Energy-dependent Processes Involved in Reduced Drug Accumulation in Multidrug-resistant Human Lung Cancer Cell Lines without P-Glycoprotein Expression

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ABSTRACT

Mechanisms contributing to reduced cytotoxic drug accumulation were studied in two multidrug-resistant (MDR) human lung cancer cell lines without P-glycoprotein expression. In these (non-small cell) SW-1573/2R120 and (small cell) GLC4/ADR MDR cells, the steady-state accumulation of [3H]daunorubicin was 30 and 12%, respectively, of that in the parent cells. When cells, at steady state, were permeabilized with digitonin, the amount of daunorubicin binding increased only in the resistant cells. The reduced accumulation of daunorubicin in the SW-1573/2R120 and GLC4/ADR cells was accompanied by a lower initial (2 min) uptake rate of this drug. No difference in initial efflux rate of daunorubicin from preloaded cells could be detected between sensitive and resistant SW-1573 cells. However, daunorubicin was extruded 5-fold faster from GLC4/ADR cells than from the parental cells. In the presence of the energy metabolism inhibitors sodium azide and deoxyglucose, the reduced daunorubicin accumulations in the SW-1573/2R120 and GLC4/ADR MDR cells were (almost) completely reversed. The effects of these inhibitors on drug uptake were already apparent during the earliest measured time points (<15 s). Also, the enhanced efflux of daunorubicin from GLC4/ADR cells was inhibited. In ATP-depleted cells, the intracellular pH was lowered by ~0.3 units in resistant as well as in sensitive cells. The lower intracellular pH, however, could not account for the increase in daunorubicin accumulation in the resistant cells. Also, for vincristine and etoposide, the increases in drug accumulation under energy-deprived conditions were more pronounced in the resistant SW-1573/2R120 cells than in the parent SW-1573 cells. These results suggest that accumulation of drugs in the non-P-glycoprotein MDR human lung carcinoma cell lines SW-1573/2R120 and GLC4/ADR is reduced by an energy-dependent drug export mechanism which prevents efficient transport of drug to the target. Since P-glycoprotein expression in lung tumors is generally low, these MDR lung cancer cell lines can be used as a model to study alternative mechanisms leading to multidrug resistance in this tumor type.

INTRODUCTION

By exposure to cytotoxic drugs, tumor cells can acquire resistance to structurally and functionally unrelated compounds. In vitro the cellular pharmacological basis for MDR often appears to be a reduced steady-state accumulation of drugs caused by the overexpression of the mdrl gene, which encodes for a plasma membrane glycoprotein of Mr 170,000—180,000 (Pgp). Pgp is thought to function as an energy-dependent drug efflux pump (1, 2). However, a series of MDR cell lines has been derived from a human non-small cell lung cancer cell line, SW-1573, by selection for resistance to doxorubicin (3, 4). During early steps of the selection, an MDR cell line (SW-1573/2R120) without any detectable expression of the mdrl gene was isolated, whereas sublines isolated at higher concentrations of doxorubicin overexpressed Pgp. The cross-resistance spectrum of the SW-1573/2R120 cells resembled that of the Pgp-expressing sublines and the cells also proved to be defective in accumulation of vincristine and daunorubicin (3).

This MDR phenotype, i.e., a broad cross-resistance spectrum and reduced cellular drug levels, has been reported recently for other cell lines that do not overexpress Pgp (5–10). The broad spectrum of drugs to which these cells are resistant makes it unlikely that alterations in a common target site are the primary cause of the resistance. An enhanced, energy-dependent efflux of daunorubicin has been reported for non-Pgp MDR HL-60/Adr (human leukemia), HT1080/Dr4 (human fibrosarcoma), and COR-L23/R (human large cell lung carcinoma) cells (6, 7, 10). Since the level of Pgp expression in lung cancer generally is low (11), other resistance mechanisms may be of importance in lung cancer. One such mechanism may be related to topoisomerase II activity (12). In a human small cell lung carcinoma cell line (GLC4/ADR), part of the resistance could be explained by reduced topoisomerase II activities (13). The possibility of alternative (non-Pgp) drug transport mechanisms operating in MDR cells has not been adequately investigated until now.

We studied the presence of putative energy-dependent drug transporters in SW-1573/2R120 (3) and GLC4/ADR (5, 13) non-Pgp-mediated MDR lung cancer cell lines. By permeabilization of the plasma membrane after steady-state accumulation of daunorubicin, we could show that the SW-1573/2R120, but not the parent cells, keep daunorubicin binding to the nuclear DNA below equilibrium at the applied extracellular concentration. Furthermore, the effects of cellular energy depletion on daunorubicin transport in these cells were studied. Since daunorubicin is a weak base, changes of intracellular pH, which might be attributed to alterations in membrane transport, were taken into account. Energy-dependent transport of vincristine and VP-16 was studied in the SW-1573 cells. From the experiments reported here, it appears that an energy-dependent mechanism leading to decreased drug accumulation is operative in non-Pgp-mediated MDR SW-1573/2R120 and GLC/ADR cells.

MATERIALS AND METHODS

Chemicals. Daunorubicin hydrochloride was obtained from Specia (Paris, France), vincristine sulfate from Sigma Chemical Co. (St. Louis, MO), and VP-16 from Bristol Myers Co. (Weesp, The Netherlands). BCECF-acetoxymethyl ester was from Molecular Probes (Eugene, OR). Sodium azide was obtained from Baker Chemicals (Deventer, The Netherlands), and nigericin and 2-deoxy-d-glucose was from Sigma. [14C]Daunorubicin (specific activity, 45 Ci/mol) and [G-3H]Hydincleristine sulfate (specific activity, 4.8 Ci/mmole) were obtained from Amer-
Cells. The human non-small cell lung carcinoma cell line SW-1573, originally isolated by Dr. A. Leibovitz (Scott and White Clinic, Temple, TX), and the doxorubicin-resistant subline SW-1573/2R120 have been described elsewhere (13, 14). The cells were cultured at 37°C in 5–6% CO₂ in a humidified incubator in Dulbecco's modified Eagle's essential medium (Flow Laboratories, Irvine, Scotland) supplemented with 7.5% heat-inactivated fetal calf serum (GIBCO Europe, Paisley, Scotland) and 20 mM HEPES (Serva, Heidelberg, Germany). The human small cell lung carcinoma cell line GLC4 and its doxorubicin-resistant subline GLC4/ADR have been described elsewhere (5, 13) and were grown as floating cells in RPMI medium (Flow Laboratories) with 7.5% fetal calf serum. The resistant cells were cultured in the presence of doxorubicin (0.12 μM for SW-1573/2R120 and 1.2 μM for GLC4/ADR cells) until 3–10 days before the experiments. All cells were screened for Mycoplasma contamination by using the Mycoplasma T.C. rapid detection system with a 1³H-labeled DNA probe from Gen-Probe Inc. (San Diego, CA) before these series of experiments and were found to be negative.

RNAse Protection. RNAse protection was carried out as described by Baas et al. (4). RNA samples (10 μg) were hybridized with a ³²P-labeled human mdr1-specific probe, a 301-nucleotide mdr1 complementary DNA fragment, or with a mdr3-specific probe, a 301-nucleotide mdr3 complementary DNA fragment (obtained from Dr. J. Smit, The Netherlands Cancer Institute, Amsterdam). The protected fragment was visualized by electrophoresis through an acrylamide/urea gel (19:1), followed by autoradiography. A γ-actin probe was included as a control for RNA recovery.

Cellular Drug Accumulation. Initial uptake studies were carried out as described before (15). Adherent cells in the logarithmic phase of growth were trypsinized, washed, and resuspended in densities of 0.5–3 × 10⁶ cells/ml in Dulbecco’s modified essential medium containing 20 mM HEPES (pH 7.3–7.4), 10% heat-inactivated fetal calf serum, and 0.025% DNase I (medium A). Cells were incubated at 37°C, radiolabeled drugs were added, and during this period, 0.2-m1 aliquots were sampled and dispersed in 1.5 ml of ice-cold PBS. After two cold washes, the cells were transferred to liquid scintillation fluid (OptiPhase III; LKB, Bromma, Sweden), and radioactivity was measured. Values were corrected for amount of cell-associated radioactivity at time zero at 0°C. Steady-state accumulation of drugs was measured in a similar way as described before (16). After steady-state accumulation of daunorubicin had been reached, some experiments 20 μM digitonin (DNase I was then not included) was added 5 min before the end of the drug accumulation period to permeabilize the plasma membrane. This method has been adapted from Ref. 17 and will be described in detail elsewhere. No daunorubicin was lost from digitonin-permeabilized cells during the washing procedures.

To study the ATP dependence of drug transport, cellular energy metabolism was inhibited by incubation of cells in medium A without glucose but containing 10 mM sodium azide and 1 mg/ml 2-deoxy-d-glucose (medium C). In this medium cellular ATP concentrations decreased very rapidly as measured by the decrease of luminescence generated by the luciferin/luciferase reaction. The bioluminescence assay kit obtained from Sigma was used. Within 15 min, the ATP concentration decreased to 10–15% of the initial concentration. A further decrease to about 5% was measured during the following 90 min. Cells were incubated for 15 min at 37°C prior to addition of drugs, to decrease cellular ATP concentrations (~90%). Viability of the cells as measured with trypan blue was always ≥94% during the incubation periods.

Daunorubicin Transport. Initially, we measured daunorubicin accumulation in SW-1573 and SW-1573/2R120 cells for 6 h, and steady-state accumulation was reached within 60 min (not shown). Therefore, 60 min was chosen for all further experiments. Compared to the parent cell lines, the steady-state accumulation of daunorubicin was 70 and 90% decreased in SW-1573/2R120 and GLC4/ADR cells, respectively (Fig. 1). To examine whether the plasma membrane formed a permeability barrier that actively kept daunorubicin out of the cell, the cells were permeabilized with digitonin after steady-state accumulation had been reached (Fig. 1). Daunorubicin binding in parent cell lines was not significantly affected by this procedure. This can be explained as follows: when daunorubicin accumulation is determined by passive diffusion, no concentration gradient of daunorubicin over the plasma membrane will be present when steady state is reached, and therefore, permeabilization of the plasma membrane is without effect. However, exposure of the resistant cells to digitonin after steady-state accumulation did result in a rapid influx of daunorubicin and binding of daunorubicin to DNA (Fig. 1). Thus, under steady-state conditions, a concentration gradient of daunorubicin over the plasma membrane was present in the resistant cells. Differences in pH over the plasma membrane could not be responsible for the increase in daunorubicin binding after permeabilization since the experiments were done at an extracellular pH of 7.35 ± 0.05 (mean ± SD) which is slightly higher than the intracellular pH (Table 2). This small difference in pH could only have resulted in a small efflux of daunorubicin during permeabilization of the plasma membrane.

Figs. 2A and 3A show the time course of [14-C]daunorubicin accumulation in parental SW-1573 and GLC4 cells compared to the sensitive lines. The resistant cells exhibited a reduced drug accumulation (Table 2). Therefore, after steady-state accumulation of 0.5 μM [14-C]daunorubicin had been reached, they were permeabilized by addition of 20 μM digitonin (C) and incubated for another 5 min. Error bar, SD of at least three independent experiments.

Intracellular pH Measurements. Intracellular pH was measured with the pH sensitive, fluorescent dye BCECF (18). Cells suspensions in medium A (1 × 10⁶ cells/ml) were loaded for 30 min at 37°C with 5 μM BCECF-acetoxymethylester. Cells were washed and fluorescence was recorded at 532 nm (emission) and 438 and 506 nm (excitation wavelengths). The ratio of the fluorescence was measured with excitation wavelengths set at 506 nm to the fluorescence measured at 438 nm was used to calculate the intracellular pH (19). The calculation was based on the ratios obtained by resuspending the cells in high K⁺ buffer (145 mM KCl, 5 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES) including 5 μM nigericin and 2 μM valinomycin. Under these conditions, the intracellular pH approached the extracellular pH (18, 19), and a linear relationship between fluorescence ratio and pH (within the range 6.8–7.5) was observed. In a control experiment, the pH was measured by a digitonin null-point technique adapted from Ref. 20. Both methods gave similar results.

**RESULTS**

**Daunorubicin Transport.** Initially, we measured daunorubicin accumulation in SW-1573 and SW-1573/2R120 cells for 6 h, and steady-state accumulation was reached within 60 min (not shown). Therefore, 60 min was chosen for all further experiments. Compared to the parent cell lines, the steady-state accumulation of daunorubicin was 70 and 90% decreased in SW-1573/2R120 and GLC4/ADR cells, respectively (Fig. 1).

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REDUCED DRUG ACCUMULATION NOT MEDIATED BY Pgp

Measurements of Intracellular pH. Daunorubicin is a weak base and is passively transported over the plasma membrane mainly in the uncharged form (21); therefore, transport of daunorubicin will be highly influenced by a pH gradient over the plasma membrane. To be able to estimate putative contributions of pH differences to the measured differences in daunorubicin accumulation, we determined the intracellular pH of parental and variant cells under the same conditions as used for drug accumulation experiments. No significant differences in intracellular pH could be measured between sensitive and resistant cells under such conditions (Table 2). Energy synthesis inhibitors, sodium azide and deoxyglucose, however, caused a rapid decrease in intracellular pH of ~0.2–0.3 units in all cell lines. In order to find out whether the increase in influx rate of daunorubicin in medium C (Fig. 2B) could be caused (in part) by this decrease in intracellular pH, the effect of nigericin on initial uptake of daunorubicin was measured. In low K⁺ medium nigericin enables electroneutral exchange of intracellular K⁺ ions for extracellular H⁺ ions (18). Nigericin lowered the intra-

to their variant sublines SW-1573/2R120 and GLC4/ADR, respectively. Cellular ATP depletion resulted in an increase of the daunorubicin accumulation in resistant and sensitive cells. However, the effects were most pronounced in the resistant SW-1573/2R120 (2.5-fold) and GLC4/ADR (10-fold) cells. Under energy-deprived conditions, the time course of daunorubicin accumulation was similar in GLC4 and GLC4/ADR cells.

The initial uptake of daunorubicin was linear with time for the first 2 min in both sensitive and MDR cells (Figs. 2B and 3B). The initial rates of daunorubicin uptake calculated from the linear curve are shown in Table 1. The rate of initial daunorubicin uptake was lower in the resistant cells than in their parental cell lines. As a result of cellular ATP depletion, the rate of initial daunorubicin uptake was increased by a factor 1.3 in the parent cell lines and a factor 2.5–3 in the resistant sublines. The effects of sodium azide and deoxyglucose on the rate of initial uptake suggest the presence of an energy-dependent efflux component that manifests its effect at a very early stage of the uptake.

Efflux of [14C]daunorubicin from drug-loaded cells was slower from cells resuspended in glucose-free medium containing sodium azide and deoxyglucose than in control medium (Figs. 4, A and B). In control medium, daunorubicin was extruded faster from GLC4/ADR cells (t½ 3 ± 0.4 min) than from the GLC4 cells (t½ 15 ± 5 min). No detectable difference in initial t½ between SW-1573 and SW-1573/2R120 cells was found, while less daunorubicin was retained in the SW-1573/2R120 cells after 30 min efflux (significant, P < 0.05, Student's t test).

Table 1 Rates of initial uptake of daunorubicin in parent and resistant cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Initial uptake rate (pmol/10⁶ cells/min)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Medium A</td>
</tr>
<tr>
<td>SW-1573</td>
<td>30 ± 4 (6)</td>
</tr>
<tr>
<td>SW-1573/2R120</td>
<td>11 ± 2 (6)</td>
</tr>
<tr>
<td>GLC4</td>
<td>19 ± 4 (3)</td>
</tr>
<tr>
<td>GLC4/ADR</td>
<td>7 ± 2 (2)</td>
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</tbody>
</table>

* Statistical different (P < 0.05) compared to control in medium A (Student's t test).
cells than in the wild-type SW-1573 cells. We also measured VP-16 and vincristine efflux after loading energy-deprived cells for 30 min with 10 μM [3H]VP-16 or 1 μM [3H]vincristine. The loss of VP-16 from cells resuspended in control medium A occurred very rapidly with equal efflux rates in parent (t½ 1.7 ± 0.2 min) and resistant cells (t½ 2.0 ± 0.4 min). However, the VP-16 efflux under ATP-depleted conditions was 2-fold slower for SW-1573/2R120 cells (t½ 4.3 ± 0.5 min) than for SW-1573 cells (t½ 2.0 ± 0.2 min). Thus, if transport of drugs under ATP-depleted conditions can be considered as a passive process (21), the passive transport (in and out) of VP-16 across the membrane is 2-fold slower in the resistant cells. Because VP-16 was extruded with equal rates from resistant and sensitive cells in normal medium, an additional energy-dependent transport mechanism should be present in the SW-1573/2R120 cells. For vincristine, the efflux was slow with no more than 10% efflux during the first 30 min.

Detection of Pgp/mdr1 mRNA. In humans, MDR is associated with the presence of Pgp, encoded by mdr1 gene (1). Recently, Herweijer et al. (22) suggested that the homologous mdr3 gene encodes for a functional drug pump only in B-cell lymphocytic leukemias. Previously, it was shown by immunostaining with monoclonal antibodies JSB-1 and C219 that the MDR cell lines SW-1573/2R120 and GLC4/ADR had no increased levels of Pgp (4, 13). Neither was any mdr1 mRNA expression detectable in the SW-1573/2R120 cells by sensitive RNase protection and polymerase chain reaction (3, 4). No mRNA overexpression of the mdr1 gene was detected in the GLC4/ADR cells by using mRNA Northern blotting (13). In order to assure the absence of Pgp in these cells, an RNase protection assay with an mdr1 probe was performed (Fig. 6). As a positive control for the sensitivity of this analysis, we used the low-resistant KB8–5 cells (23). Mdr1 mRNA expression

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**Table 2 Intracellular pH measured by BCECF fluorescence**

<table>
<thead>
<tr>
<th>Cells</th>
<th>pH (Medium A)</th>
<th>pH (Medium C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-1573</td>
<td>7.31 ± 0.11</td>
<td>6.92 ± 0.11</td>
</tr>
<tr>
<td>SW-1573/2R120</td>
<td>7.30 ± 0.04</td>
<td>7.00 ± 0.09</td>
</tr>
<tr>
<td>GLC4</td>
<td>7.30 ± 0.02</td>
<td>7.06 ± 0.06</td>
</tr>
<tr>
<td>GLC4/ADR</td>
<td>7.29 ± 0.02</td>
<td>7.08 ± 0.02</td>
</tr>
</tbody>
</table>

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**Table 3 Effect of cellular ATP depletion on accumulation of vincristine and VP-16 in SW-1573 cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Initial uptake rate (VP-16)</th>
<th>Steady-state accumulation (VP-16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-1573</td>
<td>145 ± 35</td>
<td>197 ± 18</td>
</tr>
<tr>
<td>SW-1573/2R120</td>
<td>212 ± 36</td>
<td>241 ± 78</td>
</tr>
<tr>
<td></td>
<td>151 ± 26</td>
<td>201 ± 75</td>
</tr>
<tr>
<td></td>
<td>251 ± 62</td>
<td>599 ± 238</td>
</tr>
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Fig. 4. Efflux of daunorubicin from SW-1573 (A) and GLC4 (B) cells. Cells of SW-1573 (C, G) and SW-1573/2R120 (△, △) and GLC4 (●, □) and GLC4/ADR (◇, △) were loaded for 30 min with 1 μM [14C]daunorubicin in medium C. Release of daunorubicin was measured after suspending the cells in daunorubicin-free medium A (C, △, □, ◇) or in medium C (G, △, □, △). Error bar, SD of at least three determinations.

Fig. 5. Changes in intracellular pH (pH) due to nigericin. Change of intracellular pH of SW-1573/2R120 in response to nigericin addition was recorded by using the fluorescent dye BCECF. Data are from one experiment (in duplicate), in which control pH of SW-1573/2R120 was ~0.1 pH unit lower than in SW-1573 cells.

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was clearly detectable in the KB8–5 cells, while no expression by an RNase protection assay (see "Materials and Methods"). The positions of cell lines. Also, mdr3 mRNA was not overexpressed in the absence of C2l9 staining proves the absence of mdr3 Pgp (24).

Previously, we found that, similar to Pgp MDR cells, the SW-1573/2R120 non-Pgp MDR cells had a decrease in nuclear anthracycline content compared to the parent cells (27). Assuming that the accumulation of daunorubicin is predominantly determined by binding to DNA (28, 29), we reasoned that cells with a reduced daunorubicin accumulation, caused by an active outward transport of the drug, could be detected by means of permeabilization of the plasma membrane (17, 29). Such an approach to probe putative drug transporters is required, since we are still lacking specific resistance modifiers for these non-Pgp-mediated MDR cells. We will document such a method using a low concentration of digitonin in detail elsewhere.

Here, we showed that the daunorubicin binding upon digitonin exposure was increased about 2.5-fold in the SW-1573/2R120 and 12-fold in the GLC4/ADR cells but not in the parent cells. Since daunorubicin is thought to be transported across the membrane in its uncharged form (21), the cellular accumulation of daunorubicin is highly dependent on a pH gradient across the plasma membrane (21, 29). From equations reported by Skovsgaard and Nissen (30), we calculated that the pH in the SW-1573/2R120 cells should be 0.6 units higher than in the parent cells, in order to explain the difference in daunorubicin accumulation at steady state between these cells solely by a change in intracellular pH. However, the intracellular pH, determined under conditions of drug accumulation measurements, was the same in resistant and sensitive cells. The increase of daunorubicin binding after permeabilization of the plasma membrane, therefore, strongly suggests that the reduced daunorubicin accumulation in the resistant cells is related to changes of drug transport at the plasma membrane. Therefore, the influx and efflux properties of daunorubicin transport in SW-1573/2R120 and GLC4/ADR cells were studied in more detail.

In the resistant GLC4/ADR cells, the decrease in daunorubicin accumulation was mainly caused by the 5-fold faster daunorubicin efflux. Such a large increase in efflux rate points to a high capacity of active drug transport in GLC4/ADR cells. No increased initial drug efflux from preloaded cells was apparent in SW-1573/2R120 compared to SW-1573 cells in standard medium. The relatively low resistance to daunorubicin of the SW-1573/2R120 cells (about a factor 4) might be a reason that no increased daunorubicin efflux was apparent in the SW-1573/2R120. However, in the Pgp-mediated MDR KB8–5 cells (23), which have low resistance for daunorubicin, daunorubicin was extruded 2-fold faster from the resistant KB8–5 cells than from the parent KB3–1 cells (not shown). The lower steady-state accumulation in the resistant cells was accompanied by a reduced initial uptake rate of daunorubicin. The initial uptake rates of vincristine and VP-16 were also decreased in the SW-1573/2R120 cells compared to the SW-1573 cells. Thus, in the resistant SW-1573/2R120 cells alterations in (apparent) influx rather than alterations in drug efflux can account for the drug accumulation defects.

A lower influx of drugs in resistant cells with a Pgp-related accumulation defect is not an uncommon feature of this phenotype. Sirotnak et al. (31) showed that the influx of vincristine in MDR Chinese hamster cells was 24-fold lower compared to the parental cells. Also, for other drugs, including doxorubicin belonging to a superfamily of ATP-dependent transporters have been identified in organisms from bacteria to eukaryotes (25, 26). Since Pgp seems to play a minor role in lung cancer (11), we searched for yet unidentified drug transporters in these two MDR lung tumor cell lines.

The characteristics of daunorubicin transport in two resistant lung cancer cell lines, SW-1573/2R120 and GLC4/ADR, were studied in detail because these cells have a reduced drug accumulation and display a multidrug cross-resistance pattern and lack mdr1 mRNA expression. Therefore, MDR in these cells seems to be based at least partly on other drug handling mechanisms than Pgp. An increasing number of membrane proteins

Fig. 6. P-glycoprotein expression. Expression of the mdr1 gene was measured by an RNase protection assay (see "Materials and Methods"). The positions of the protected fragments for mdr1 and γ-actin are indicated.
REDUCED DRUG ACCUMULATION NOT MEDIATED BY Pgp

(17), daunorubicin (32), and VP-16 (33, 34), a reduced influx in MDR cells has been reported. Thus, a reduced drug accumulation caused by overexpression of Pgp might manifests itself not only by an increase in drug efflux but also by a decrease in apparent drug influx rate (17, 21, 33). Model calculations for daunorubicin transport in Ehrlich ascites cells have shown that the apparent uptake of daunorubicin can be influenced immediately after offering the drug to cells with an outwardly directed drug pump with high affinity ($K_m = 10^{-8}$ m) (35). Blocking of this pump would then result in an increase in apparent uptake of daunorubicin. In the present experiments, the initial uptake of daunorubicin increased as a result of cellular energy depletion. However, we were unable to measure saturation of influx rates in SW-1573 and SW-1573/2R120 cells, since the apparent influx rate was linear with external daunorubicin concentrations from $10^{-4}$ to $4 \times 10^{-6}$ m in both cell lines (not shown). Thus, a putative ATP-dependent drug efflux mechanism in these cells would have a higher capacity for daunorubicin. An alternative explanation for an apparent slower drug influx in Pgp MDR cells might manifest itself in the same way by a decrease in apparent drug influx rate.

Another indication for the presence of an active drug transporter can be obtained by studying the ATP dependence of drug transport as was initially performed by Danø et al. (37). In both non-Pgp-mediated MDR lung cancer cell lines, we found a large increase in daunorubicin accumulation in response to cellular ATP depletion. In the resistant cells, the increase in daunorubicin was accompanied by a 2.5- to 3-fold faster uptake of daunorubicin. In the GLC4/ADR cells, the enhanced daunorubicin extrusion was inhibited as result of the energy depletions. A much smaller effect was detectable in the parental cell lines. This small increase in daunorubicin accumulation in the parental cells could be explained by the lowered intracellular pH in energy-deprived cells. The lower intracellular pH results in a lower concentration of the uncharged form of daunorubicin and, therefore, in a slower passive efflux of daunorubicin.

In the SW-1573/2R120 cells, the increase in steady-state accumulation of VP-16 during energy depletion is larger than the increase in initial uptake rate (6- versus 2.5-fold) but not in the SW-1573 cells (a 2-fold increase in steady-state accumulation and in initial uptake rate). The efflux of VP-16 was 2-fold slower in energy-deprived SW-1573/2R120 cells than in the energy-deprived SW-1573 cells, while no significant difference in efflux rates between resistant and parent cells was observed under normal conditions. These results suggest that the VP-16 accumulation in SW-1573/2R120 cells is reduced as a result of an energy-dependent efflux of VP-16. Also, alterations in VP-16 binding in resistant cells, as suggested by Polit and Sinha (34), might be involved.

In line with the criteria from Skovsgaard (21), the following observations support the existence of an active outward drug transport mechanism in SW-1573/2R120 and GLC4/ADR MDR cells: (a) the steady-state accumulation of daunorubicin in the resistant SW-1573/2R120 and GLC4/ADR cells is below equilibrium binding of daunorubicin to the nucleus, (b) inhibition of energy metabolism resulted in a marked increase in drug accumulation in the resistant cell lines, and (c) efflux of daunorubicin was faster from GLC4/ADR cells than from parent GLC4 cells and the efflux rate was decreased by energy depletions. Such a rapid efflux was not found in SW-1573/2R120 cells compared to the SW-1573 cells, pointing to possible differences in energy-dependent drug transport mechanisms between the two non-Pgp MDR cell lines. For three other MDR cell lines without Pgp expression, a human leukemia cell line, HL-60/ADR (7), a human fibrosarcoma cell line, HT1080/Dr4 (6), and a human large cell lung carcinoma cell line, COR-L23/R (10), an enhanced efflux of doxorubicin and daunorubicin has been reported. For HL-60/ADR cells, it has been suggested that an $M_r$ 190,000, ATP-binding membrane protein was involved in efflux of the drugs out of the cells (8).

Recently, we developed an IgG2b antibody (LRP56) against SW-1573/2R120 cells (38). The antibody showed intense staining of the non-Pgp MDR SW-1573/2R120 and the GLC4/ADR cells during acetone fixation, while the parental cell lines SW-1573 and GLC4 stained only weakly. We speculate that the protein recognized by this antibody is involved in the mechanisms of cytotoxic drug accumulation in lung cancer cells.

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REFERENCES


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