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ACTIVE OUTWARD TRANSPORT OF DAUNOMYCIN IN RESISTANT EHRLICH ASCITES TUMOR CELLS

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SUMMARY

The mechanism of a previously reported decreased accumulation of daunomycin in resistant compared to wild-type Ehrlich ascites tumor cells was investigated *in vitro*.

Several findings indicated an active extrusion of daunomycin from resistant cells: (1) Over a certain range of daunomycin concentrations, the accumulation was considerably higher in isolated nuclei from resistant cells than in the corresponding whole cells. (2) The distribution ratio at steady state of daunomycin in resistant whole cells to that in the medium increased with concentration, in contrast to a decrease for isolated resistant nuclei. (3) Accumulation of daunomycin in resistant cells was enhanced by structural analogs (*N*-acetyldaunomycin and daunorubicinol) and by metabolic inhibitors (2-deoxyglucose and iodoacetate).

In wild-type cells the accumulation of daunomycin was also increased by 2-deoxyglucose, suggesting an active extrusion of daunomycin from these cells too. The initial rate of net daunomycin uptake was lower in resistant than in wild-type cells, and the decreased accumulation in resistant cells may be due either to a higher rate of active efflux, a lower rate of influx (in the presence of active efflux), or both. The nuclear binding capacity for daunomycin was about the same in the two cell types.

Vincristine and vinblastine increased the accumulation of daunomycin in resistant cells. Together with previous findings of reciprocal cross-resistance between daunomycin and the vinca alkaloids, and a decreased accumulation of daunomycin in cells selected for resistance to vincristine and vinblastine, this effect suggests that these drugs are transported by the same extrusion mechanism as daunomycin.

INTRODUCTION

Daunomycin** is a cytotoxic antibiotic isolated from *Streptomyces peucetius*. It is an anthracycline, and the chemical constitution of the drug and of some related compounds used in this study is shown in Fig. 1. Daunomycin is an inhibitor of cellular synthesis of DNA and RNA[6,7], probably by binding to DNA by intercala-

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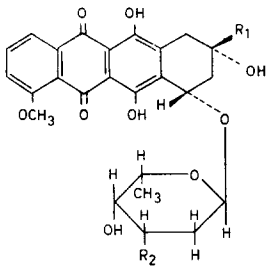
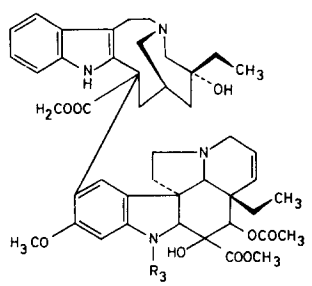
** Daunomycin is identical with rubidomycin and daunorubicin.

tion between the bases in the two strands of DNA[8–10]. Daunomycin inhibits the growth of several animal tumors[11–13] and plays an important role in the treatment of acute leukemia[14,15].



Daunomycin	$R_1 = COCH_3$	$R_2 = NH_2$
Adriamycin	$R_1 = COCH_2OH$	$R_2 = NH_2$
Daunorubicinol	$R_1 = CHOHCH_3$	$R_2 = NH_2$
N-acetyl daunomycin	$R_1 = COCH_3$	$R_2 = NHCOCH_3$



Vincristine $R_3 = CHO$

Vinblastine $R_3 = CH_3$

Fig. 1. Chemical structure of the anthracyclines daunomycin[1], adriamycin[2], daunorubicinol[3] and *N*-acetyldaunomycin, and the vinca alkaloids vincristine and vinblastine[4,5].

Previously, resistance to daunomycin was developed *in vivo* in an originally sensitive strain of Ehrlich ascites tumor by treatment with the drug[16]. The daunomycin-resistant tumor line showed cross-resistance to the antibiotic adriamycin[17] which is closely related to daunomycin (see Fig. 1). Furthermore, cross-resistance was found to the vinca alkaloids vincristine[16] and vinblastine[18] (Fig.1). The vinca alkaloids arrest mitosis during metaphase due to binding to microtubules, and the drugs play an important role in cancer chemotherapy[19,20]. Sublines of the Ehrlich ascites tumor resistant to each of the drugs adriamycin, vincristine and vinblastine have also been obtained by treatment with the respective drugs, and reciprocal cross-resistance has been demonstrated between each of these drugs and daunomycin[17,18].

In vitro investigations on the inhibition of nucleic acid synthesis by daunomycin

in wild-type and resistant Ehrlich ascites tumor cells were previously reported[7] and showed that approximately five times the concentration of daunomycin was needed to give the same degree of inhibition of DNA and RNA synthesis in resistant cells as in wild-type cells. One reason for this difference, but not the whole explanation, was that considerably less daunomycin was accumulated in resistant cells than in wild-type cells[7]. The present study was undertaken to elucidate the mechanism of the decreased daunomycin accumulation in resistant cells. Several findings indicate that there is an active outward transport of daunomycin in resistant cells. It also appears that other anthracyclines and the vinca alkaloids are transported by the same mechanism. Abstracts on part of this study have been published[21,22].

MATERIALS AND METHODS

Tumor cells

The cells investigated were Ehrlich mouse ascites tumor strain EHR 2 (ref. 16) and the corresponding resistant tumor lines EHR 2/DM+ (ref. 16), EHR 2/ADR+ (ref. 17), EHR 2/VCR+ (ref. 18), and EHR 2/VLB+ (ref. 18), in which resistance was developed and maintained *in vivo* by daunomycin, adriamycin, vincristine, and vinblastine, respectively. The tumors were propagated and ascitic fluid collected as described previously[7,16]. No drug treatment was given to the resistant tumor lines in the last passage before the experiments.

Drugs and isotopes

Daunomycin, adriamycin, and [^{3}H]daunomycin (16.6 μCi/mg), all as hydrochlorides, and *N*-acetyldaunomycin, were kindly supplied by Farmitalia Co., Milan, Italy. The radiochemical purity of [^{3}H]daunomycin was assayed by thin-layer chromatography as described below, and more than 89% of the radioactivity was found in the daunomycin spot. In experiments in which [^{3}H]daunomycin was used in concentrations higher than 10 μg/ml, the specific activity was lowered by addition of unlabeled daunomycin. Daunorubicinol was a gift from Dr Nicholas Bachur, National Cancer Institute, Baltimore Cancer Research Center, Baltimore, U.S.A. Vincristine and vinblastine, both as sulphates, were obtained from Lilly Co., Minneapolis, U.S.A. 2-Deoxyglucose was supplied by B.D.H. Chemicals, Poole, England.

Incubation

Unless otherwise stated, incubation of cells was performed as described in detail previously[7] in a medium composed of 90% Earle's solution and 10% calf serum, in a shaking incubator at 37 °C in atmospheric air with 5% CO_2. The pH of the medium remained between 7.0 and 7.4 during the incubations. In all experiments (except those described in Fig. 9) the relative cell volume of the suspensions was 10 μl packed cells/ml as determined by centrifugation in a hematocrit centrifuge. After incubation the suspensions were cooled to 0–4 °C and the cells were pelleted by centrifugation at 900$\times g$ for 4 min and washed twice with a 0.15 M NaCl solution, still at 0–4 °C.

Nuclei

Isolated nuclei were prepared by swelling of the cells in a hypotonic buffer

and homogenization with a close-fitting ball homogenizer as described previously[7]. Using light microscopy the nuclei looked intact with varying but small amounts of cytoplasm attached. The preparations contained 1–3% apparently intact cells. DNA and RNA in cells and nuclei were determined as described[7]. Preliminary experiments showed that there was a loss of 20–30% of the nuclei during preparation as judged by the DNA content. For comparison of uptake in nuclei and cells, nuclei were prepared from 1.2–1.3 times the amount of cells in corresponding cell suspensions. Incubation of nuclei was performed as described for cells.

Determination of daunomycin

The volume of supernatant medium and wash fluid was measured. After addition of one-tenth (v/v) 1.0 M HCl the concentration of daunomycin was determined either by spectrophotometry at 480 nm or by spectrofluorimetry with a Zeiss PMQ II spectrophotometer with ZFM 4 fluoresence attachment (excitation 480 nm, emission 585 nm). In experiments with [^{3}H]daunomycin the radioactivity was determined in a Beckman L 250 liquid scintillation spectrometer (100 μl sample + 10 ml dioxane–methanol–naphthalene scintillation fluid). The possible influence of quenching was ruled out by using appropriate standard solutions of [^{3}H]daunomycin. In all the determinations spectrophotometrically adjusted daunomycin standards were used, assuming $E^{1\%}_{1\,cm}$ in methanol at 490 nm to be 218. The weight statements for daunomycin refer to the hydrochloride. In all cases the combined daunomycin content of the wash fluid was less than 5% of that of the medium.

Calculation of daunomycin uptake in cells and nuclei

In all experiments (except those shown in Fig. 9) daunomycin uptake was calculated by subtraction of the daunomycin content in medium and wash fluid from the amount added to the suspension. The uptake is stated as μg daunomycin/ml suspension. In experiments with cells (except those in Fig. 9) 1 μg/ml suspension = 100 μg/ml packed cells = 177 nmoles/ml packed cells.

Filter disc technique for rapid sampling

In order to measure the rate of daunomycin uptake, cell suspensions were sucked through cellulose acetate filter discs (Millipore Corp., Mass., U.S.A.) 25 mm in diameter, with pore size 1 μm. This filter was chosen because of low adsorption of daunomycin and the adsorption was further reduced by pre-wetting the filters with the medium. Immediately after filtering the suspensions the filters were washed by sucking 25 ml 0.15 M NaCl solution at 0–4 °C through them. Daunomycin was extracted from the cells retained on the filters by shaking for 15 min in a mixture of 0.12 ml 7.5 M HCl and 2.88 ml 96% (v/v) alcohol at 37 °C. The daunomycin concentration was determined spectrofluorimetrically. As blanks, extracts were used from filters through which medium with daunomycin but without cells had been sucked.

Recovery of daunomycin

Previously it was found that all of the daunomycin added to suspensions of cells could be accounted for in the medium and in the extractions from the cells

spectrophotometrically[7]. However, several metabolites of daunomycin have been described which have absorption spectra almost identical with the drug itself: the quantitatively important ones being daunoumycin aglycone, daunorubicinol (Fig. 1), and daunorubicinol aglycone[23].

To evaluate the possible role of metabolism in the uptake, daunomycin was extracted from cells and characterized by thin-layer chromatography using a modification of a technique which has been reported to separate daunomycin from these metabolites[24]. Wild-type and daunomycin-resistant cells or isolated nuclei were incubated with [^{3}H]daunomycin for 60 min. [^{3}H]Daunomycin was extracted from cells and isolated nuclei by homogenization in chloroform–methanol (2:1, v/v), an equal volume of water was added, the emulsion was centrifuged, the organic phase collected and evaporated to dryness, the residue dissolved in a mixture (6:1, v/v) of an 0.02 M acetate buffer (pH 3.8) and *n*-butanol, and after centrifugation the *n*-butanol phase was removed. [^{3}H]Daunomycin was extracted from medium and wash solutions with chloroform and processed as described for the cell extracts. All extractions were repeated 2–4 times. The *n*-butanol solutions were used for thin-layer chromatography on silica gel plates with chloroform–methanol–acetic acid (80:20:4, by vol.) as the mobile phase. The daunomycin spot was localized under 366 nm light by comparison with spots obtained with the authentic compound. The spot was scraped from the plate and mixed with scintillation fiuid. Radioactivity was determined by liquid scintillation spectrometry. The chromatogram remaining after the removal of the daunomycin spot was divided into portions and the radioactivity was determined as above.

There were no major differences between the different preparations, either in recovery during extraction or in the chromatographic pattern of extracted [^{3}H]daunomycin. In all four cases 74–82% of the radioactivity added to the suspensions was recovered in the butanol extract and after chromatography 85–89% of the radioactivity in the butanol was found in the daunomycin spot. (In parallel control chromatograms with authentic [^{3}H]daunomycin, 89–92% of the radioactivity was found in the daunomycin spot.) This makes it unlikely that metabolism of daunomycin plays any major role in the cellular or nuclear uptake under the conditions used in the present study.

In order to test whether, after incubation of resistant cells, daunomycin might be present in the medium in a conjugated form which was transported differently from daunomycin itself, resistant cells were incubated with daunomycin at 20 μg/ml for 60 min. Cells were pelleted and the supernatant was used for suspending wild-type cells after dilution to a concentration of spectrophotometrically active compounds corresponding to daunomycin 10 μg/ml. Authentic daunomycin at 10 μg/ml was added to parallel controls and cellular uptake determined spectrophotometrically. There was no difference in the steady-state uptake in the two cases, and thus no indication of the presence of metabolites or conjugates of daunomycin with transport characteristics different from daunomycin itself.

To evaluate the possible role of binding of daunomycin to protein in the medium, the uptake in wild-type and daunomycin-resistant cells was determined when incubated in medium prepared without calf serum. No difference was observed from control incubations in standard medium (experimental conditions were as in the experiments described in Fig. 2).

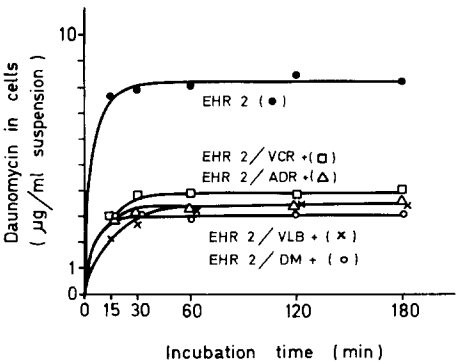


Fig. 2. Uptake of daunomycin in cells from a wild-type Ehrlich ascites tumor (EHR 2) and from sublines in which resistance was developed *in vivo* by treatment with daunomycin (EHR 2/DM+), adriamycin (EHR 2/ADR+), vincristine (EHR 2/VCR+), and vinblastine EHR 2/VLB+). Suspensions of cells (10 μl/ml) were incubated at 37 °C. Daunomycin at 10 μg/ml was added at time 0. Daunomycin remaining in the medium and in two batches of wash fluid obtained at 0–4 °C was determined spectrophotometrically and the cellular drug uptake was calculated by subtraction from the total amount added. 1 μg/ml suspension= 100 μg/ml packed cells.

RESULTS

Uptake of daunomycin in various cell lines

Fig. 2 shows the cellular uptake of daunomycin with time in different cell types when 10 μg/ml is added at time zero. In all the cases a steady state is reached within 15–30 min. Daunomycin is highly concentrated in all the cell types, but less so in resistant than in wild-type cells. The mean cellular steady-state concentration of daunomycin, which was not washed out by two washings at 0 °C, was 810 μg/ml in wild-type cells compared to a concentration of 1.8 μg/ml in the surrounding medium (ratio 450); for the daunomycin-resistant cells the corresponding figures were 310 μg/ml and 6.8 μg/ml (ratio 46). The cells selected for resistance to adriamycin, vincristine, and vinblastine, which were cross-resistant to daunomycin[17,19], showed the same low degree of accumulation of daunomycin as cells selected by treatment with the drug itself.

Steady-state level in isolated nuclei and whole cells

Daunomycin is probably mainly bound to DNA in the nuclei[9,25]. To study whether the difference in cellular uptake was due to a difference in nuclear binding capacity, whole cells and isolated nuclei were incubated with daunomycin and the uptake determined with time (Fig. 3).

In all the cases the uptake of daunomycin reached a steady state within 15–30 min. Slightly less daunomycin was taken up at steady state in the suspensions of resistant than of wild-type isolated nuclei. However, the DNA contents indicate that the yield was lower in the preparation of isolated nuclei from resistant cells, and calculated in relation to DNA content the uptake is a little higher in the suspension of resistant than of wild-type isolated nuclei. The daunomycin uptake in relation to DNA content cannot be taken as an absolute measure for the binding capacity as the daunomycin concentration in the medium at steady state is different in the

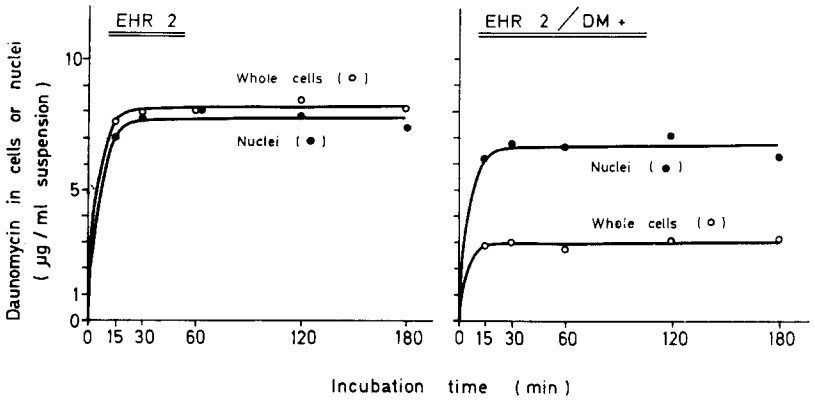


Fig. 3. Uptake of daunomycin in whole cells and isolated nuclei from a wild-type Ehrlich ascites tumor (EHR 2) and a daunomycin-resistant subline (EHR 2/DM+). Suspensions of whole cells (10 µl/ml) or isolated nuclei prepared from 13 µl whole cells/ml (EHR 2) and 12 µl whole cells/ml (EHR 2/DM+) were incubated at 37 °C with daunomycin at 10 µg/ml. The uptake of daunomycin was determined as described in Fig. 2 and is given as µg after two washings in cells or nuclei from 1 ml suspension. DNA, RNA content (nmoles/ml suspension) was as follows: EHR 2 whole cells: 3.80, 4.91; EHR 2 isolated nuclei: 4.19, 0.63; EHR 2/DM+ whole cells: 3.75, 4.68; EHR 2/DM+ isolated nuclei: 3.25, 0.58. Daunomycin uptake at steady state in relation to DNA content (µg/nmoles DNA) was: EHR 2 whole cells: 2.18; EHR 2 isolated nuclei: 1.86; EHR 2/DM+ whole cells: 0.77; EHR 2/DM+ isolated nuclei : 2.13.

two cases, but the findings indicate that there is little difference in the nuclear binding capacity in the same packed cell volume of the two cell types.

The uptake of daunomycin in isolated resistant nuclei was considerably higher than in the corresponding whole cells, despite the fact that the DNA content indicates that the number of isolated resistant nuclei was lower than the number of cells in the corresponding cellular suspension. This finding is in contrast to the daunomycin uptake in isolated wild-type nuclei which was slightly less than in the wild-type whole cells, despite the indication from the DNA content that in this case the number of nuclei in the suspension is higher than the number of cells in the corresponding cellular suspension.

Fig. 4 shows the relation between the steady-state daunomycin concentration in the medium and the uptake of daunomycin in whole cells and isolated nuclei. At low daunomycin concentrations the uptake in wild-type nuclei is about the same as in whole cells, whereas at higher daunomycin concentrations more drug is taken up by whole cells than by nuclei. In resistant nuclei, however, more daunomycin is taken up than in the corresponding whole cells when the steady-state concentration in the medium is below about 30 µg/ml, whereas at higher daunomycin concentrations the uptake is highest in whole cells.

To evaluate whether the binding capacity of the resistant nuclei was lower in their natural intracellular milieu than when suspended in incubation medium, the binding of daunomycin to resistant nuclei was investigated in different media. (1) In 10% calf serum and 90% of the following solution: 71 mM Na^+, 80 mM K^+, 0.4 mM Mg^{2+}, 58 mM Cl^-, 0.4 mM SO_4^{2-}, 35 mM HPO_4^{2-}, 28 mM $H_2PO_4^-$, 5.5 mM glucose (pH 7.2–7.4 in presence of 5% CO_2), a concentration of Na^+, K^+

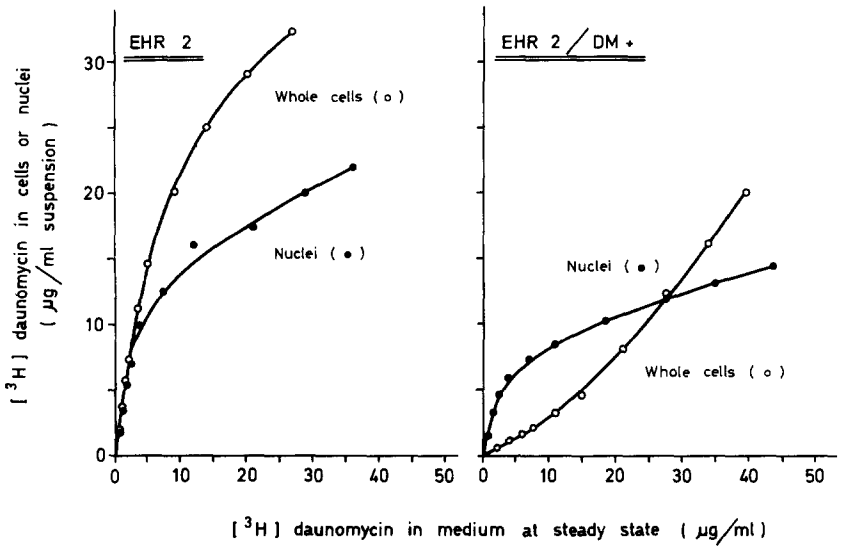


Fig. 4. [^{3}H]Daunomycin in whole cells and isolated nuclei from a wild-type Ehrlich ascites tumor (EHR 2) and a daunomycin-resistant subline (EHR 2/DM+) *versus* concentration in medium at steady state. Experimental conditions as described in Fig. 3, except that [^{3}H]daunomycin (2.5–60 µg/ml, 16.6–2.8 µCi/mg) was added, and the drug optake was determined by liquid scintillation spectrometry after 60 min. Separate experiments showed that at this time steady state was reached even with the highest concentrations. The uptake is given as µg after two washings in cells or nuclei from 1 ml suspension.

and Cl^- similar to that reported[26] for the water phase of Ehrlich ascites tumor cells. (2) In a medium of the same composition except that pH in presence of 5% CO_2 was adjusted by addition of H_3PO_4 to 6.7–6.9, which is less than the intracellular pH reported[27] for Ehrlich ascites tumor cells when incubated in a buffer with pH between 7.2 and 7.4. In both cases the steady-state uptake of daunomycin in resistant nuclei was not significantly different from what was found in parallel incubations in standard medium.

The results in Figs 3 and 4 indicate that at low daunomycin concentrations a three compartment model for the distribution of daunomycin at steady state in suspensions of whole cells may be a good approximation : daunomycin in the medium; daunomycin dissolved in cytoplasm; and daunomycin bound in the nucleus. The above findings make it most likely that at steady state, the distribution of daunomycin between the cytoplasm and the nuclei *in situ* in resistant whole cells is similar to that found between medium and suspended isolated resistant nuclei. With these assumptions, the lower uptake of daunomycin in resistant whole cells than in the corresponding isolated nuclei indicates that the concentration in the cytoplasm of intact resistant cells at steady state is lower than in the medium.

This cannot be explained solely by the existence of a passive diffusion barrier to daunomycin in the cell membrane. A diffusion barrier would influence the time of reaching equilibrium, but at equilibrium the electrochemical potential of daunomycin in the cytoplasm would be equal to that in the medium, and from electrochemical considerations it is likely that the cytoplasmic concentration of free dauno-

mycin at a passive equilibrium would be even higher than the concentration in the medium*. Neither can a lower concentration of daunomycin in the cytoplasm of the resistant cells than in the medium at steady state be explained solely by a decreased active inward transport.

However, the findings could be explained by an active outward transport of daunomycin in resistant cells. As a leak-pump system this outward transport would then at steady state be in equilibrium with an inward transport, which might be carrier-mediated transport, simple diffusion, or both. The shape of the graphs in Fig. 4 supports this hypothesis. In contrast to the findings for wild-type whole cells and isolated nuclei, and resistant isolated nuclei, the graph for resistant whole cells is convex downward, implying that the distribution ratio of daunomycin in resistant cells to that in the medium increased with concentration.

Effect of anthracyclines and vinca alkaloids on accumulation of daunomycin

Competitive inhibitors may provide the opportunity for verifying that a carrier-mediated extrusion is present in resistant cells, as inhibition of an active extrusion of daunomycin in resistant cells would cause an increase in the cellular steady-state level of the drug.

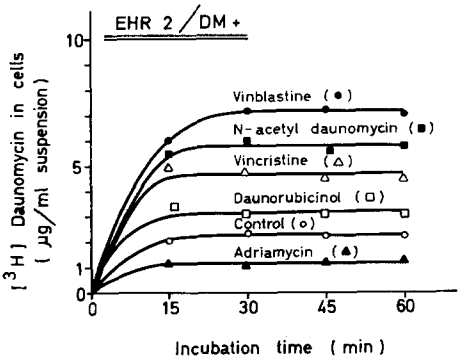


Fig. 5. Effect of various compounds on uptake of [^{3}H]daunomycin in resistant whole cells. Suspensions of cells (10 μl/ml) were incubated at 37 °C. At time 0 [^{3}H]daunomycin (10 μg/ml, 16.6 μCi/mg) was added and simultaneously the compounds indicated were added to give concentrations at 30 μg/ml. [^{3}H]Daunomycin in medium and two batches of wash fluid were determined by liquid scintillation spectrometry and the cellular uptake was calculated by substraction of this from the total amount added.

* Daunomycin·HCl has a pK_A value for the amino group of about 8.2, and in medium and cytoplasm about 90% will be present as daunomycin-H$^+$. Ehrlich ascites tumor cells have been reported[28] to have a membrane potential of about −26 mV, the interior of the membrane being negative in relation to the exterior. This means that at a passive equilibrium the concentration of daunomycin-H$^+$ would be higher in the cytoplasm than in the medium. To explain a concentration in the cytoplasm of resistant cells lower than in the medium, as due to an electrochemically passive equilibrium, requires a membrane potential on the interior which is positive relative to the exterior. It is very unlikely that this is the case in the resistant cells. The membrane transport of daunomycin may actually take place in the form of the uncharged base, due to the higher lipid solubility of the latter, but this would not affect the steady-state levels at a passive equilibrium across the membrane. However, it should be noted that the influence of membrane potential on daunomycin accumulation at steady state will depend on the form in which daunomycin is bound to the nuclei.

Possible competitive inhibitors of daunomycin transport would be structurally related compounds such as adriamycin, daunorubicinol, and *N*-acetyldaunomycin, a derivative of daunomycin with low affinity to DNA (ref. 9) (Fig. 1). Although the chemical structures are different (see Fig. 1), other possible competitive compounds would be the vinca alkaloids vincristine and vinblastine, because of the common mechanism of uptake, suggested by the reciprocal cross-resistance between these drugs and daunomycin[16,18], and by the decreased uptake of daunomycin in cells with resistance developed to vinca alkaloids (Fig. 2).

Experiments were performed as shown in Fig. 5 with measurement of the effect of the above mentioned compounds on uptake of [^{3}H]daunomycin in resistant cells. Vinblastine, *N*-acetyldaunomycin, vincristine, and daunorubicinol all caused an increase of varying degree in the accumulation of [^{3}H]daunomycin at steady state in resistant cells; vinblastine being most effective and daunorubicinol least effective. On the other hand adriamycin caused a decrease in the steady-state level of [^{3}H]-daunomycin.

The effect of vincristine on the accumulation of [^{3}H]daunomycin at steady state was investigated at different concentrations of daunomycin. The results are shown in Fig. 6. In resistant cells, vincristine at 30 μg/ml increased the steady-state level

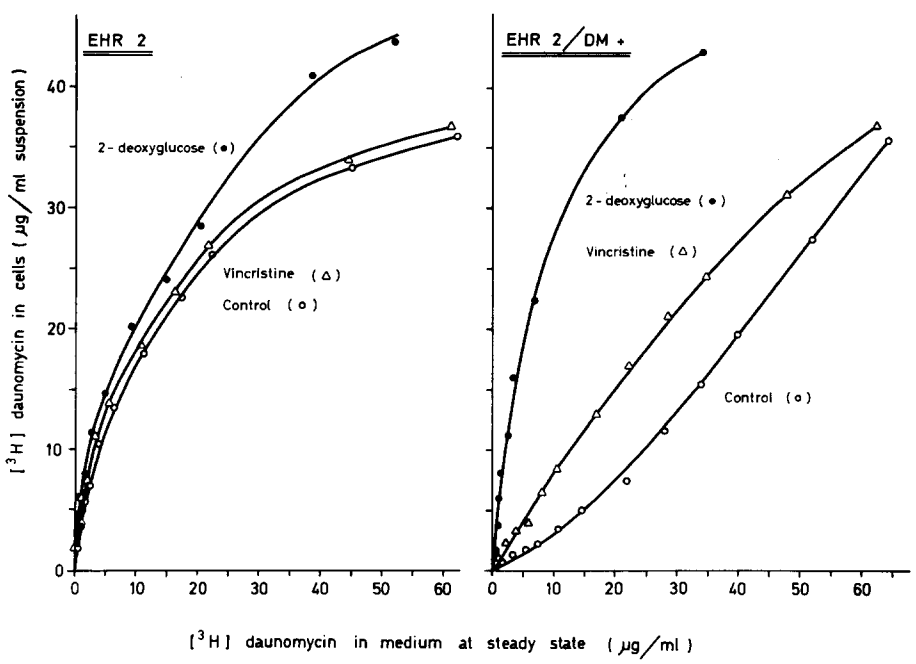


Fig. 6. The effect of vincristine and 2-deoxyglucose on intracellular *versus* extracellular concentration of [^{3}H]daunomycin at steady state in cells from a wild-type Ehrlich ascites tumor (EHR 2) and a daunomycin-resistant subline (EHR 2/DM+). Suspensions of cells (10 μl/ml) were incubated at 37 °C. [^{3}H]Daunomycin (2.5 μ 100 μg/ml, 16.6–1.66 μCi/mg) was added at time 0. Vincristine at 30 μg/ml was added simultaneously with daunomycin. 2-Deoxyglucose at 10 mM was added at −5 min to cells which were preincubated for 60 min in a medium prepared with Earle's solution from which glucose and phosphate were omitted. Further experimental conditions were as described in Fig. 4.

of daunomycin at low daunomycin concentrations. The effect of vincristine decreased with increased daunomycin concentrations, and with an initial daunomycin concentration at 100 μg/ml which gave a steady-state concentration in the medium at about 60 μg/ml, there was almost no effect of vincristine 30 μg/ml on the cellular steady-state level of daunomycin. These findings are compatible with a competitive inhibition by vincristine of an active saturable outward daunomycin transport, and support the hypothesis that anthracyclines and vinca alkaloids share the same transport system. The different effectiveness of daunorubicinol, *N*-acetyldaunomycin, vincristine and vinblastine in increasing uptake of [^{3}H]daunomycin may reflect differences in affinity to the outward transport mechanism.

The decrease in steady-state level of [^{3}H]daunomycin caused by adriamycin does not exclude that adriamycin is transported by the same transport system as daunomycin, but may suggest that also the inward transport of daunomycin is carrier-mediated (but not uphill). The effect of adriamycin on the daunomycin-pump might then be an example of competitive acceleration as discussed by Rosenberg and Wilbrandt[29].

The effect of several other compounds on the steady-state level of [^{3}H]daunomycin in resistant cells was investigated under conditions identical to those described in Fig. 5: tetracycline chloride, streptomycin sulphate, colchicine, atropine sulphate, ethylmorphine, allantoin, urea, β-alanine and caffeine. At a concentration of 30 μg/ml none of these compounds affected the steady-state level of [^{3}H]-daunomycin. This suggests that the hypothetical extrusion mechanism is rather specific, in spite of the indications that it transports such chemically different compounds as anthracyclines and vinca alkaloids.

An alternative explanation for the increased daunomycin uptake induced by *N*-acetyldaunomycin, daunorubicinol, and vinca alkaloids is damage to the cell membrane, increasing the leakage of daunomycin and thus diminishing the concentration gradient maintained by an active outward transport. Another explanation would be that the compounds affected the extrusion of daunomycin in a way which was not competitive or affected another cell function which was necessary for outward transport of daunomycin. These explanations all presuppose an active extrusion of daunomycin from resistant cells.

An explanation which does not imply an active extrusion of daunomycin is that the compounds increase the intracellular binding capacity for daunomycin. Experiments similar to those in Fig. 5 were therefore performed with suspensions of nuclei from resistant cells, but no effect on the daunomycin uptake in nuclei was found, either with *N*-acetyldaunomycin and daunorubicinol or with the vinca alkaloids. This result does not exclude that the drugs affect the binding capacity of the nuclei *in situ*, but makes it unlikely. It should be noted that all the alternative explanations require that these effects are caused by *N*-acetyldaunomycin, daunorubicinol, and the vinca alkaloids, but not by the above mentioned compounds which did not affect the daunomycin uptake.

Effect of metabolic inhibitors on accumulation of daunomycin

2-Deoxyglucose was found to increase the accumulation of daunomycin considerably in resistant cells which were preincubated in a medium where glucose and phosphate were omitted from Earle's solution (Fig. 7). Control resistant cells in-

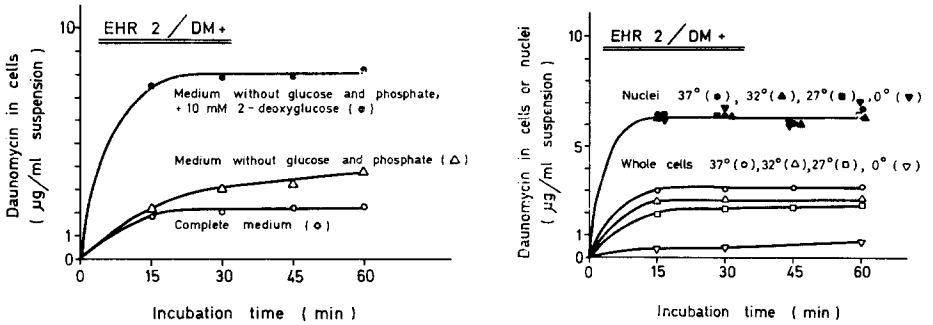


Fig. 7. Effect of 2-deoxyglucose on uptake of daunomycin in resistant cells. Suspensions of cells (10 μl/ml) were incubated at 37 °C in medium prepared with Earle's solution or with Earle's solution from which glucose and phosphate were omitted. Incubation was started at −60 min, and at −5 min. 2-Deoxyglucose was added at 10 mM. Daunomycin at 10 μg/ml was added at time 0 and the uptake determined spectrophotometrically as described in Fig. 2.

Fig. 8. Temperature dependence of uptake of daunomycin in whole cells and isolated nuclei from a daunomycin-resistant Ehrlich ascites tumor. Experimental conditions were as described in Fig. 3, except that the incubations were performed at various temperatures, as indicated.

cubated in a medium where glucose and phosphate were omitted from Earle's solution showed a moderate increase in daunomycin uptake, compared to cells incubated in complete medium. This was observed in several experiments and may indicate a progressive impairment of the efficiency of an extrusion mechanism under these conditions.

The effect of 2-deoxyglucose on the steady-state level of [^{3}H]daunomycin in resistant cells was also investigated in experiments where the concentration of [^{3}H]daunomycin was varied. The results in Fig. 6 show that in contrast to the findings with vincristine, the effect of 2-deoxyglucose was not diminished when the daunomycin concentration was raised. This suggests a different mechanism of inhibition of daunomycin efflux with the two compounds.

When resistant cells were preincubated with iodoacetate at 0.035 mM for 60 min before addition of daunomycin at 10 μg/ml, the cellular uptake of daunomycin after another 60 min was increased from 2.7 ± 0.2 to 4.6 ± 0.4 μg/ml suspension*.

These findings are compatible with an active extrusion of daunomycin, dependent on energy metabolism, but the alternative explanations mentioned for the action of anthracyclines and vinca alkaloids on daunomycin uptake (unspecific damage to the cell membrane or the daunomycin-pump, increase of intracellular binding capacity) can also be applied in the case of the action of 2-deoxyglucose and iodoacetate.

In wild-type cells it was found (Fig. 6) that 2-deoxyglucose caused a considerable increase in the steady-state level of daunomycin, while a small, but repeated increase in the daunomycin level was seen with vincristine. This suggests that there is an active extrusion of daunomycin also from these cells.

The steady-state level of daunomycin in 2-deoxyglucose-poisoned cells may be taken as a measure of the intracellular binding capacity for daunomycin, and Fig. 6 then shows that the intracellular binding capacity of daunomycin-resistant cells is about the same as that of the same packed volume of wild-type cells.

* Mean of five parallel incubations ± S. E. of the mean.

Temperature dependence of steady-state level

The uptake of daunomycin in resistant cells and isolated nuclei was investigated at different temperatures and the results are shown in Fig. 8. For nuclei, the uptake at steady state appears to be unchanged by a change in temperature from 37 to 0 °C. For resistant whole cells there was a moderate but significant decrease in the steady-state level when the temperature was decreased from 37 to 27 °C. At 0 °C the rate of uptake of daunomycin in resistant whole cells was strongly decreased and a steady state was not reached within 60 min. The rate-limiting process in the cellular uptake of daunomycin at 0 °C is most probably the transport across the membrane, as the binding to nuclei at 0 °C reached the steady-state level within 15 min.

The steady-state level of daunomycin will be dependent of the relation between the rate of inward and outward transport. The small decrease with temperature in the steady-state level of daunomycin in resistant cells indicates that there is a slightly higher degree of temperature dependence of the inward than of the outward transport. Generally, carrier-mediated transport is expected to have a high degree of temperature dependence and diffusion a lower degree. Provided outward daunomycin transport is carrier mediated, the findings therefore indicate that the inward transport is also carrier mediated. However, as pointed out by Danielli and Davson[30], in some instances diffusion may be expected to have a high degree of temperature dependence, and the conclusions as to the nature of the transport processes for daunomycin which may be drawn on the basis of the temperature dependence of the steady-state level, are therefore limited.

Rate of daunomycin elution

The rate of elution of daunomycin from resistant cells was measured in experiments (not shown) where the cells were incubated at 37 °C with daunomycin, pelleted by centrifugation and transferred to medium without daunomycin. There was an increase in the concentration of daunomycin in the medium which was linear for 5 min. However, when similar experiments were performed with nuclei, the rate of elution was of the same order of magnitude. It could not be excluded that the rate-limiting process measured by elution from whole cells was the release from the nuclei. Therefore the system is not suitable for direct studies of the extrusion of daunomycin.

Rate of daunomycin uptake

The rate of uptake of daunomycin was determined in experiments where the cells for rapid sampling were collected on filter discs. In order to keep the fall of the daunomycin concentration in the medium at a minimum during the sampling period, the cells were incubated in suspension with a cell density (1 μl/ml) which was one-tenth of the usual. Results in Fig. 9 (inset) show that the uptake was linear with time for 5 min, and the slope of this line was taken as the rate of uptake. The uptake curves do not pass through the point of origin. This can partly be due to trapping of medium between the cells and the filter, but this cannot explain the whole difference. These findings suggest that before the linear phase of uptake there is a phase of rapid binding of daunomycin, which has been completed within 15 s. The experiments were performed with initial daunomycin concentrations between 0.2 and 10 μg/ml, and in this range the rate of uptake was directly proportional to the

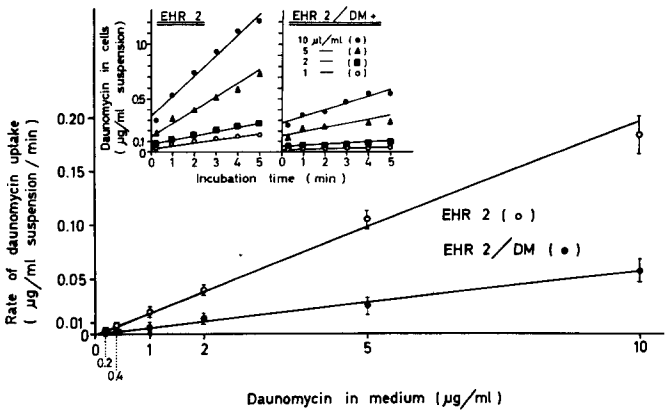


Fig. 9. Initial rate of daunomycin uptake in cells from a wild-type Ehrlich ascites tumor (EHR 2) and a daunomycin-resistant subline (EHR 2/DM+) *versus* concentration in medium. Suspensions of cells (1 μl/ml) were incubated at 37 °C. Daunomycin was added and samples of 5 ml of the suspension were taken 15 s and 1, 2, 3, 4, and 5 min later, the cells were collected on filter discs, the filters were washed by suction with 25 ml of a 0.15-M NaCl solution at 0–4 °C, and daunomycin was extracted as described in Materials and Methods. Daunomycin was measured spectrofluorimetrically and the rate of uptake was calculated as the slope of a line fitted by the method of least squares. Vectors indicate 95% confidence limits. Inset, the cellular daunomycin uptake with time for the four highest initial concentrations. Note that the relative cell volume in these experiments was one-tenth of the usual. 1 μg daunomycin/ml suspension= 1 mg daunomycin/ml cells in this case.

daunomycin concentration for both wild-type and daunomycin-resistant cells; the rate being more than three times higher for wild-type than for resistant cells.

With rates of daunomycin uptake as calculated, the mean overall intracellular daunomycin concentration reached the extracellular concentration in 3 s in wild-type cells and in 10 s in resistant cells. This raises the questions whether the rate-limiting process in the daunomycin uptake is the membrane transport or the binding in the nuclei and whether during the period of measurement there is an outward flow of daunomycin, so that the rate of net uptake measured is smaller than the influx. The lower uptake in resistant cells despite the indications that the intracellular binding capacity is as high as in the wild-type cells (Fig. 6), argues for the membrane transport being rate limiting in resistant cells. The linearity of the uptake with time argues for the efflux in both cell types being negligible in the first 5 min. A final answer to these questions requires further investigations.

Provided that the membrane transport is rate limiting for daunomycin uptake, that the net flux is equal to the influx, and that the cytoplasmic concentration of free daunomycin is practically zero during the first 5 min, then the linearity of the uptake rate with increasing concentration in the medium is in agreement both with a diffusion mechanism of the inflow, and with a carrier mechanism in which the carrier is well below saturation within the investigated range of concentrations.

With the assumption that outward flow in resistant cells is negligible in the first 5 min, it is further indicated that the inward permeability to daunomycin of resistant cells is lower that that of wild-type cells.

DISCUSSION

Several findings on the steady-state level of daunomycin support the tentative conclusion that in resistant cells there is an outward flow of daunomycin which is against an electrochemical gradient, carrier mediated, and dependent on the energy metabolism. These are:

(1) Over a certain range of daunomycin concentrations, the accumulation was considerably higher in isolated nuclei from resistant cells than in the corresponding whole cells (Figs 2 and 3).

(2) The distribution ratio of daunomycin in resistant whole cells to that in the medium, increased with concentration, in contrast to a decrease for isolated resistant nuclei (Fig. 3).

(3) Accumulation of daunomycin in resistant cells was enhanced by some structural analogs (Fig. 5) and by metabolic inhibitors (Figs 6 and 7).

It should be noted that the supposed daunomycin-pump may be localized not only in the cell membrane but also in the nuclear membrane. If this is the case, the pump may be impaired in the isolated nuclei, because they are deprived of their energy supply.

In wild-type cells an active extrusion of daunomycin may be indicated by an increase in accumulation of daunomycin by 2-deoxyglucose treatment (Fig. 6), but the influence of a hypothetical daunomycin extrusion is much less pronounced in wild-type cells.

Data on the daunomycin uptake in 2-deoxyglucose-poisoned cells indicate that the intracellular binding capacity is about the same in wild-type and resistant cells (Fig. 6). The rate of net daunomycin uptake during the first 5 min was lower in resistant than in wild-type cells, suggesting a lower influx (Fig. 9). Thus the lower accumulation in resistant compared to wild-type cells may be due either to a higher efficiency of the active daunomycin extrusion, a decreased influx (in presence of active extrusion), or both.

A linear increase of the rate of daunomycin uptake with concentration (Fig. 9) suggests that daunomycin influx is either diffusion or carrier mediated with a carrier which is not saturated at 10 μg/ml. The steady-state level of daunomycin in resistant cells decreased slightly with temperature (Fig. 8), combined with the indications of carrier-mediated efflux this suggests that influx is either carrier mediated or the result of diffusion with high degree of temperature dependence. A decrease in accumulation of daunomycin by adriamycin (Fig. 5) argues for the daunomycin influx being carrier mediated.

Vincristine and vinblastine increased the steady-state level of daunomycin in resistant cells (Figs 5 and 6), suggesting that these drugs are transported by the same active extrusion mechanism as daunomycin.

The hypothesis of a common transport mechanism for daunomycin and the vinca alkaloids is supported by reciprocal cross-resistance between these drugs[16,18] and by the decreased uptake of daunomycin in cells selected for resistance to vincristine and to vinblastine (Fig. 2).

Similarly, it was suggested that *N*-acetyldaunomycin and daunorubicinol are transported by the daunomycin-pump (Fig. 5). Adriamycin decreased the daunomycin accumulation, but reciprocal cross-resistance between daunomycin and adria-

mycin-resistant cells, and a previously reported[21] decreased accumulation of adriamycin in daunomycin-resistant cells, still make it likely that adriamycin is transported by the same efflux mechanism as daunomycin.

The present study is based on observations on the steady-state level of daunomycin. The system is well suited for such observations, as the high nuclear binding capacity for daunomycin will amplify changes in the cytoplasmic concentration of free daunomycin at steady state up to a 100-fold and make them easily recognizable. On the other hand, the nuclear binding complicates direct measurements on the membrane transport of daunomycin: the cytoplasmic concentration of daunomycin cannot be measured directly, and on measurement of the efflux of daunomycin it is not known whether the rate-limiting process is in liberation from the binding in the nuclei or the membrane transport. Other compounds transported by the same mechanism but less firmly bound in the nuclei may therefore be more suitable for direct measurements.

The data presented permit a rough estimate of the magnitude of extrusion of daunomycin from resistant cells. From Fig. 6 it is suggested that with a steady-state concentration of daunomycin in the medium at 10 μg/ml, the cytoplasmic concentration in resistant cells will be about 1 μg/ml, *i.e.* a gradient of 9 μg/ml. Fig. 9 suggests that with this gradient the daunomycin influx will be 0.06 μg/μl cells per min = approx. 6 mmoles/l cells per h, and because of the steady state the efflux will be equal to the influx. Fig. 6 indicates that the extrusion mechanism is not saturated at this concentration and the capacity of the mechanism may be a few fold higher. Maizels *et al.*[47] have found that efflux of Na^+ in Ehrlich cells at steady state was 300–600 mmoles/l cells per h. From the point of view of energetics the hypothesis of active daunomycin efflux therefore seems to be reasonable.

Experiments implying active extrusion of drugs from mammalian cells have been reported for methotrexate[31] and folic acid[32] but not for daunomycin. Therapeutic response of several transplantable mouse tumors to daunomycin has been reported[33] to be correlated with retention of the drug, and development of resistance to daunomycin has in several cases been found to be associated with a decreased drug uptake[34,35]. Similar findings have been reported for other cytotoxic drugs, *e.g.* methotrexate[36–38], nitrogen mustard[39–41], glucocorticoids[42,43], and actinomycin D[44–46]. Active extrusion has not been proposed as the mechanism of decreased drug uptake in resistant cells in any of the cited cases, but might well appear to play a role in some of these systems.

The increase in daunomycin uptake in resistant Ehrlich cells achieved by structural analogs implies possibilities of reversing that part of the resistance which is due to decreased drug uptake, by treatment with daunomycin in combination with such analogs. However, the therapeutic value of combination treatment may be limited by toxicity: even if the analog itself is atoxic in the necessary concentration, it may enhance the toxicity of daunomycin by increasing the uptake also in normal cells, should these also have an active extrusion of daunomycin. Furthermore, it has been found previously that in Ehrlich ascites tumor cells, decreased uptake is only one of several factors in the mechanism of resistance to daunomycin[7].

The selective effect of daunomycin on malignant cells might possibly be related to the existence of a daunomycin pump which is more effective in normal than in some malignant cells, and exploration of this and of the efficiency of this

pump for transport of daunomycin analogs might be of importance for development of more selective drugs. Similar considerations are possibly relevant for the vinca alkaloids.

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