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 confers 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 \([10-20 \times 10^{-10} \text{sec/(cells/ml)}]\). 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 \( k_a \) \([2 \times 10^{-10} \text{sec/(cells/ml)}]\) than for the MRP. The apparent Michaelis-Menten constants \( K_m \) and maximal efflux rates \( V_{max} \) for the active transport were within a narrow range for both transporters, except for OH-DOX and OH-DNR, which had a lower \( V_{max} \) 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

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'-hydroxy-daunorubicin; \( C_{ex} \), extracellular free drug concentration; \( C_i \), cytosolic free drug concentration; \( V_{max} \), 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; Priebé, 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; Borrel et al., 1995; Borrel et al., 1994a, 1994b; Pereira et al., 1994). In order to determine the Pgp-mediated efflux of anthracycline derivatives, Cells (1 × 10^6/ml; 2 ml per cuvette) were 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_t 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 anthracyclines. 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_t = C_i 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).

**Materials and Methods**

**Cell culture**

K562 leukemia cells and the Pgp-expressing K562/ADR cells (Mankhetkorn et al., 1996), as well as GLC and the MRP-expressing GLC/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/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, 1985). 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%/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 anthracyline derivatives.** Cells (1 × 10^6/ml; 2 ml per cuvette) were 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_t 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 anthracyclines. 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_t = C_i 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):

\[ V_a = V_m C_i^n / (K_m^n + C_i^n) \]

(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; Speelstra et al., 1992) we have defined a mean active efflux coefficient (k_a) according to the equation:

\[ V_a = k_a n C_i \]

(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:

\[ dV_a/dC_i = (V_m n K_m^n) / (C_i^n + K_m^n) \]

(3)

When n_H = K_m^n / (C_i^n + K_m^n) = 1 or alternatively when

\[ C_i = K_m / (n_H - 1)^{1/n_H} \]

(4)
eq. 1 [which can be written as \( V_a/C_i = V_m \cdot C_i^{n-1}/(K_m^{n-1} + C_i^{n-1}) \)] becomes

\[
V_a/C_i = (V_m/n_H \cdot K_m) \cdot (n_H - 1)(1 - \frac{1}{n_H})
\]

or \( h_a \cdot n = (V_m/n_H \cdot K_m) \cdot (n_H - 1)(1 - \frac{1}{n_H}) \)

(5)

When \( n_H = 2 \), it follows that \( h_a \cdot n = V_m/2 K_m \); in other words, \( h_a \cdot n \) is equal to the slope of the tangent to the curve \( V_a = f(C_i) \) when \( C_i = K_m \). Thus \( h_a \) 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 \( \psi \) 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 \( c \log P = \log P_0 + \sum \psi_i \), where \( P_0 \) is the partition coefficient for DNR in \( n \)-octanol/water and \( \psi_i \) 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 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, \( C_i = C_o \) was calculated from the (nonquenched) fluorescence (Frézard and Garnier-Suillerot, 1991a, 1991b; Mankhetkorn et al., 1996) and \( V_a \) was calculated as \( C/T \cdot F_0 \cdot (dF/dt) \), where \( F_0 \) is the fluorescence of a \( C/T \) \( \mu \)M 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).

![Fig. 1. Structures, \( pK_a \) and relative hydrophobicity values of the anthracyclines used. Variation of hydrophobicity evaluated as sum of fragmental values \( \psi_i \), referred to DNR (the calculated \( \log P \), \( c \log p = \log P_o \) + \( \sum \psi_i \) where \( \log P_o \) is the partition coefficient for DNR in \( n \)-octanol/water).](image-url)
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 GLC4/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 GLC4/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 GLC4/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 GLC4/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. 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).

Fig. 3. Kinetics of the Pgp-mediated efflux of DNR and Br-DNR plotted as a function of $C_i$. GLC4/ADR cells ($1 \times 10^6$/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 GLC4/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 μmol/10^6 cells/min, the conversion being 1 μmol/10^6 cells/min = 6 × 10^14 molecules/cell/sec.

<table>
<thead>
<tr>
<th>Anthracline</th>
<th>$k_a \times 10^{10}$ molecules/cell/sec</th>
<th>$V_M \times 10^5$ molecules/cell/sec</th>
<th>$K_m \mu M$</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>eso-DNR</td>
<td>17</td>
<td>16.2 ± 1.2</td>
<td>0.8 ± 0.1</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>DNR</td>
<td>13</td>
<td>12.0 ± 1.2</td>
<td>0.8 ± 0.2</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>PIRA</td>
<td>14</td>
<td>15.0 ± 1.8</td>
<td>0.9 ± 0.2</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Br-DNR</td>
<td>15</td>
<td>7.2 ± 0.6</td>
<td>0.42 ± 0.03</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>OH-DNR</td>
<td>2</td>
<td>1.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>OH-DOX</td>
<td>2</td>
<td>2.4 ± 1.2</td>
<td>1.3 ± 1.1</td>
<td>1.3 ± 0.7</td>
</tr>
</tbody>
</table>

TABLE 2

Efflux parameters of anthracycline derivatives by K562/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 μmol/10^6 cells/min, the conversion being 1 μmol/10^6 cells/min = 6 × 10^14 molecules/cell/sec.

<table>
<thead>
<tr>
<th>Anthracline</th>
<th>$k_a \times 10^{10}$ molecules/cell/sec</th>
<th>$V_M \times 10^5$ molecules/cell/sec</th>
<th>$K_m \mu M$</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>eso-DNR</td>
<td>14</td>
<td>26.4 ± 5.4</td>
<td>1.7 ± 0.7</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>DNR</td>
<td>8</td>
<td>20.4 ± 1.8</td>
<td>2.1 ± 0.3</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>PIRA</td>
<td>32</td>
<td>15.0 ± 3.0</td>
<td>0.4 ± 0.2</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Br-DNR</td>
<td>28</td>
<td>11.4 ± 3.0</td>
<td>0.4 ± 0.2</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>OH-DNR</td>
<td>5</td>
<td>15.6 ± 3.6</td>
<td>2.4 ± 0.7</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>OH-DOX</td>
<td>6</td>
<td>21.0 ± 2.4</td>
<td>2.4 ± 0.3</td>
<td>2.3 ± 0.5</td>
</tr>
</tbody>
</table>

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.
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 $k_3$. We have also seen that after depletion of cellular GSH by 20 hr of pretreatment of the GLC/ADR cells with 25 $\mu$m 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 anthracyline and vincal 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 (Loo 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 anthracyline 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 anthracylines occurs in tumor cells (e.g., to glucuronides). In this respect, it will be important to identify the anthracyline binding site(s) of MRP and their connection with the binding site for organic anions.

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