Anthracycine Resistance in P388 Murine Leukemia and Its Circumvention by Calcium Antagonists

David Kessel and Cynthia Wilberding

ABSTRACT

Daunorubicin transport was compared in P388 murine leukemia and in P388/Adriamycin (ADR), an anthracycline-resistant subline. We can demonstrate an energy-dependent outward transport system in P388/ADR which limits drug accumulation. Although no calcium requirement for the outward transport process could be shown, several calcium antagonists inhibited outward transport of daunorubicin in P388/ADR and modified the drug resistance pattern. But these agents failed to alter calcium fluxes in either cell line, suggesting that their mode of action in these studies, was not related to interactions with calcium-dependent processes. Accumulation differences could not account for the level of daunorubicin resistance observed in the P388/ADR cell line, nor could resistance be wholly circumvented by calcium antagonists.

INTRODUCTION

Resistance to anthracyclines and certain other drugs is associated with an energy-dependent outward transport process demonstrable in ADR-resistant P388/ADR (9-12, 35-37) and in other anthracycline-resistant cell lines (3, 6, 7, 16, 24, 28-30). The structural specificity of this transport system was exploited by Skovsgaard, who used N-acetyldaunorubicin to compete for outward transport, thereby promoting DNR cytotoxicity in a drug-resistant cell line (30).

Recent reports have described the ability of calcium antagonists (22-24, 31, 32, 34-36) and calmodulin inhibitors (9, 35) to promote responsiveness to anthracyclines and other natural products in drug-resistant cell lines, apparently via inhibition of the outward drug transport system. In this study, we have examined interactions between anthracycline resistance, calcium fluxes, and resistance circumvention by verapamil and other calcium antagonists.

MATERIALS AND METHODS

Cell Lines. The ADR-resistant P388/ADR cell line was derived from the P388 murine leukemia; drug responsiveness patterns of both cell lines have been described (13, 14). P388 and P388/ADR cells were maintained in cell culture using Fischer’s medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% horse serum. This medium contains 1 mM CaCl2.

Chemicals. [14-3H]Daunorubicin (31 Ci/mol) was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH. Purity was >98% by thin-layer chromatography assay (6, 26) carried out as described below. 44CaCl2 (45 Ci/g) was purchased from New England Nuclear, Boston, MA. Verapamil was obtained from Knoll Pharmaceutical Co., Whippany, NJ, and prenylamine was from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ. Pregnyline maleate was provided by Dr. W. J. Hudak, Merrell Dow Pharmaceuticals, Cincinnati, OH.

DNR Transport Studies. DNR accumulation was measured using cell densities of 2 x 10⁶/ml (7 mg/ml, wet weight) in FHS medium (this medium contained 1 mg/ml glucose unless otherwise specified) at 37 °C. The initial DNR level was usually 0.1 μM, corresponding to approximately 7000 cpm/ml of radioactive drug. In no experiment was >20% of total radioactivity accumulated by cells. In some experiments, drug accumulation was also measured from an extracellular concentration of 2 or 10 μM. Long-term incubations were terminated by dilution with ice-cold medium and centrifugation (30 s; 200 x g). The cells were then washed once with 150 mM NaCl at 0 °C. For some studies, other agents were added 5 min before labeled DNR, and their effects on accumulation of radioactivity were assessed. Influx at times <3 min was measured using the “stopping” solution described by Dalmark and Hofmann (5). This involves a 5-fold dilution of cell suspensions with an isotopic solution containing albumin (10 mg/ml) plus DNA (25 μg/ml). Cell pellets were collected by centrifugation and washed twice with this solution. Some uptake studies were carried out in glucose-free FHS medium containing 10 mM sodium azide to provide an estimate of unidirectional drug influx (6, 26-29).

For studies on outward transport, P388 cells were loaded in medium containing 2 μM labeled DNR during 30-min incubations at 37 °C. So that the initial drug concentration in the 2 cell lines was similar (approximately 30 pmol DNR/10⁹ cells), P388/ADR cells were loaded in medium containing 10 μM DNR. The cells were then collected by centrifugation and suspended in growth medium containing specified levels of calcium antagonists, or in glucose-free medium containing 10 mM sodium azide. At intervals, aliquots of the cell suspension were removed and diluted with chilled buffer. The cells were collected by centrifugation (30 s; 200 x g) for determination of intracellular drug levels.

Purity of labeled DNR and intracellular DNR metabolism were examined by thin-layer chromatography using silica sheets (Eastman 6061). Chloroform:methanol (2:1) extracts of drug-loaded cells were prepared as described previously (6, 26). Appropriate standards were simultaneously run, using a chloroform:methanol:water solvent (80:20:3). After development, fluorescence was quantitated with an Amino scanning fluorometer (excitation, 460 nm; emission, 570 nm). This solvent system can resolve daunorubicinol, daunorubicin, and the corresponding aglycones. The silica sheets were then sectioned for determination of radiolabel associated with each band by liquid scintillation counting.

mRNA Synthesis. To measure export of mRNA from nucleus to cytoplasm (20), cells (10⁶/ml) were incubated with specified levels of DNR for 60 min, then [3H]uridine was added (0.1 μCi). After an additional 30 min, the cells were collected and suspended in fresh medium containing 0.1 mM nonlabeled uridine. After 2 h at 37 °C, the cells were collected and lysed, and mRNA was isolated using oligodeoxythymidylic acid columns. The application buffer contained 500 mM NaCl, and the elution buffer was 10 mM Tris, pH 7.5. In other details, the procedure...
followed the method described previously (20).

Calcium Exchange. Studies of calcium exchange were carried out using cells grown in Fischer’s media which contain 1.0 mM CaCl₂. This was supplemented with 45CaCl₂ (0.1 μCi/ml). After 24 h, the cells were collected by centrifugation and washed once with 0.9% NaCl at 0 °C to remove extracellular radioactivity. Cell pellets (approximately 80 mg) were suspended in 1 ml of 0.9% NaCl at 0 °C, and an aliquot (10 mg of cells) was used for an initial determination of intracellular radioactivity. Time constants and pool sizes were estimated using a reiterative curve-fitting computer program.

Growth Studies. Cells were treated for 4 h with 0.1 to 10 μM DNR and other agents as specified, then washed and diluted appropriately into growth medium plus 0.2% agar. Colonies of control versus treated cells were counted after 7 days.

RESULTS

The time-course of uptake of labeled DNR (extracellular level, 0.1 μM) by P388 and P388/ADR cells is shown in Chart 1 (left). Uptake of 0.35 pmol of DNR/10⁶ cells represents a distribution ratio of 1. These data illustrate the impaired DNR accumulation by P388/ADR cells. The kinetics of DNR accumulation by the 2 cell lines was not altered by omission of Ca²⁺ or addition of 2 mM EGTA in the presence or absence of Ca²⁺. A study of DNR uptake by P388/ADR cells (extracellular level, 10 μM) is also shown (Chart 1, right).

Addition of verapamil promoted DNR accumulation by P388/ADR cells (Chart 1, left) while not affecting drug uptake by P388. The extent of this effect was related to the verapamil concentration. At an extracellular verapamil concentration of 3 μM, DNR accumulation by P388/ADR cells, after 60 min, was increased to 200% of the control value. We define this as an ED₂₀₀ concentration of verapamil. When a verapamil concentration of 20 μM was used, the rate of DNR accumulation by P388/ADR cells resembled that of a control P388 experiment.

Three other calcium antagonists, nifedipine, prenylamine, and perhexiline maleate, and 2 phenothiazine calmodulin inhibitors, triflupromazine and trifluoperazine, also promoted DNR accumulation by P388/ADR cells. Table 1 shows the corresponding concentrations of different agents required to promote the accumulation of the labeled DNR (after 60 min) to 200% of the control value in P388/ADR cells.

A thin-layer chromatography assay was used to examine intracellular drug pools formed when P388 or P388/ADR cells were incubated for 60 min in medium containing 0.1 or 2 μM DNR. The extracted material was >90% DNR, and this was not changed by the addition of verapamil, nifedipine, or trifluoperazine at levels shown in Table 1.

We also examined the effect of 20 μM verapamil on DNR influx. Drug influx over 2 min was markedly different in P388 versus P388/ADR cells (Chart 2, bottom); addition of verapamil selectively promoted DNR influx in P388/ADR cells. In the presence of 10 mM NaN₃ (glucose-free medium), influx of DNR was not significantly different in P388 versus P388/ADR cells (Chart 2, top), nor did 20 μM verapamil have any detectable effect on DNR accumulation.

Chart 3 shows DNR efflux from P388 and P388/ADR cells.
initially loaded with labeled drug. The loading concentrations varied, as described above, to provide similar DNR intracellular levels in both cell lines. In both cell lines, the $t_{1/2}$ of DNR loss into glucose-free medium containing NaH$_2$PO$_4$ was approximately 60 min. This result was not altered when DNR-loaded P388 cells were suspended in FHS medium (containing glucose). However, addition of glucose markedly promoted DNR exodus from P388/ADR cells; 82% of the accumulated drug was rapidly lost ($t_{1/2}$ = 1.6 min) with the remainder more tightly bound ($t_{1/2}$ = 62 min). All agents listed in Table 1 inhibited DNR exodus from P388/ADR cells. When DNR-loaded cells above, were suspended in FHS containing glucose plus 3 mM verapamil, the half-life of the loosely bound drug pool was prolonged to 4.5 min, and the size of this pool was decreased to 64% of total drug accumulated. Prenylamine and perhexiline were only slightly less effective than verapamil as an antagonist of DNR exodus; nifedipine, trifluoperazine and triflupromazine antagonized DNR exodus, but 35% of the 99% lethal level of DNR in P388 cells was 0.1 mM, no nontoxic level of verapamil could yield a 99% cell kill of P388/ADR using 0.1 mM DNR. But 20 mM verapamil plus 0.8 mM DNR was a 99% lethal dose combination.

At the extracellular 99% lethal dose DNR concentrations (0.1 mM for P388 and 10 mM for P388/ADR, the corresponding steady-state intracellular drug levels were 12 and 150 pmol/10^6 cells. P388/ADR cells can therefore proliferate in the presence of an intracellular DNR level which is lethal to P388 cells. This phenomenon is also illustrated by studies on mRNA synthesis. Under conditions described in "Materials and Methods," an extracellular level of 0.3 mM DNR inhibited mRNA synthesis by 50%. In contrast, a similar inhibition of mRNA synthesis in P388/ADR required an extracellular DNR level of 33 mM. The corresponding intracellular DNR concentrations were 40 and 300 pmol/10^6 cells, respectively.

**DISCUSSION**

Dalmark and Hoffmann (5) have compared anthracycline transport in the erythrocyte and in Ehrlich ascites tumor cells and concluded that a diffusional model could account for the observed transport kinetics. Drug resistance in P388/ADR and certain other drug-resistant cells is associated with an additional (unidirectional) outward transport process which recognizes anthracyclines (6, 7, 11, 12), actinomycin D (10), emetine (4), and m-4'n(9-acridinylamino)methanesulfon-m-anisidide (17), a cytotoxic acridine derivative (2). Energy dependence is suggested by inhibition of the exodus process by metabolic inhibitors (3, 6, 10–12, 17, 26–29).

**Table 2**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Additions</th>
<th>% of fast</th>
<th>Fast</th>
<th>Slow</th>
</tr>
</thead>
</table>
| P388/ADR  | 49 ± 2  
| P388     | 51 ± 3  
| P388/ADR  | 35 ± 2  
| P388/ADR  | 50 ± 2  
| P388/ADR  | 50 ± 2  

*Mean ± SD of 5 experiments.*

**Table 3**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>P388</th>
<th>P388/ADR</th>
</tr>
</thead>
</table>
| DNR      | 0.1 mM        | 1.3 ± 0.2  
| DNR      | 0.8 mM        | <0.1  
| DNR      | 10 mM         | <0.1  
| Verapamil| 20 mM         | 95 ± 3  
| DNR      | 0.1 mM        | 1.2 ± 0.1  
| Verapamil| 20 mM         | <0.1  

*Mean ± SD.*

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**ANTHRACYCLINE RESISTANCE**

Chart 3. Exodux at 37° of labeled DNR from P388 and P388/ADR cells initially loaded with the drug. The extracellular loading drug concentrations were 2 µM for P388, and 10 µM for P388/ADR cells. DNR; P388, 0, P388 plus 20 µM verapamil; △, P388/ADR; □, P388/ADR plus 3 µM verapamil; ◊, P388/ADR plus 20 µM verapamil. All agents listed in Table 1 inhibited DNR exodus from P388/ADR cells.

**DISCUSSION**

Dalmark and Hoffmann (5) have compared anthracycline transport in the erythrocyte and in Ehrlich ascites tumor cells and concluded that a diffusional model could account for the observed transport kinetics. Drug resistance in P388/ADR and certain other drug-resistant cells is associated with an additional (unidirectional) outward transport process which recognizes anthracyclines (6, 7, 11, 12), actinomycin D (10), emetine (4), and m-4'n(9-acridinylamino)methanesulfon-m-anisidide (17), a cytotoxic acridine derivative (2). Energy dependence is suggested by inhibition of the exodus process by metabolic inhibitors (3, 6, 10–12, 17, 26–29).
In the present study, we examined the effect of extracellular Ca$^{2+}$ on anthracycline accumulation by P388 and P388/ADR cells. Neither the absence of Ca$^{2+}$ nor addition of the calcium chelating agent EGTA, affected DNR accumulation by either cell line, suggesting that Ca$^{2+}$ does not play a role in DNR influx or efflux. A similar conclusion was reached by Ramu et al. (23) in studies involving the calcium antagonist perhexiline maleate. Studies on DNR influx in P388/ADR cells are complicated by the 

The rate of calcium found 2 approximately equal pools of exchangeable intracellular line, suggesting that Ca$^{2+}$ does not play a role in DNR influx or transport system. The results are, however, consistent with competition for an outward analogue W-acetyldaunorubicin to promote DNR accumulation onized by antimetabolites. In the case of the anthracyclines, it is difficult to reconcile Beck's model with the ability of the DNR fraction, and that decreased DNR binding by P388/ADR cells, alterations in calcium fluxes (32,34). But we found no relationship between calcium fluxes and anthracycline transport. The intra lines. It was suggested that these effects were mediated via mechanisms which may mediate drug resistance. We have recently reported on competition between verapamil and DNR for outward transport in P388/ADR cells to resemble data obtained with P388 (Charts 1 to 3), this did not wholly reverse the degree of DNR resistance observed in P388/ADR (Table 3). Growth of P388/ADR proceeded at intracellular DNR levels which are toxic to P388 cells. Such an observation suggests the presence of multiple modes of drug resistance, a possibility which has been made before (7, 28, 29). Studies on radical detoxification systems (21), and on drug-lipid binding (33) illustrate additional mechanisms which may mediate drug resistance.

Although 20 µM verapamil could change the kinetics of DNR transport in P388/ADR cells to resemble data obtained with P388 (Charts 1 to 3), this did not wholly reverse the degree of DNR resistance observed in P388/ADR (Table 3). Growth of P388/ADR proceeded at intracellular DNR levels which are toxic to P388 cells. Such an observation suggests the presence of multiple modes of drug resistance, a possibility which has been made before (7, 28, 29). Studies on radical detoxification systems (21), and on drug-lipid binding (33) illustrate additional mechanisms which may mediate drug resistance.

The data reported here are in general agreement with a similar study involving measurement of intracellular DNR pools via fluorescence microscopy (37), but these authors concluded that the major difference between P388 and an anthracycline-resistant subline was the relative size of a slowly exchanging drug fraction, and that decreased DNR binding by P388/ADR cells, and not enhanced exodus, was associated with drug resistance. A similar hypothesis has been proposed by Beck et al. (1), who interpreted the enhanced exodus of Vinca alkaloids from drug-resistant cells in terms of intracellular binding which was antagonized by antimetabolites. In the case of the anthracyclines, it is difficult to reconcile Beck's model with the ability of the DNR analogue N-acetyl-4-doxorubicin to promote DNR accumulation and cytotoxicity in an anthracycline-resistant cell line (30). These results are, however, consistent with competition for an outward transport system.

Tsuruo et al. (34–36) and Slater et al. (32) have demonstrated that verapamil and certain other calcium antagonists promote vincristine, ADR, and DNR responsiveness in drug-resistant cell lines. It was suggested that these effects were mediated via alterations in calcium fluxes (32, 34). But we found no relationship between calcium fluxes and anthracycline transport. The intra-cellular calcium level in P388 and P388/ADR cells was 0.2 mmol/kg cells. A similar value was reported for HeLa cells (8). We found 2 approximately equal pools of exchangeable intracellular calcium with different time constants, suggesting the presence of both tightly and loosely bound Ca$^{2+}$. The rate of calcium exchange in these compartments was not altered by calcium antagonists, but was modified by the calcium-chelating agent EGTA. EGTA affected kinetics of calcium exchange (Chart 3) and reduced the rate of 45Ca$^{2+}$ uptake in both cell lines by >90% (results not shown), but did not affect DNR transport in either cell line.

We interpret the data shown in Chart 2 to suggest that verapamil and other calcium antagonists alter neither DNR influx in P388 or P388/ADR cells, nor drug exodus from P388 cells. We conclude that these agents promote DNR toxicity (Table 3) via impairment of DNR exodus (data for verapamil shown in Chart 3) in P388/ADR cells. Rogan et al. (24) reported that verapamil could circumvent anthracycline resistance in tumors of human origin, suggesting a clinical role for this drug combination. But data shown here suggest that additional resistance modes may limit the clinical usefulness of anthracycline plus verapamil.

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REFERENCES


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