Circumvention of Resistance to Daunorubicin by N-Acetyl-Daunorubicin in Ehrlich Ascites Tumor

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ABSTRACT

Experimental evidence indicates that Ehrlich ascites tumor cells resistant to daunorubicin (DNR) have a higher active drug extrusion than do wild-type cells. In the present study, the possibility of circumventing this mechanism of resistance by addition of an analog of DNR, N-acetyl-Daunorubicin (N-acetyl-DNR), was investigated in vitro and in vivo. The affinity of N-acetyl-DNR was 7 times lower than that of DNR in the lysates of both wild-type and resistant cells. In agreement with this finding, N-acetyl-DNR reduced the binding of [3H]DNR to lysate from the two cell lines only to a minor extent. On the other hand, N-acetyl-DNR exerted a marked inhibition on both the active efflux and the unidirectional influx of [3H]DNR in both cell lines. Within certain limits, addition of N-acetyl-DNR resulted in increased steady-state uptake of [3H]DNR; in wild-type cells, the maximal obtainable elevation was 18%, compared to 142% in resistant cells. In vivo addition of N-acetyl-DNR, even at 80 mg/kg for 4 consecutive days, did not influence the toxicity of DNR in mice. In a therapeutic experiment, addition of N-acetyl-DNR increased the antitumor activity of DNR upon the resistant tumor line significantly, but no change was observed in the wild-type tumor. These data indicate that N-acetyl-DNR or related analogs may be used as adjuvants to circumvent acquired resistance to DNR.

INTRODUCTION

Several studies have confirmed that the development of resistance to the anthracycline antibiotics is accompanied by reduced cellular uptake (4, 5, 9, 10, 17, 22, 25, 27, 32, 33, 35). Consequently, the attempts to overcome cellular resistance, natural or acquired, were intended in some way to increase the cellular drug uptake. The first compound used for this purpose was the detergent Tween 80. In vivo, Tween 80 increased both cellular drug uptake and the cytotoxicity of DNR. In vivo, Tween 80 (27) and other detergents (12) were able to increase the therapeutic activity of the anthracyclines. However, the toxicity data in these studies do not exclude the fact that the higher inhibition of the tumor growth was a result of a generally higher cytotoxicity of the combination than that of the drug alone. Increased uptake of ADR has also been demonstrated by hyperthermia (15). In vivo, local hyperthermia increased the therapeutic activity of ADR, whereas the therapeutic gain by whole-body hyperthermia in combination with ADR was doubtful (24). The lysosomotropic therapy principle introduced by Trouet et al. (36) assumes another mechanism of drug uptake and therefore a possible circumvention of resistance to anthracyclines. However, as previously shown, cross-resistance was observed between DNR and DNR-DNA in Ehrlich ascites tumor (34). In the present study, our previously obtained knowledge of the cellular mechanisms of resistance in Ehrlich ascites tumor has been utilized in an attempt specifically to reverse the anthracycline resistance. The 2 major findings on DNR-resistant cells have been a decreased influx (32) and an increased active efflux (9, 32) of DNR. A specific increase in drug uptake might be obtained if the efflux could be reduced by competitive inhibition by a nontoxic substrate. Several findings suggest that N-acetyl-DNR could serve as substrate in this purpose. Thus, N-acetyl-DNR is a substrate for the mechanism of active efflux in resistant cells (32), and it has a very low affinity to DNA (40); consequently, a higher concentration of free drug in cytoplasm might be expected for N-acetyl-DNR compared to analogs with a high affinity to DNA at equal drug concentrations in the medium. Finally, no acute toxicity for this drug could be found in toxicity experiments (39).

MATERIALS AND METHODS

The wild-type tumor line was a hypotetraploid Ehrlich ascites tumor, and the tumor resistant to DNR was a hyperdiploid subline (16). The origin and maintenance of the 2 tumor lines has been described by Danø (6). In experiments with the resistant subline, no treatment was given in the last passage before use.

Preparation of cells, incubation, sampling procedure, and washings were performed as described previously (31). The standard medium contained 57.0 mM NaCl, 5.0 mM KCl, 1.3 mM MgSO4, 9 mM NaH2PO4, 51 mM Na2HPO4, and 1.0 mM glucose (pH adjusted to 7.45). Calf serum (5%, by volume) was added. Experiments on the initial rate of uptake were performed in a medium without glucose containing 10 mM sodium azide; no calf serum was added in these experiments. Preparation of lysed cells has been described previously (31). Microscopic control confirmed that all cells were lysed but that the nuclei remained intact. Incubation of lysed cells was performed in a buffer containing 250 mM sucrose, 5 mM CaCl2, 0.1% (v/v) Nonidet P-40, and 25 mM Tris-HCl (pH adjusted to 7.45). Drug uptake in cells and lysate was determined by measuring the total drug fluorescence extracted from the drained pellet with 0.3 N HCl:50% ethanol as described previously (31). Inasmuch as there was no washing in determination of the binding of drug to lysate, the total fluorescence in the pellet was corrected for remaining extracellular supernatant liquid. Fluorescence of the extractions was determined in an Amino-Bowmann spectrofluorometer (excitation, 470 nm; fluorescence, 585 nm). Fluorescence of samples extracted from untreated cells was subtracted from the experimental samples. Drug concentration was determined in all cases by comparison with spectropho-
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tomically adjusted standards. Using the maximum at 490 nm, $E_{1	ext{cm}}^{1	ext{cm}}$ was 218 for DNR and 186 for N-acetyl-DNR.

For determinations of $[^3H\text{]DNA}$, aliquots of 200 µl cell extract were transferred to the scintillation solution (Packard Insta-Gel; Packard Instrument Co., Downer’s Grove, Ill.), and the vials were counted in a Beckman LS-250 liquid scintillation spectrometer. In experiments in which $[^3H\text{]DNA}$ and N-acetyl-DNR were used simultaneously, the influence of quenching was negligible.

Animal Experiments. For toxicity experiments, mice were given daily doses for 4 days and were observed for a period of 30 days after the first injection. The LD$_{10}$, with 95% confidence limits, was calculated as described previously (6). For chemotherapy studies, 15 x 10$^6$ cells were inoculated i.p. on Day 0, and the mice were randomized. Mice were treated i.p. for 4 consecutive days starting 24 hr after the inoculation.

The mice were weighed before each injection, and the appropriate dose was administered. Results were evaluated by comparing the median survival time of the dead mice of the treated groups with that of an untreated control group.

Chemicals. DNR as hydrochloride was obtained from Farmitalia, Milan, Italy. $[^3H\text{]DNA}$ (0.5 mCi/mg) was kindly supplied by Rhône-Poulenc Research Laboratory, Paris, France.

The radiochemical purity of $[^3H\text{]DNA}$ was determined to be 90%. N-Acetyl-DNR was synthesized according to the method described by Yamamoto et al. (39). To remove remaining DNR (5 to 10%), the final product was dissolved and shaken in chloroform:0.01 N HCl (1:1, by volume). The organic phase was separated, and the procedure was repeated twice with a fresh volume of 0.01 N HCl. After evaporation of the organic phase to dryness, thin-layer chromatography of the drug in 3 different systems as described previously (9, 30) revealed only a single spot. A solution of N-acetyl-DNR was prepared as a stock just before use by dissolving the drug in 0.9% NaCl solution until a saturated solution was obtained at 37°C. When administered in vivo, the stock was used undiluted, and the volume per g mouse was adjusted according to the drug administered in vivo, the stock was used undiluted, and the solution until a saturated solution was obtained at 37°C. When administered in vivo, the stock was used undiluted, and the volume per g mouse was adjusted according to the drug administered. Results were evaluated by calculating the mean of 3 determinations. The units of $r$ are pmol/µl packed cells, corresponding to $r$-pmol/200-µl suspension of lysate C, concentration of free drug in the medium at equilibrium. The lines were fitted by the method of least squares.

RESULTS

After cellular uptake, the anthracycline antibiotics are mainly bound to DNA in the nuclei (3, 13, 30). Consequently, the essential binding characteristics of DNR and N-acetyl-DNR in nuclei may be obtained by determinations of drug uptake in a suspension of lysed cells at equilibrium. To avoid removal of drug from the cellular components, no washing procedure was included in these experiments.

Charts 1 and 2 show a Scatchard plot of the data obtained for DNR and N-acetyl-DNR, respectively. A linear relationship was demonstrated between $r$ and $r/C$, where $r$ represents mol of bound drug to lysate originating from 1 µl packed cells and C is the molar concentration of free antibiotic in the medium at equilibrium.

The binding constants derived from the plot, the apparent association constant ($K_{app}$), and the total numbers of binding sites (n) are presented in Table 1. Comparing the values of $K_{app}$ with those determined when pure DNA was used as substrate (40), it appears that the affinity to lysate, which mainly is composed of nuclei, is about 5- to 7-fold higher than affinity to DNA. No significant difference was observed between $K_{app}$ of DNR and lysate originating from wild-type cells and resistant cells. On the other hand, the number of binding sites for DNR was a little lower in lysate from resistant cells than in lysate from wild-type cells. The data show that the acetylation of DNR results in a considerable reduction of both binding parameters in both cell lines. Thus, the number of binding sites was about 9 times lower and the affinity was about 7 times less for N-acetyl-DNR than for DNR.

Chart 3 shows the unidirectional efflux of N-acetyl-DNR in wild-type cells and resistant cells loaded to different levels of drug content. In all cases, linear functions were obtained when the data were presented in a semilogarithmic plot. This finding indicates that the efflux was a result of a first-order process. In wild-type cells, at least 2 lines are required to fit the data: an initial phase in which $t_{1/2}$ decreased when the loading level decreased; and a subsequent phase in which $t_{1/2}$ was inde-
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Dependent of loading level. A corresponding finding was observed in
resistant cells at lower loading levels. These data indicate
that the second phase reflects the period in which the rate-
limiting step is the release of drug from the intracellular binding
sites. Consequently, the activity of the efflux mechanism in
the membrane is probably represented by the initial phase only. At
loading levels below 800 pmol/μl packed cells, the t1/2 of the
initial phase was significantly lower in resistant cells than in the
wild-type cells. At higher levels of drug content, the t1/2’s of
the initial phase seemed to approach each other in the 2 cell
lines.

If DNR and N-acetyl-DNR are extruded from the cells by the
same mechanism, the efflux of DNR may be suppressed by N-
acetyl-DNR, provided that the free concentration of N-acetyl-
DNR is sufficiently high compared to DNR at the inner surface.
The effect of N-acetyl-DNR on the active efflux of [3H]DNR
was therefore investigated in both wild-type tumor cells and resist-
ant tumor cells. As shown previously, determination of unidi-
rectional efflux of DNR is an unsuitable method to measure the
capacity of the active efflux, because the release from the
nucleus rapidly becomes the rate-limiting step in the efflux

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Kd (10⁶ M⁻¹)</th>
<th>n (pmol/μl packed cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNR</td>
<td>13.82</td>
<td>5107</td>
</tr>
<tr>
<td>N-Acetyl-DNR</td>
<td>2.18</td>
<td>566</td>
</tr>
</tbody>
</table>

The apparent binding constant calculated as the negative of the slope lines
shown in Charts 1 and 2.

The apparent number of binding sites in lysate corresponding to 1 μl packed
cells, equal to 200 μl of the suspension, calculated as the intercept of the lines
on the abscissa in Charts 1 and 2.

**Chart 2.** Scatchard plots for the binding of N-acetyl-DNR to lysate from wild-
type cells (○) and resistant cells (●). Methods are as described in Chart 1.

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**Chart 3.** Efflux of N-acetyl-DNR in wild-type cells (○) and in resistant cells (●) were loaded with N-acetyl-DNR for 15 min at
different drug concentrations (5 to 200 μM) in standard
medium at 37° and were centrifuged. After being washed
twice with Ringer’s solution at 4°, the cells were resus-
pended in standard medium at 0°. Samples (25 to 50 μl)
containing 5 μl packed cells were added to 3000 μl
standard medium at 37°. Incubations were terminated at the
time indicated by rapid injection of 9 ml ice-cold
Ringer’s solution into the cell suspension, and the cells
were separated by rapid centrifugation. In controls, no
significant efflux could be demonstrated at 0° within 5
min. Cellular drug content was determined as described
under “Materials and Methods.” The lines were fitted by
the method of least squares.
process (32). On the other hand, the net efflux of DNR could be followed by reestablishing the energy synthesis in cells which have been loaded with drug while the sources of cellular energy were blocked. In the experiments shown in Chart 4, glycolysis was stopped by omission of glucose from the medium, and oxidative phosphorylation was inhibited by the poison sodium azide. It appears that when both sources of energy are deprived from the cells the uptake at steady state in resistant cells was nearly equal to that of wild-type cells. At 30 min, glucose was added to the medium at a concentration of 10 mM. Initiation of glycolysis in this way resulted in an efflux of $[^3H]$DNR from both cell lines, but most pronouncedly from the resistant cells. When N-acetyl-DNR at 30 μM was added at the same time as was glucose, the efflux was reduced by about 50% in both cell lines.

In Chart 5, the initial uptake of DNR and N-acetyl-DNR at 27°C is shown for the 2 cell types. To prevent active efflux during uptake, glycolytic energy was suppressed by omission of glucose, and respiratory energy was inhibited by sodium azide. For both drugs, the initial uptake displayed a biphasic course with a rapid component within 5 sec (probably implying drug adsorption at the surface), and a subsequent linear component of at least 60 sec duration. It appears that the intercept which represents the rapid component was significantly lower for N-acetyl-DNR than for DNR, indicating a lower affinity of N-acetyl-DNR to membrane structures in the surface. The finding of a linear course of uptake in spite of a concentrative drug uptake indicates that the influx across the membrane is rate limiting to the intracellular binding at this temperature. Therefore, the rate of the unidirectional influx may be determined from the slope of the linear component. On the other hand, preliminary experiments revealed that the duration of the linear component for N-acetyl-DNR lasted for only 15 to 20 sec at 37°C; i.e., the limitation of the technique prohibits a comparison of the rate of influx between the 2 drugs at 37°C. At 27°C, the rate of influx of DNR was 2 and 3 times higher than that of N-acetyl-DNR in wild-type cells and resistant cells, respectively.

To investigate the effect of N-acetyl-DNR on influx of DNR, the initial rate of $[^3H]$DNR was measured in the presence of increasing concentrations of N-acetyl-DNR. Chart 6 shows that N-acetyl-DNR exerts a marked substrate-dependent inhibitory effect on the influx of $[^3H]$DNR; the effect was a little more pronounced in wild-type cells than in resistant cells. Since indications of carrier-mediated influx of several anthracyclines in Ehrlich ascites tumor have been demonstrated previously (31), it seems most likely that the effect of N-acetyl-DNR reflects competitive inhibition of the inward transport of $[^3H]$-DNR.

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**Chart 4.** Efflux of $[^3H]$DNR induced by glucose in wild-type cells (α) and in resistant cells (β) in suspensions corresponding to 5 μl packed cells per ml. The suspensions were incubated in standard medium without glucose containing 10 mM sodium azide. Cells were loaded with $[^3H]$DNR by the addition of 1 μM at time 0. At the arrow, 10 mM glucose or 10 mM glucose plus 30 μM N-acetyl-DNR was added to the suspensions. In controls, a corresponding volume of 0.9% NaCl solution was added. Serial samples were withdrawn at the times indicated, and the cellular drug content after 2 washings was determined as described in "Materials and Methods."

**Chart 5.** Initial uptake of DNR and N-acetyl-DNR in wild-type cells (O, △) and resistant cells (●, ▲) at 27°C. A cell suspension of 10.0% (v/v) was incubated in standard medium without glucose, containing 10 mM sodium azide for 10 min at 27°C. The experiment was started by transferring the cell suspension to a medium of the same composition and temperature including 25 μM drug. During rapid stirring, a final suspension of 0.5% (v/v) was formed, and serial samples of 2000 μl were withdrawn at 5-sec intervals. The flux reaction was terminated by rapid injection of the cell suspension into 8 ml ice-cold Ringer's solution, and the cells were then separated by centrifugation at 3000 × g for 1 min. The cellular drug content after 2 washings was determined as described in "Materials and Methods." The lines were fitted by the method of least squares.
Table 2 shows the therapeutic effect of DNR, N-acetyl-DNR and the combination of the 2 drugs on the wild-type tumor and the subline resistant to DNR. In the wild-type tumor, no significant therapeutic activity could be demonstrated by N-acetyl-DNR, and no difference in response was observed between DNR solely and in combination with N-acetyl-DNR. With this tumor no long-term survivors were observed. The data show that the subline EHR 2/DNR+ was completely resistant to DNR and to N-acetyl-DNR, whereas a prolongation in the life span corresponding to 53% was evoked by a combination of the 2 compounds, and 22% of the mice were long-term survivors.

DISCUSSION

The present data indicate that N-acetyl-DNR is capable of increasing net uptake of DNR by inhibition of the mechanism of active efflux. As both DNR and N-acetyl-DNR are substrates for the mechanism of active outward transport, the inhibition of the efflux of DNR by N-acetyl-DNR is probably an expression of competitive inhibition. On the other hand, the inhibition could also be a result of an impairment of the energy metabolism induced by N-acetyl-DNR, since an inhibitory effect on cell respiration and electron transport systems in mitochondria has
been shown for other anthracyclines (1, 14, 18, 23). However, as shown in Chart 4, glycolytic energy alone is sufficient to permit the active efflux to operate. Furthermore, the very low toxicity of N-acetyl-DNR does not support a specific effect of this compound on the energy metabolism. However, a complete clarification of this point demands unfeasible measurements of the unidirectional efflux of DNR as a function of the cytoplasmic concentration of free N-acetyl-DNR and DNR.

Inasmuch as the capacity of the mechanism of active efflux is considerably higher in resistant cells than in sensitive cells, the maximal increment in net uptake obtainable is significantly higher in the resistant cells.

In vivo, addition of N-acetyl-DNR to DNR in toxicity experiments, in the ratio of 20:1, did not influence the toxicity of DNR. This finding indicates that addition of N-acetyl-DNR did not increase uptake of DNR in normal cells and suggests that normal cells are not capable of extruding anthracyclines by a significant active efflux.

In interpreting the results of therapy experiments, 2 different possibilities for the mechanism of action of N-acetyl-DNR exist: an antitumor activity of the drug itself or the effect this compound exerts on the activity of DNR. A weak antitumor activity of N-acetyl-DNR has been demonstrated in the L1210 tumor (39). Furthermore, Di Marco et al. (11) demonstrated that N-acetyl-DNR may exert a sizable antimitotic activity, even if the DNA synthesis proceeded. The results obtained in this study indicate that N-acetyl-DNR possesses no significant antitumor activity against Ehrlich ascites tumor at the dose level used. Thus, the improvement in the therapeutic effect of DNR obtained when N-acetyl-DNR was added in therapy of the resistant subline can most probably be attributed to the effect which N-acetyl-DNR exerts on uptake of DNR in the tumor cells.

The applicability of N-acetyl-DNR as an adjuvant to therapy with DNR against resistant tumor cells may be ascribed to its low affinity to DNA, as well as its ability to serve as substrate for the mechanism of active efflux. There is no reason to expect that N-acetyl-DNR is unique with respect to these characteristics or that it represents the optimal compound to overcome resistance to DNR. Thus, the influx of N-acetyl-DNR was significantly lower than that of DNR; i.e., to obtain a substrate concentration of N-acetyl-DNR at the inner surface sufficient to inhibit efflux of DNR, the external concentration of N-acetyl-DNR had to be considerably higher than that of DNR. However, as shown in Chart 6, the external concentration of N-acetyl-DNR exerts a concentration-dependent reduction of the influx of [3H]DNR, which in itself results in lower net uptake of DNR. As indicated in a previous study, the influx of anthracyclines may be carrier mediated (31), but little is known about the consequences of modifications of the structure of the anthracyclines for the influx characteristics. According to the present results, the ideal compound to increase net uptake of DNR in resistant cells specifically should possess low affinity to DNA and to the carrier which mediates the inward transport of DNR, whereas the affinity to the mechanism of the active outward transport of DNR should be as high as possible. Furthermore, to obtain a high substrate concentration of the inhibitor at the inner surface, compared to that of DNR, the permeability should be high compared to DNR. By use of screening procedures for the characteristics of inhibition on influx and efflux of DNR of the different anthracyclines, compounds could be selected which are much more effective than N-acetyl-DNR in circumventing resistance to DNR.

To achieve the optimal effect of the adjuvant in vivo, the pharmacological aspects of the regimen also must be consid-

![Chart 8. Log-probit plot of toxicity of DNR given alone (○) and in combination with N-acetyl-DNR at a dose of 30 mg/kg (●). Both drugs were administered i.p., and 10 mice were tested for each dose. Arrows pointing down and up indicate that either no mice or all mice in the group died.](chart8.png)

**Table 2**

Comparison of the effects of DNR and DNR plus N-acetyl-DNR on the life span of mice bearing sensitive or resistant Ehrlich ascites tumor cells

Each group is composed of 18 to 20 mice.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Wild-type tumor</th>
<th>DNR-resistant tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs</td>
<td>Dose (mg/ kg i.p. daily for 4 days)</td>
<td>MSTb (days)</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>10 (9—25)</td>
<td>17 (14—19)</td>
</tr>
<tr>
<td>DNR</td>
<td>1.5</td>
<td>21 (15—43)</td>
</tr>
<tr>
<td>N-acetyl-DNR</td>
<td>30.0</td>
<td>10 (9—23)</td>
</tr>
<tr>
<td>DNR + N-acetyl-DNR</td>
<td>1.5</td>
<td>19 (16—48)</td>
</tr>
<tr>
<td>N-acetyl-DNR</td>
<td>30.0</td>
<td></td>
</tr>
</tbody>
</table>

* Drugs were administered i.p. 24 hr after inoculation of 15 x 10⁶ cells i.p.

b MST, median survival time; ILS, percentage of increase in life span relative to untreated controls.

c Number of mice that survived 60 days after receiving the drug.

d Numbers in parentheses, range.
nered. In this study, the mice received N-acetyl-DNR immediately prior to DNR, equivalent to the situation shown in Chart 7. However, the results obtained in vitro which reflect uptake in a closed compartment may possibly not be analogous to the situation in vivo which is an open compartment. Furthermore, the pharmacological fate of N-acetyl-DNR as well as the influence of N-acetyl-DNR on the pharmacology of DNR is unknown. The confusion on these points may imply that the interval as well as the succession for the 2 drugs chosen in this study are far from optimal.

In several studies, mutual cross-resistance has been shown between the anthracycline derivatives, indicating a mechanism of resistance common to these drugs (2, 7, 19, 20, 29, 37). The findings that reduced cellular drug uptake in resistant cells is also demonstrated for anthracyclines, apart from the analog used in the development of resistance, support this concept (9, 17, 35). Cross-resistance has also been described between anthracyclines and Vinca alkaloids (6–8, 19, 21, 29, 38). In a previous study, the findings indicated that increased active outward transport was a common mechanism of resistance for DNR and vincristine in Ehrlich ascites tumor (33). These findings and the circumscription of resistance to DNR shown in this study by competition of the specific mechanism of efflux may serve as a model for circumscription of resistance to several anthracyclines and Vinca alkaloids.

ACKNOWLEDGMENTS

The author is indebted to S. Olesen Larsen for statistical assistance. The technical assistance of Marianne Knudsen and John Petersen is gratefully acknowledged.

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