. Fig. 4 shows a representation of V_M as a function of the percentage of neutral form of the anthracycline. In the case of K562/ADR, no trend could be seen, whereas for the GLC_4/ADR cells, an apparently negative correlation between V_M and the percentage of neutral form of the anthracycline was seen.

The mean active efflux coefficients K_a were calculated using eq. 5 and are shown in Tables 1 and 2. This parameter had a somewhat lower value for the two analogues in which the amino group had been replaced by a hydroxy group (OH-DOX and OH-DNR), particularly for the GLC_4/ADR cells. The efficiency of efflux of PIRA and Br-DNR seemed to be slightly higher than for DNR in the case of K562/ADR cells but not GLC_4/ADR cells.

Discussion

Overexpression of Pgp or MRP in tumor cells, either resulting from selection with cytostatic agents or from transfection with the *MDR1* or *MRP* gene leads to resistance to a spectrum of anticancer drugs, represented by some major clinically active drugs, such as DOX, DNR, vincristine, and etoposide. Resistance to the anticancer agents taxol and mitoxantrone is high in Pgp-overexpressing cells but less or absent in MRP-overexpressing cells (Zaman *et al.*, 1994; Cole *et al.*, 1994; Broxterman *et al.*, 1995b). It has been established that both proteins belong to the superfamily of ATP-binding cassette transmembrane transporter proteins (Hughes, 1994), which have ATP-binding sites (Cornwell *et*

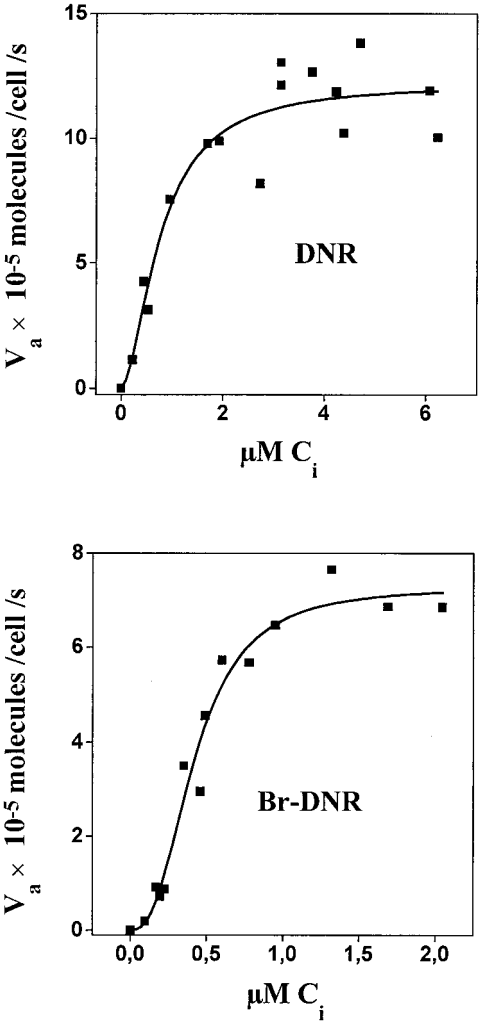


Fig. 3. Kinetics of the Pgp-mediated efflux of DNR and Br-DNR plotted as a function of C_i. GLC4/ADR cells (1×10^6/ml) were incubated in the presence of various concentrations of drug ranging from 0 to 12 µM. V_a and C_i were determined as described. Data are from 3–5 independent experiments on different days.

TABLE 1

Efflux parameters of anthracycline derivatives by GLC_4/ADR cells

Data are mean ± standard deviation from three to five independent experiments on different days. k_a was calculated using eq. 5, where V_M was expressed in M/sec/(cells/ml), the conversion being 1 M/sec/(cells/ml) = 6×10^{14} molecules/cell/sec.

Anthracycline	$k_a \times 10^{10}$	V_M	K_m	n_H
	s^{-1} (cells/ml)$^{-1}$)	$\times 10^{-5}$ molecules/cell/sec	μM	
eso-DNR	17	16.2 ± 1.2	0.8 ± 0.1	1.9 ± 0.5
DNR	13	12.0 ± 1.2	0.8 ± 0.2	1.8 ± 0.5
PIRA	14	15.0 ± 1.8	0.9 ± 0.2	1.8 ± 0.4
Br-DNR	15	7.2 ± 0.6	0.42 ± 0.03	2.6 ± 0.4
OH-DNR	2	1.6 ± 0.2	0.6 ± 0.1	1.9 ± 0.8
OH-DOX	2	2.4 ± 1.2	1.3 ± 1.1	1.3 ± 0.7

TABLE 2

Efflux parameters of anthracycline derivatives by K562/ADR cells

Data are mean ± standard deviation of three to five independent experiments on different days. k_a was calculated using eq. 5, where V_M was expressed in M/sec/(cells/ml), the conversion being 1 M/sec/(cells/ml) = 6×10^{14} molecules/cell/sec.

Anthracycline	k_a	V_M	K_m	n_H
	$\times 10^{10}$ s^{-1} (cells/ml)$^{-1}$	$\times 10^{-5}$ molecules/cell/sec	μM	
eso-DNR	14	26.4 ± 5.4	1.7 ± 0.7	1.2 ± 0.4
DNR	8	20.4 ± 1.8	2.1 ± 0.3	1.9 ± 0.4
PIRA	32	15.0 ± 3.0	0.4 ± 0.2	1.3 ± 0.4
Br-DNR	28	11.4 ± 3.0	0.4 ± 0.2	1.3 ± 0.7
OH-DNR	5	15.6 ± 3.6	2.4 ± 0.7	1.8 ± 0.6
OH-DOX	6	21.0 ± 2.4	2.4 ± 0.3	2.3 ± 0.5

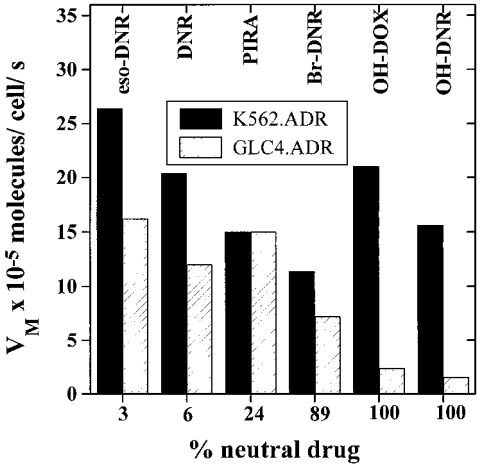


Fig. 4. Relation between the maximum rate of efflux V_M and the percentage of drug in the neutral form for GLC4/ADR and K562/ADR cells.

al., 1987), ATPase activity (Hooijberg, 1997), and use the energy from ATP hydrolysis, probably to directly translocate their substrates (Broxterman and Pinedo, 1991).

Despite considerable overlap in substrate specificity between Pgp and MRP, major differences have now been established, mainly related to the charge of putative substrates. Whereas Pgp seems to transport neutral and positively charged substrates, MRP has clearly been shown to have the properties of an organic anion transporter (cMOAT or GS-X pump) (Loe *et al.*, 1996a; Leier *et al.*, 1994). Whereas the presence of a positive charge on such molecules as rhodamine 123 or SYTO 16 may be the reason for their highly efficient Pgp-mediated transport, it may preclude their efficient transport by MRP (Twentyman *et al.*; 1994; Broxterman *et al.*, 1997).

In this article, we present data on the kinetics of ATP-dependent transport of a series of anthracyclines in the MRP-overexpressing GLC4/ADR cells and compare the kinetic parameters with new and previous data on Pgp-mediated transport. Basically, we blocked the energy-dependent transport of the anthracyclines by MRP and Pgp by depleting ATP in the cells until steady state was reached. After applying glucose, the initial efflux rate of the fluorescent anthracyclines can be measured, because the quenching by DNA is released (Frézard and Garnier-Suillerot, 1991b; Mankhetkorn *et al.*, 1996; Garnier-Suillerot, 1995; Borrel *et al.*, 1994a, 1994b; Pereira *et al.*, 1994). This allows the accurate estimation of efflux kinetics from intact viable cells with the transporter proteins in their native membrane environment. The value we found for the V_M of active DNR transport in the GLC_4/ADR cells was 2 nM/sec, which corresponds to 1.2 million molecules of DNR per cell per second, which is in good agreement with the V_M for DNR transport from the GLC4/ADR cells, as has been determined previously by a radioactive method (200 pmol/10^6 cells/min or about 2 million molecules DNR per cell per second) (Versantvoort *et al.*, 1994). The parameter V_M contains both the turnover number of the relevant transporter protein as well as the density of transporter protein molecules on the cellular plasma membrane. Thus, in comparing the data recorded for the two drug-resistant cell lines in Tables 1 and 2, a difference in V_M for any substrate could arise from a difference in either the turnover number of the transporter or the density of the transporter molecules.

We show that the mean active efflux coefficient for the present series of anthracyclines is very similar for Pgp- and MRP-mediated transport, except for a tendency for a slightly higher k_a for PIRA and Br-DNR in the case of Pgp-mediated efflux. A further similarity between Pgp- and MRP-mediated anthracycline transport follows from what are apparently not simple hyperbolic curves of V_a versus C_i, with many Hill coefficients close to 2. This means that for both proteins, a positive cooperative transport of two molecules of anthracycline may be hypothesized, as was previously already suggested for Pgp using a different methodology (Spoelstra *et al.*, 1992).

Substitution of a hydroxyl group for the amine group as in

OH-DOX and OH-DNR did not seem to abolish its transport by Pgp (Borrel *et al.*, 1994b) or MRP, but it did result in a similar reduction of the k_a. We have also seen that after depletion of cellular GSH by 20 hr of pretreatment of the GLC_4/ADR cells with 25 μM buthionine sulfoximine (Versantvoort *et al.*, 1995), the efflux of OH-DOX or OH-DNR was abolished (not shown). Thus, the theory that the amino group in the sugar portion of doxorubicin might be an important but not essential part of the anthracycline molecule for recognition by Pgp (Priebe *et al.*, 1993) also seems to hold for MRP. However, we also found a remarkable decrease in the V_M of the transport of both permanently neutral derivatives in the case of MRP-mediated transport only (Fig. 4). This would suggest that the presence of the amino group in the anthracycline structure is more important in the case of MRP-mediated transport. As pointed out before, other evidence is not in favor of efficient transport of permanently positively charged substrates by MRP.

On the other hand, because the transport by MRP of the two negatively charged substrates, oxidized glutathione (Heijn *et al*. 1997) and azidophenylglutathione (Shen *et al.*, 1997), can be competitively inhibited by DNR, it has been hypothesized that the anthracycline binding site of MRP may be the same as or near the organic anion binding site (Heijn *et al.*, 1997). Thus it may be suggested that the anthracycline and a negatively charged molecule interact in a (partly overlapping) binding site of MRP. Further indications for that suggestion are the GSH dependence of anthracycline and vinca alkaloid transport by MRP in intact cells (Versantvoort *et al.*, 1995; Davey *et al.*, 1995) and the finding that GSH can stimulate the ATP-dependent transport of the anticancer drug vincristine into inside-out vesicles of MRP-transfected cells (Loe *et al.*, 1996b). Because GSH is present in the intact cells but not in some other reported vesicle experiments (Paul *et al.*, 1996), it may be that GSH by a (cooperative) interaction decreases the K_M for the binding of DNR to its transporter site.

In conclusion, our data show that the kinetics of anthracycline transport by MRP are very similar to those for Pgp, and we are therefore in favor of the idea that MRP transports these molecules predominantly in their unmetabolized form. This does not exclude the idea that these species may be MRP substrates if some metabolism of anthracyclines occurs in tumor cells (e.g., to glucuronides). In this respect, it will be important to identify the anthracycline binding site(s) of MRP and their connection with the binding site for organic anions.

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References

Borrel M-N, Fiallo M, Veress I. and Garnier-Suillerot A (1995) The effect of crown ethers, tetraalkylammonium salts, and polyoxyethylene amphiphiles on pirarubicin incorporation in K562 resistant cells. *Biochem Pharmacol* **50**:2069–2076.

Borrel M-N, Pereira E, Fiallo M, and 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 and 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 PF and Ling V (1988) Mechanism of multidrug resistance. *Biochim Biophys Acta* **948:**87–128.

Broxterman HJ and Pinedo HM (1991) Energy metabolism in multidrug resistant tumor cells: a review. *J Cell Pharmacol* **2:**239–247.

Broxterman HJ, Jansen G, Linn SC, and Lankelma J (1995a) The impact of transport-associated resistance in anticancer chemotherapy, in *Drug Transport in Antimicrobial and Anticancer Chemotherapy* (NH Georgopapadakou, ed). Dekker, New York, 21–62.

Broxterman HJ, Giaccone G, and Lankelma J (1995b) Multidrug resistance proteins and other drug transport-related resistance to natural product agents. *Curr Opin Oncol* **7:**532–540.

Broxterman HJ, Heijn M, and Lankelma J (1996) How does the MRP/GS-X pump export doxorubicin? *J Natl Cancer Inst* **88:**466–467.

Broxterman HJ, Schuurhuis GJ, Lankelma J, Oberink JW, Eekman CA, Claessen AME, Hoekman K, Poot M, and Pinedo HM (1997) Highly sensitive and specific detection of P-glycoprotein function for hematological and solid tumor cells using a novel nucleic acid stain. *Br J Cancer*, in press.

Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, and Deeley RG (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* **54:**5902–5910.

Cornwell MM, Tsuruo T, Gottesman MM, and Pastan I (1987) ATP-binding properties of P-glycoprotein from multidrug-resistant KB cells. *FASEB J* **1:**51–54.

Davey RA, Longhurst TJ, Davey MW, Belov L, Harvie RM, Hancox D, and Wheeler H (1995) Drug resistance mechanisms and MRP expression in response to epirubicin treatment in a human leukaemia cell line. *Leukemia Res* **19:**275–282.

Frézard F and 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 and 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.

Feller N, Broxterman HJ, Währer DCR, and Pinedo HM (1995) ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): no inhibition by intracellular glutathione depletion. *FEBS Lett* **308:**385–388.

Garnier-Suillerot A (1995) Impaired accumulation of drug in multidrug resistant cells. What are the respective contributions of the kinetics of uptake and of P-glycoprotein-mediated efflux of drug? *Curr Pharmaceut Design* **1:**69–82.

Gekeler V, Ise W, Sanders KH, Ulrich WR, and Beck J (1995) The leukotriene LTD_4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* **208:**345–352.

Heijn M, Hooijberg JH, Scheffer GL, Szabó G, Westerhoff HV, and Lankelma J (1997) Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. *Biochim Biophys Acta* **1326:**12–22.

Hill T (1985) *Cooperative Theory in Biochemistry*. Springer-Verlag, Berlin, 63–67.

Hooijberg JH, Heijn M, Fles DLA, Lankelma J, Pinedo HM and Broxterman HJ (1997). Substrate interaction and ATPase activity of multidrug resistance protein (MRP). *Proc Am Assoc Cancer Res* **38:**392.

Hughes AL (1994) Evolution of the ATP-binding cassette transmembrane transporters of vertebrates. *Mol Biol Evol* **11:**899–910.

Ishikawa T, Akimaru K, Tien Kuo M, Priebe W, and Suzuki M (1995) How does the MRP/GS-X pump export doxorubicin? *J Natl Cancer Inst* **87:**1639–1640

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

Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG, and Keppler D (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J Biol Chem* **269:**27807–27810.

Loe DW, Deeley RG, and Cole SPC (1996a) Biology of the multidrug resistance-associated protein. *Eur J Cancer* **32A:**945–957.

Loe DW, Almquist KC, Deeley RG, and Cole SPC (1996b) Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapeutic agents in membrane vesicles. *J Biol Chem* **271:**9675–9682.

Paul S, Breuninger LM, and Kruh GD (1996) ATP-dependent transport of lipophilic cytotoxic drugs by membrane vesicles prepared from MRP-overexpressing HL60/ADR cells. *Biochemistry* **35:**14003–14011.

Mankhetkorn S, Dubru F, Hesschenbrouck J, Fiallo M, and 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;

Pearce HL, Safa AR, Winter NJ, Cirtain MC and Beck WT (1989) Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. *Proc Natl Acad Sci (USA)* **86:**5128–5132.

Pereira E, Borrel M-N, Fiallo M and Garnier-Suillerot A (1994) Non-competitive inhibition of P-glycoprotein-associated efflux of THP-adriamycin by verapamil in living K562 leukemia cells. *Biochem Biophys Acta* **1225:**209–216.

Priebe W, Van NT, Burke TG and Perez-Soler R (1993) Removal of the basic center from doxorubicin partially overcomes multidrug resistance and decreases cardiotoxicity. *Anti-cancer Drug Des* **4:**37–48.

Priebe W (1995) Mechanism of action-governed design of anthracycline antibiotics**:** a "turn-off/turn-on" approach. *Curr Pharmaceut Des* **1:**51–68.

Rekker RF (1977) The hydrophobic fragmental. Its derivation and applications. A means of characterizing membrane systems, in *Pharmacochemistry Library*, vol 1 (Nauta W and Rekker R, eds) pp 39–103, Elsevier, Amsterdam.

Shen H, Paul S, Breuninger LM, Ciaccio PJ, Laing NM, Helt M, Tew KD, and Kruh GD (1996) Cellular and *in vitro* transport of glutathione conjugates by MRP. *Biochemistry* **35:**5719–5725.

Spoelstra E, Westerhoff H, Dekker H, and Lankelma J (1992) Kinetics of daunorubicin transport by P-glycoprotein of intact cells. *Eur J Biochem* **207:**567–579.

Twentyman PR, Rhodes T, and Rayner S (1994) A comparison of rhodamine 123 accumulation and efflux in cells with p-glycoprotein-mediated and MRP-associated multidrug resistance phenotypes. *Eur J Cancer* **30A:**1360–1369.

Versantvoort CHM, Broxterman HJ, Feller N, Dekker H, Kuiper CHM, and Lankelma J (1992) Probing daunorubicin accumulation defects in non-P-

glycoprotein expressing multidrug-resistant cell lines using digitonin. *Int J Cancer* **50:**906–911.
Versantvoort CHM, Broxterman HJ, Lankelma J, Feller N, and Pinedo HM (1994) Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact MRP overexpressing human small cell lung cancer cells. *Biochem Pharmacol* **48:**1129–1136.
Versantvoort CHM, Broxterman HJ, Bagrij T, Scheper R,J and Twentyman P R (1995). Regulation by glutathione of drug transport in multidrug resistant human tumour cell lines overexpressing MRP. *Br J Cancer* **72:**82–89.
Zaman GJR, Flens MJ, van Leusden MR, de Haas M, Mülder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ, and Borst P (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci USA* **91:**8822–8826.
Zijlstra JG, de Vries EGE, and Mulder NH (1987) Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* **47:**1780–1784.

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