

Mechanism of Cross-Resistance between Vincristine and Daunorubicin in Ehrlich Ascites Tumor Cells¹

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ABSTRACT

An investigation was undertaken of the mechanism of a previously reported cross-resistance between vincristine (VCR) and daunorubicin (DNR) in Ehrlich ascites tumor cells. No significant difference was demonstrated for the time course of [³H]VCR uptake in cells resistant to VCR (EHR 2/VCR+) and in cells resistant to DNR (EHR 2/DNR+), whereas wild-type cells accumulated nearly 6-fold more drug at steady state. The energy dependence of [³H]VCR and of DNR transport was investigated by the metabolic inhibitors sodium azide and iodoacetic acid. These studies revealed that uptake of [³H]VCR and of DNR was depressed in both resistant sublines by an energy-dependent process that mostly requires energy from glycolysis. If glucose was omitted from the medium together with addition of sodium azide, the uptake of [³H]VCR and of DNR in EHR 2/VCR+ reached a level nearly equal to that of wild-type cells. If glycolysis was restored by addition of glucose to the resistant cells loaded with drug in this way, a pronounced extrusion of [³H]VCR and of DNR was induced. In a similar experiment with wild-type cells, a slight but significant extrusion of [³H]VCR could be induced.

The studies showed that, for nearly unidirectional influx, the cells must be incubated in the medium without glucose but with sodium azide. In this medium the influx of [³H]VCR and of DNR was significantly higher in wild-type cells than in cells from the resistant sublines. The flux of DNR was not competitively inhibited by VCR either in wild-type cells or in resistant cells. The data indicate that the mechanism of cross-resistance between VCR and DNR in Ehrlich ascites tumor cells is a result of at least two different mechanisms: (a) an energy-dependent drug extrusion common to VCR and DNR; and (b) unspecific changes in the membrane, which reduce the influx of both compounds.

INTRODUCTION

In several studies, cross-resistance between anthracycline derivatives and *Vinca* alkaloids has been shown (3, 10-12, 17, 24, 30). These observations are surprising as they belong to 2 different classes of drugs, with different cellular targets and mechanisms of action. The findings of cross-resistance suggest a common mode of resistance for *Vinca* alkaloids and the anthracyclines. In VBL²-resistant Ehrlich

ascites cells, Creasey (9) had demonstrated both reduced uptake of [³H]VBL and slower conversion of the drug to alkali-labile material. In a VCR-resistant subline of P388, Bleyer *et al.* (4) demonstrated decreased accumulation and binding of [³H]VCR. As regards the anthracycline antibiotics, it has been confirmed in several tumor systems that decreased cellular drug uptake is an important mechanism of resistance (7, 8, 13, 14, 18, 23, 28). For DNR the mechanism of decreased drug uptake in Ehrlich ascites tumor cells has been shown to be multifactorial, including decreased influx, increased efflux, and a lower affinity for intracellular binding sites (13, 27).

The present study was undertaken to elucidate the cellular mechanism of resistance to *Vinca* alkaloids in Ehrlich ascites tumor cells and the mechanism of cross-resistance between *Vinca* alkaloids and anthracyclines. As compounds, VCR was chosen as representative of the *Vinca* alkaloid group, and DNR was chosen as representative of the anthracycline group.

MATERIALS AND METHODS

[³H]vincristine sulfate was obtained from the Radiochemical Centre, Amersham, England, and was stored at -20° in the dark. The compound was supplied as a solution in methanol (adjusted to pH 4.3 with 0.05 N sulfuric acid) with a specific activity of 2.6 to 9.1 Ci/mmol. The radiochemical purity of the product was determined by thin-layer chromatography on silica gel plates. A mixture of 0.4 × 10⁻⁷ mmol [³H]VCR and 1 mmol VCR was dissolved in 1.0 ml methanol and chromatographed in the solvent systems: (a) diethyl ether:toluene:methanol:diethylamine (100:5:5:5), and (b) chloroform:methanol:formic acid (70:20:5). The radioactivity in spots identified as the authentic compound (viewed under UV) as well as in the residue of the chromatogram divided into sections was determined by liquid scintillation. In both systems, >93% of the counts were within the VCR spot. Vincristine sulfate (Oncovin) was obtained from Eli Lilly & Co., Indianapolis, Ind. DNR as hydrochloride was obtained from Farmitalia, Milan, Italy. Sodium azide was obtained from Merck, Darmstadt, Germany; and iodoacetic acid from Sigma Chemical Co., St. Louis, Mo.

Experiments were performed with Ehrlich ascites tumor cells maintained in first-generation hybrids of female random-bred Swiss mice and male inbred DBA mice by weekly transplantation. Development of the tumor lines resistant to VCR (EHR 2/VCR+) and to DNR (EHR 2/DNR+) was performed *in vivo* by treatment with increasing doses of drug during weekly passages of the tumor (10, 12). The resistant tumor lines were maintained by daily treatment for 4 days in each passage (total dose = 10% lethal dose); no drug treatment was given in the last passages before the experi-

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² The abbreviations used are: VBL, vinblastine; VCR, vincristine; DNR, daunorubicin.

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ments. Unless otherwise stated, the preparation of cells, incubation, and the sampling procedure were performed as previously described (26, 27). The standard medium contained 57.0 mM NaCl, 5.0 mM KCl, 1.3 mM MgSO₄, 9 mM NaH₂PO₄, 51 mM Na₂HPO₄, and 10 mM glucose (pH adjusted to 7.45). In all experiments, calf serum constituting 5% v/v was added.

Determination of Drug Uptake. For determination of the cellular content of [³H]VCR, the pellet was digested in 0.8 ml 0.5 N KOH at 70° for 1 hr. After cooling to room temperature, 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]VCR and DNR were used simultaneously, the influence of quenching was negligible. Cellular uptake of DNR was determined by measuring the total drug fluorescence extracted from the drained cell pellet as previously described (26).

The metabolism of DNR in wild-type cells of Ehrlich ascites tumor has previously been evaluated in this laboratory (13, 25). By the thin-layer chromatography technique, the metabolism was found to be <10% during a 1-hr incubation. By the same technique the metabolism of DNR in EHR 2/VCR+ was determined to be <5%. The metabolism of [³H]VCR was determined by a modification of the method of Castle and Mead (6). One μM [³H]VCR was added to 25 ml suspension of cells (0.5% v/v) of wild-type tumor cells, EHR 2/VCR+, and EHR 2/DNR+. Controls consisted of a corresponding suspension of the same tumor without drug. After incubation for 60 min at 37° in standard medium, the cells were homogenized by ultrasonification at 0°, and the suspensions were mixed with an equal volume of ice-cold ethanol including 1 mg VCR carrier. To the controls, 2.5 nmol [³H]VCR were also added. The solutions were adjusted to pH 8 with ammonium hydroxide and centrifuged. The supernatant was decanted off, and the pellet was extracted twice more with 5 ml ice-cold ethanol. The pooled ethanol fractions were extracted twice with an equal volume of methylene chloride, and the organic phase from each of the extractions was evaporated to dryness. The residue was redissolved in methanol and chromatographed as described previously. The VCR spot was identified under UV, scraped off from the plate, and assayed for tritium. The chromatogram remaining after removal of the VCR spot was divided into equal sections and assayed for tritium. No major differences were observed between the 3 tumor lines. In all cases, >94% of the radioactivity of the VCR spot of controls were recovered from the VCR spot of the tested extracts.

RESULTS

Chart 1 illustrates the time course of uptake of [³H]VCR in cells of the wild-type tumor and in cells of sublines resistant to VCR or to DNR. Both the rate of net uptake and the steady state were considerably higher in the wild-type tumor than in the 2 resistant sublines. At steady state the cell:medium ratio in wild-type tumor cells was calculated as 10. No significant difference could be demonstrated between the steady-state levels of EHR 2/VCR+ and EHR 2/

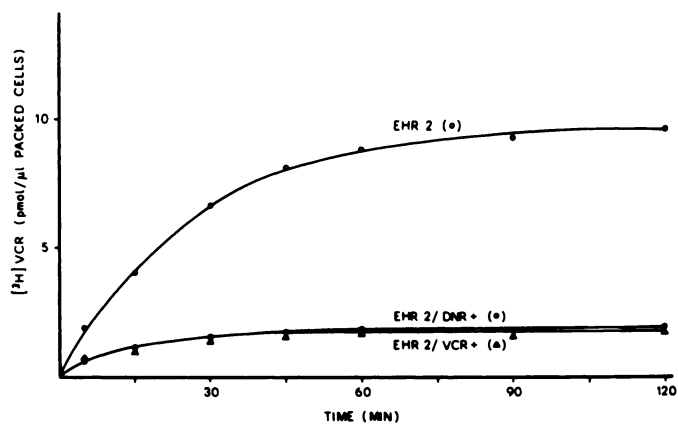


Chart 1. Time course of the uptake of [³H]VCR in cells from the wild-type Ehrlich ascites tumor (EHR 2) and from sublines resistant to VCR (EHR 2/VCR+) and to DNR (EHR 2/DNR+). Suspension of cells (5 μl packed cells/ml) were incubated in standard medium at 37°. [³H]VCR at 1 μM was added at 0 time, and serial samples were withdrawn at the times indicated. The cellular drug content after 2 washings was determined as described in "Materials and Methods."

DNR+; the cell:medium ratios for these tumor lines had been determined as 1.8.

Table 1 shows the influence of the energy metabolism on the uptake of [³H]VCR and DNR in wild-type tumor cells and in tumor cells resistant to the 2 drugs. The influence of glycolysis on uptake was determined by addition of iodoacetic acid to the suspension in a concentration sufficient to obtain inhibition of glycolysis (22); the influence of oxidative phosphorylation on uptake was evaluated by the addition of the specific inhibitor sodium azide (31). The exposure of the tumor cells to the metabolic poisons results in the same effect on the uptake of [³H]VCR and of DNR. A selective inhibition of oxidative phosphorylation had nearly no effect on drug uptake. The selective inhibition of glycolysis with iodoacetic acid in most cases resulted in a moderate increase in drug uptake. However, if both sodium azide and iodoacetic acid were added to the cell suspension, a synergistic increase in drug uptake was observed. The findings indicate that the uptake of both [³H]VCR and DNR in all of the tested sublines of Ehrlich ascites tumor cells is depressed by an energy-dependent process. Furthermore, the results suggest that the energy derived from glycolysis may substitute completely for oxidative phosphorylation, whereas oxidative phosphorylation may only partially substitute for glycolysis as energy supplier. In a comparison of the effect of energy blockade on drug uptake in the different tumor lines, it appeared that the increment in uptake for both drugs was considerably more pronounced for the resistant sublines than for the wild-type tumor. When both sources of energy were inhibited, the uptake of [³H]VCR in resistant cells corresponded to 62 to 82% of that in wild-type cells, and the uptake of DNR in the resistant cells corresponded to 87 to 98% of that in wild-type tumor cells.

Because Ehrlich ascites tumor cells are incapable of glycogenesis (1), glycolysis in these cells occurs only in the presence of exogenous glucose (19, 32); *i.e.*, the total energy metabolism of these cells may also be stopped by the omission of glucose from the medium and addition of sodium azide. Chart 2 shows the uptake of [³H]VCR in EHR

Table 1
Effect of metabolic inhibitors on uptake of [³H]VCR and DNR

[³H]VCR at 1 μM and DNR at 5 μM were added at 0 time. Cellular drug content after 2 washings was determined at incubation for 60 min, as described in "Materials and Methods."

Tumor	[³ H]VCR (pmol/μl packed cells)				DNR (pmol/μl packed cells)			
	Control ^a	Azide ^b	Iodoacetic acid ^c	Azide + iodoacetic acid	Control	Azide	Iodoacetic acid ^c	Azide + iodoacetic acid
EHR 2	8.55 ± 0.07 ^d	8.74 ± 0.02	8.48 ± 0.04	10.05 ± 0.12	589 ± 4	601 ± 2	681 ± 4	725 ± 7
EHR 2/VCR+	2.16 ± 0.16	2.16 ± 0.04	4.01 ± 0.18	8.26 ± 0.16	209 ± 2	200 ± 4	393 ± 0	711 ± 2
EHR 2/DNR+	1.95 ± 0.02	1.94 ± 0.10	2.66 ± 0.06	6.26 ± 0.05	175 ± 3	171 ± 4	289 ± 6	631 ± 9

^a Standard medium.

^b Standard medium with 10 mM sodium azide; the cells were exposed to the poison 10 min prior to exposure to the drug.

^c Standard medium with 0.2 mM iodoacetic acid; the cells were exposed to the poison 30 min prior to exposure to the drug.

^d Mean ± S.E. of 3 determinations.

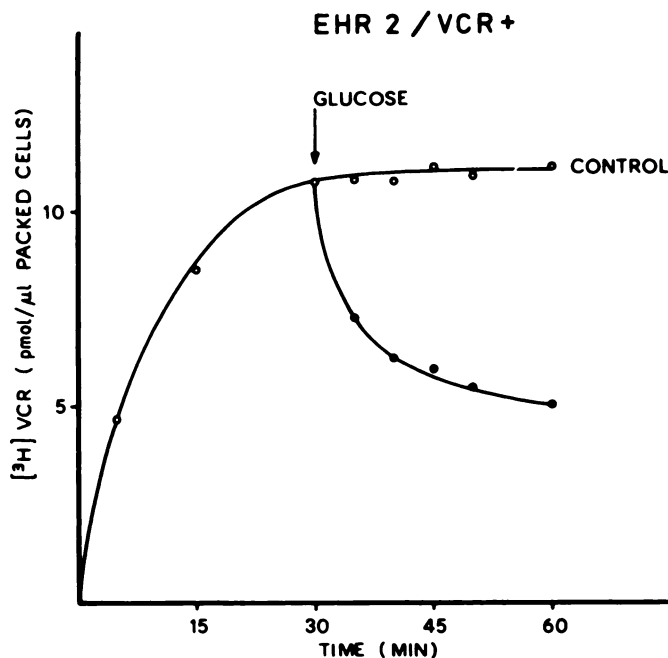


Chart 2. Efflux of [³H]VCR induced by glucose in cells resistant to VCR. The cell suspension (0.5% v/v) was incubated in standard medium without glucose, containing 10 mM sodium azide (○). Cells were loaded with [³H]VCR by addition of 1 μM at 0 time. At the arrow, 10 mM glucose were added to the suspension, and serial samples were withdrawn at the times indicated. In controls a corresponding volume of 0.9% NaCl solution was added.

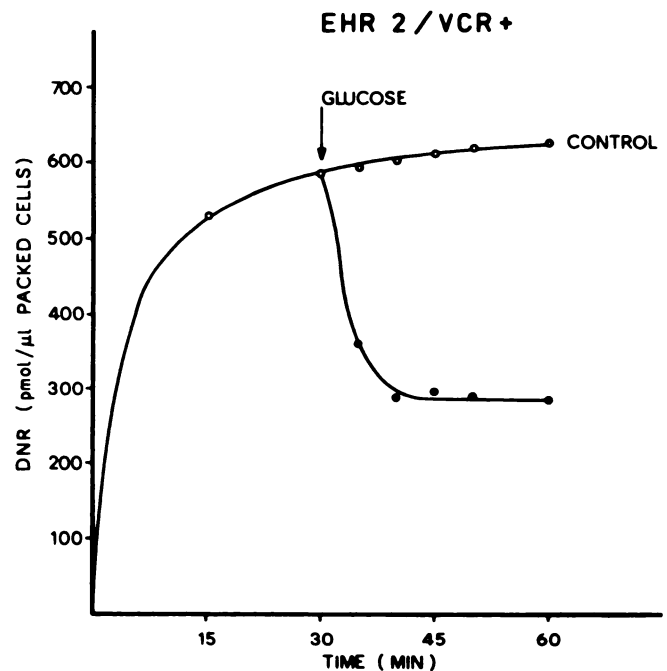


Chart 3. Efflux of DNR induced by glucose in cells resistant to VCR. Cells were loaded with DNR by addition of 5 μM at 0 time. Experimental conditions are the same as those described in the legend to Chart 2.

2/VCR+ incubated in the medium containing sodium azide without glucose. At 30 min, glucose was added to the medium to a concentration of 10 mM. It appears that glucose induced a pronounced extrusion of [³H]VCR to the surrounding medium. Chart 3 shows a corresponding experiment in which the uptake of DNR instead of [³H]VCR was determined. Together, these experiments indicate that energy derived from glycolysis may be used to extrude [³H]VCR or DNR from EHR 2/VCR+. Extrusion of [³H]VCR was also demonstrated in cells resistant to DNR in a corresponding experiment (not shown). Chart 4 shows that, when a corresponding energetic manipulation was performed with wild-type tumor cells, only a slight extrusion of [³H]VCR occurred.

Charts 5 and 6 show the initial uptake of [³H]VCR and of DNR, respectively, in the tumor cells resistant to VCR. For

both drugs the uptake experiments were performed in standard medium and in a medium without glucose but with 10 mM sodium azide. In all cases the uptake was linear for at least 55 sec, indicating that the concentration gradient was nearly constant within that period. When extrapolated to 0 time, the lines for both drugs showed a positive intercept, which was only slightly influenced by the composition of the medium. This component is thought mainly to reflect an initial adsorption of drug on the cell surface components. For both drugs, inhibition of the cellular energy metabolism results in a significant increment of the slope of the linear phase. This finding indicates that the component of active efflux exerts its effect so early in the uptake process that determination of unidirectional influx is not obtainable in the standard medium. Charts 5 and 6 also show that during the linear phase both drugs accumulate in the cells in the medium without glucose but with sodium azide. Thus, at 60 sec the overall cell:medium

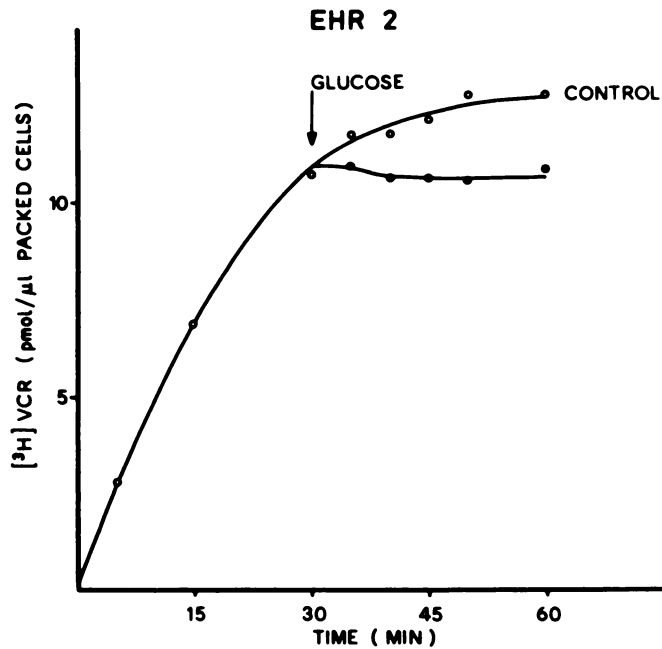


Chart 4. Efflux of $[^3\text{H}]\text{VCR}$ induced by glucose in wild-type tumor cells. Cells were loaded with $[^3\text{H}]\text{VCR}$ by addition of $1\ \mu\text{M}$ at 0 time. Experimental conditions are the same as those described in the legend to Chart 2.

distribution ratios were calculated as 2.3 and 35 for $[^3\text{H}]\text{VCR}$ and DNR, respectively.

Table 2 shows the result of the initial rate of uptake of $[^3\text{H}]\text{VCR}$ and of DNR in the 3 tumor lines in the medium without glucose, containing 10 mM sodium azide. The rates of uptake of both compounds were significantly higher in the wild-type tumor cells than in the resistant cells; on the other hand, nearly equal results were obtained for the 2 resistant cell lines.

For clarification of whether a common specific inward transport mechanism exists for VCR or DNR, the influence of VCR on the initial rate of uptake of DNR was determined in the 3 tumor lines. Table 3 shows that, even if the concentration of VCR was 50-fold higher than that of DNR, no significant inhibition of the rate of DNR uptake could be demonstrated.

DISCUSSION

Indications of an active outward transport of DNR in cells resistant to DNR have previously been demonstrated in our laboratory (13, 27). This study demonstrates an active extrusion of DNR in cells resistant to VCR and an active extrusion of $[^3\text{H}]\text{VCR}$ in cells resistant to VCR as well as to DNR. Together, these findings indicate that an energy-dependent drug extrusion is a common mechanism of resistance to VCR and DNR.

Previous experiments indicate that the mechanism of active extrusion of DNR in cells resistant to DNR most probably attributes to a primary active transport (27). According to this concept a specific binding of the drug to a reactive site of a carrier is expected to occur at the inner surface of the membrane. In consideration of the chemical structure of VCR and DNR, no obvious similarity is visible. VCR is a dimeric periwinkle alkaloid; DNR is a glycoside

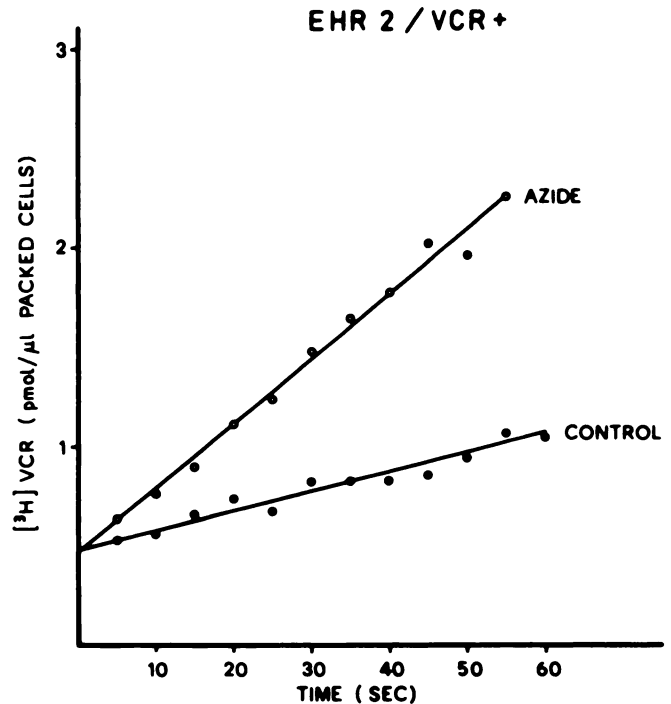


Chart 5. Effect of sodium azide upon initial uptake of $[^3\text{H}]\text{VCR}$ in cells resistant to VCR. A cell suspension (10% v/v) was exposed to standard medium (●) or to standard medium without glucose, containing 10 mM sodium azide (○) for 10 min at 37° . The experiment was started by transferring the cell suspension to the drug solution (37°) during rapid stirring, forming a final cell suspension of 0.5% v/v. Serial samples of $2000\ \mu\text{l}$ 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 $2350 \times g$ for 1 min. The cellular content after 2 washings was determined as described in "Materials and Methods."

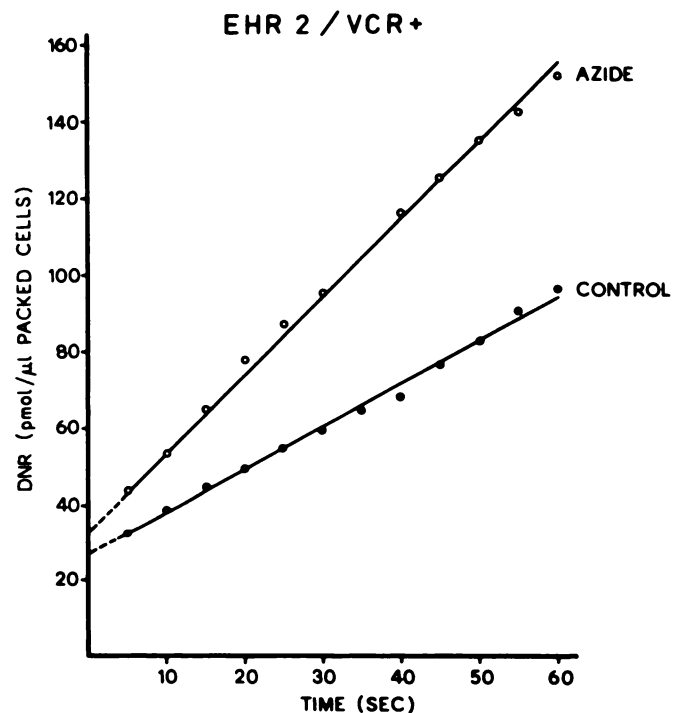


Chart 6. Effect of sodium azide upon initial uptake of DNR in cells resistant to VCR. Five μM DNR were added at 0 time. Other experimental conditions are the same as those described in the legend to Chart 5.

Table 2
Initial rate of uptake of [³H]VCR and of DNR

[³H]VCR at 1 μM and DNR at 5 μM were added at 0 time. Prior to exposure to the drug, the cells were incubated for 10 min in standard medium without glucose, containing 10 mM sodium azide. The sampling technique was the same as that described in the legend to Chart 5. The initial rate of uptake was determined by the slope of the uptake during the initial 50 sec.

Tumor	[³ H]VCR (pmol/μl packed cells/min)	DNR (pmol/μl packed cells/min)
EHR 2	2.85 ± 0.04 ^a	169.6 ± 3.1
EHR 2/VCR +	1.90 ± 0.04	103.5 ± 5.7
EHR 2/DNR+	1.56 ± 0.05	103.4 ± 1.6

^a Mean ± S.E. in 3 experiments performed on 3 different days.

Table 3
Effect of VCR on initial rate of uptake of DNR

The experimental conditions were identical with those described in the legends to Chart 5 and Table 2. When VCR was added, cells were exposed to VCR and DNR simultaneously.

Tumor	1 μM DNR	1 μM DNR + 50 μM VCR
EHR 2	37.4 ± 0.7 ^a	40.4 ± 0.7
EHR 2/VCR+	24.8 ± 0.5	25.7 ± 0.5
EHR 2/DNR+	27.2 ± 0.2	27.8 ± 0.7

^a Mean ± S.E. in 3 experiments performed on 3 different days.

composed of a tetracyclic aglycone, daunomycinone, and an amino sugar, daunosamine. The similarity could arise in the cells by structural modifications of DNR, VCR, or both. However, the experimental data on metabolism in this study and in previous studies (13, 25, 27) from this laboratory do not support significant conversion of VCR or DNR within the experimental period. Thus, most probably some parts of the 2 molecules do, from a steric point of view, look very similar, although this is not obvious from the structural formulas.

The mechanism of active drug efflux is not a property exclusively restricted to the resistant Ehrlich ascites tumor cells. In an earlier study (25), indications of an energy-dependent efflux of DNR from wild-type tumor cells were demonstrated. The results shown in Table 1 and Chart 4 support this finding. The much more pronounced efflux from the resistant sublines might suggest that these sublines have arisen by a selection of a small population with a high capacity of active efflux in the wild-type tumor.

The drug extrusion demonstrated in Ehrlich ascites tumor cells probably also includes several derivatives of VCR and DNR. Thus, mutual cross-resistance has been demonstrated between VCR, VBL, DNR, Adriamycin, and rubidazone *in vivo* (10–12, 24). Furthermore, *in vitro* a lower net uptake of DNR has been demonstrated in cells resistant to VBL and to Adriamycin (13), and a reduced net uptake of rubidazone has been demonstrated in cells resistant to DNR, VCR, and VBL (28).

The findings of a decreased drug uptake as a mechanism of resistance to *Vinca* alkaloids (4, 9) as well as to anthracyclines (7, 8, 18, 23) in several other tumor systems may suggest that the active extrusion mechanism reflects a more general phenomenon.

As previously shown for DNR in DNR-resistant cells, a slower inward transport of [³H]VCR in cells resistant to VCR

also plays a role in the resistance to VCR. Furthermore, both influx of DNR in EHR 2/VCR+ and influx of [³H]VCR in EHR 2/DNR+ were reduced, compared with wild-type cells. As indications of carrier-mediated influx have been demonstrated for *Vinca* alkaloids (2, 4) as well as for anthracyclines (8, 26), the lower influx in the resistant cells could reflect a change in a common carrier system. However, the absence of a competition for influx of DNR by VCR does not support this concept. A remaining active efflux in spite of the inhibition of the energy metabolism could account for the lower inward transport in the resistant cell. If so, the steady-state level of drug uptake is expected to be reduced, compared with wild-type cells. However, in comparison of Charts 2 and 4 it appears that the level of [³H]VCR in resistant cells at equilibrium (at 60 min) contributes 86% of that of wild-type cells. Thus, probably only very little active drug extrusion remains if the cells are inhibited by omission of glucose and of sodium azide in the medium. Another possibility could be a reduced rate of binding to common intracellular binding sites. However, several experimental findings indicate that the anthracyclines mostly bind to DNA in the nucleus (5, 15, 20, 33), whereas the *Vinca* alkaloids bind with high affinity to protein subunits of microtubules (16, 21, 29). Furthermore, the findings of a linear initial phase in uptake in spite of intracellular drug accumulation (Charts 5 and 6) indicate that, when the extrusion process is eliminated, the membrane transport is rate limiting to binding to intracellular binding sites. Thus, most probably the reduced influx of the drugs in the resistant cells expresses unspecific changes in the membrane structure, which reduce the rate of specific influx mechanisms for each of the 2 classes of cytostatics.

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