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KINETICS OF DAUNORUBICIN TRANSPORT IN EHRLICH ASCITES TUMOR CELLS WITH DIFFERENT EXPRESSION OF P-GLYCOPROTEIN

INFLUENCE OF VERAPAMIL

DORTE NIELSEN,* CHRISTIAN MAARE and TORBEN SKOVSGAARD
Department of Oncology R, Herlev Hospital, University of Copenhagen, Denmark

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Abstract—The classical multidrug resistance (MDR) phenotype is characterized by a decrease in the intracellular drug concentration in resistant cells as compared to sensitive cells. P-glycoprotein (P-gp) is thought to be responsible for an active efflux of lipophilic drugs. Four Ehrlich ascites tumor cell lines selected *in vivo* for resistance to daunorubicin (DNR) and their sensitive counterpart were investigated. The resistant sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and EHR2/0.8 were developed by treatment of tumor bearing mice with DNR 0.1, 0.2, 0.4, and 0.8 mg/kg × 4 weekly, respectively. One passage from EHR2/0.1, EHR2/0.2, and EHR2/0.4 and two passages from EHR2/0.8 were investigated. Western blot analysis showed significantly different amounts of P-gp (a 6-fold variation). Efflux of DNR in a drug free medium was investigated with and without presence of verapamil (VER). Efflux from sensitive and resistant cells was described by mono- and bi-exponential kinetics, respectively. In all cases but one, a correlation between resistance, expression of P-gp, P-gp mediated efflux capacity and effect of VER was established. In passage No. 12 of EHR2/0.8, however, a high expression of P-gp was found in spite of a low degree of resistance and a low efflux capacity. In this subline the effect of VER did not correlate to the expression of P-gp. Active efflux seemed to be saturable and was suggested to constitute the major route of efflux in MDR cells. A dose–response relationship was established for the effect of VER on efflux. In conclusion, the results support that P-gp acts as a drug efflux pump. No simple correlation, however, could be established between P-gp and drug transport in all the investigated cell lines. Other factors which might influence transmembranous transportation of DNR are suggested. The active efflux capacity of the cell lines seemed to determine the degree of resistance and the sensitivity to circumvention by VER.

Key words: cell line; cytotoxicity; daunorubicin; drug resistance; membrane glycoprotein; pharmacokinetics; verapamil

Effective cancer chemotherapy is often limited by the presence of drug resistant cells within a tumor population. Some malignant tumors are intrinsically resistant to anti-neoplastic agents, while others respond initially to chemotherapy and then, subsequently, relapse after development of resistance. MDR† describes the simultaneous expression of cellular resistance to a range of structurally and functionally unrelated drugs. The phenotype is characterized by (1) cross resistance, (2) decrease in drug accumulation, (3) expression of a 170 kDa plasma membrane glycoprotein, P-gp, (4) as a result of increased transcription or amplification of the *mdr*1 gene, and (5) reversal of the phenotype by a variety of different compounds (e.g. VER) [for a review, see Refs 1–4]. The most striking feature of P-gp is its similarity to a variety of transport proteins. P-gp is related to a superfamily of transport systems including bacterial transporters, the cystic fibrosis transmembrane conductance regulator, and the *Plasmodium falciparum* drug resistance gene [5]. A model for the function of P-gp has been proposed in which it acts as an ATP-dependent hydrophobic vacuum cleaner extruding hydrophobic drugs from the plasma membrane of MDR cells [6]. The precise mechanism, however, by which MDR cells maintain a lower intracellular cytotoxic drug level than their corresponding sensitive cell lines is poorly understood. To determine the role of P-gp in the active outward transport of antitumor drug in MDR cells it is necessary to understand the mechanism of efflux.

In the present study, Ehrlich ascites tumor cell lines selected *in vivo* for resistance to DNR were used as an experimental model. In the resistant sublines, western blot analysis showed significantly different amounts of P-gp. The drug efflux from the wild-type cell line and the drug resistant sublines were described by exponential kinetics. In four resistant sublines a reasonable correlation between resistance, P-gp expression, efflux capacity, and effect of VER was established. In one subline, however, a high expression of P-gp was not correlated with high degree of resistance or high efflux capacity.

* Corresponding author: Dorte Nielsen M.D., Department of Oncology ONK 5074, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark.

† Abbreviations: DNR, daunorubicin; MDR, multidrug resistance; P-gp, P-glycoprotein; VER, verapamil; EHR2, wild-type Ehrlich ascites tumor.

Active efflux was found to be saturable and suggested to constitute a major route of efflux in MDR cells. A dose–response relationship was established for the effect of VER on efflux.

MATERIALS AND METHODS

Tumor cells. The wild-type Ehrlich ascites tumor, EHR2, and its DNR resistant sublines were used in the experiments. All cell lines were maintained in first generation hybrids of female NRMI and male inbred DBA/2 mice by weekly transplantation of 1.5×10^7 cells per mouse. The resistant sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and EHR2/0.8 were developed *in vivo* by intraperitoneal treatment with DNR 0.1, 0.2, 0.4, and 0.8 mg/kg × 4 weekly corresponding to 6.25, 12.5, 25, and 50% of the LD_{10} dose (1.6 mg/kg × 4 weekly). Cells from passage No. 72 of EHR2/0.1, No. 24 of EHR2/0.2, No. 54 of EHR2/0.4, and No. 12 of EHR2/0.8 were used for *in vitro* experiments. These passages were chosen as a previous study had shown that cells from these passages at steady state accumulated approximately the same amount of DNR [7]. For comparison the highly resistant passage No. 72 of EHR2/0.8 was investigated.

The original resistant subline EHR2/DNR+ was used for calibration of the western blot assay. This subline was maintained by treatment with the LD_{10} dose of DNR [8, 9].

Chemicals. DNR as hydrochloride (Farmatalia Carlo Erba, Milan, Italy), sodium azide (Merck, Darmstadt, Germany), and VER as hydrochloride (Ercopharm Ltd, Copenhagen, Denmark) were all of analytical grade.

Drug resistance. Chemosensitivity was determined by the clonogenic assay described by Roed *et al.* [10]. A single cell suspension with the desired drug concentration was plated in soft agar on top of a feeder layer containing sheep red blood cells. A continuous incubation was used. Each experiment was performed in triplicate and repeated 8–12 times. Counting was performed after 10 days of incubation. An image analysis system from Scan Beam (Hadsund, Denmark) was used for counting. Surviving fraction was calculated by dividing No. of colonies on the treated plates by No. of colonies on the untreated control plates. The IC_{50} was defined as the drug concentration which inhibited 50% of colony formation. Relative resistance was the ratio between IC_{50} of the resistant cell lines and IC_{50} of parental. Fold reversal was the ratio between IC_{50} for DNR in absence and presence of VER.

Antibodies. The primary antibody used was C219 (Centocor Diagnostics, Philadelphia, PA, U.S.A.). As secondary antibody was applied peroxidase conjugated $F(ab')_2$ fragments of affinity purified sheep anti-mouse IgG (Medac, Hamburg, Germany).

P-gp expression. Western blot analysis was used for quantitation of P-gp [7]. Cells were suspended in hypotonic buffer (NaCl (10 mM), $MgCl_2$ (1.5 mM), Tris (50 mM), and phenylmethylsulphonylfluoride (2 mM), pH 7.4) for 10 min and disrupted. After centrifugation (4000 *g*, 10 min), supernatants were collected and centrifuged at 40,000 *g* for 60 min. The membrane-enriched pellets were harvested, diluted, and centrifuged (40,000 *g*, 60 min). The pellets were resuspended in buffer and loaded on gels consisting of a stacking gel zone (5%) and a separation zone with an 8–18% polyacrylamide gradient. Following electrophoresis proteins were transferred to nitrocellulose paper. The paper was blocked, incubated overnight with C219, and for 2 hr with the secondary antibody. H_2O_2 and diaminobenzidine tetrahydrocloride (Sigma Chemical Company) were used as substrates. Controls were performed by omission of primary antibody.

Reflectance photometry was performed using a CS-9000 scanning densitometer (Shimadzu, Kyoto, Japan). The content of P-gp was calculated using a standard curve composed of membrane preparations with defined concentrations of P-gp (mixtures of EHR2 and EHR2/DNR+).

DNR efflux. The method described by Skovsgaard [11] was used. In one series of experiments, tumor cells were preloaded by incubating for 30 min at 37° in medium (NaCl (57 mM), KCl (5 mM), $MgSO_4$ (1.3 mM), NaH_2PO_4 (9 mM), Na_2HPO_4 (51 mM), pH 7.45) with 5% (v/v) calf serum and DNR (25 μM) resulting in an uptake of approximately 2000 pmol DNR/10^6 cells. Subsequently, the cells were washed twice at 4°, a final cell suspension of 8×10^7 cells/mL was formed, and 25 μL of this suspension corresponding to 2×10^6 cells was transferred to 10 mL of standard medium with glucose (10 mM) at 37°. In all the resistant cell lines, the effect of VER on the efflux was investigated by addition of either 1.1, 5.5 or 11 μM VER. Furthermore, dose–response of VER on efflux was evaluated in EHR2/0.8 passage No. 72 by addition of either 0.11, 0.33, 0.55 or 3.3 μM VER. After 6, 20, 40, 60, 80 and 100 sec then, 2, 3, 4, 5, 10 and 15 min, the flux reaction was terminated by cooling and centrifugation at 4360 *g* for 5 min at 4°. The drug was extracted from the drained cell pellet with 0.3 N HCl: 50% ethanol. Fluorescence of the extractions was determined in a spectrofluorometer (Hitachi F 3010, Tokyo, Japan) at excitation 500 nm and emission 583 nm. A curve-fitting computer programme from Sigma plot was used for calculations of kinetics parameters.

Determination of DNR release from nuclei of sensitive and resistant cells The efflux from nuclei of sensitive and resistant cells (passage No. 72 of EHR2/0.8) was determined by incubating the cells for 30 min in standard phosphate medium with 5% (v/v) calf serum and DNR (25 μM). Cells were washed twice and a suspension of 8×10^7 cells/mL was formed. The buffer used was sucrose (250 mM), $CaCl_2$ (5 mM), and Tris–HCl (25 mM), pH adjusted to 7.45. Fifty microliters (4×10^6 cells) of the cell suspension was transferred to 10 mL buffer with glucose at 37°. Isolated nuclei were prepared by addition of Nonidet P40 0.1% (v/v) to the medium. Termination of the flux reaction, extraction, and determination of fluorescence were performed as described previously.

RESULTS

P-gp expression and in vitro *resistance to DNR*

Table 1 shows the IC_{50} values achieved when EHR2 and the resistant sublines were continuously

Table 1. Cytotoxicity of DNR and expression of P-gp in Ehrlich ascites tumor cells

Cell line	Passage No.	IC_{50} DNR (μM)	No. of assays	P-gp (units ± SD)	No. of assays
EHR2	—	0.019 ± 0.001	12	12.0 ± 4.0	8
EHR2/0.1	72	0.117 ± 0.006 (6.2)*	8	75.6 ± 15.8	8
EHR2/0.2	24	0.105 ± 0.023 (5.5)*	8	38.4 ± 6.9	8
EHR2/0.4	54	0.617 ± 0.100 (32.5)*	8	210.6 ± 16.8	8
EHR2/0.8	12	0.097 ± 0.027 (5.1)*	8	165.6 ± 14.8	8
EHR2/0.8	72	0.667 ± 0.067 (35.1)*	8	255.0 ± 15.0	8

* Relative resistance = ratio between IC_{50} of the resistant cell lines and IC_{50} of the wild-type.

exposed to DNR. The cell lines EHR2/0.1, EHR2/0.2, and passage No. 12 of EHR2/0.8 were 6.2, 5.5, and 5.1-fold resistant to DNR, respectively ($P > 0.05$). The sublines EHR2/0.4 and passage No. 72 of EHR2/0.8 were 32.5 and 35.1-fold resistant, respectively ($P > 0.05$).

P-gp was quantitated by western blot analysis. The relative content of P-gp in EHR2 and the resistant cell lines is given in Table 1. A 6-fold variation in P-gp was observed in the resistant cell lines. In four of the sublines, a reasonable correlation between expression of P-gp and degree of resistance was established. However, passage No. 12 of EHR2/0.8 was only five-fold resistant to DNR in spite of a high expression of P-gp.

Efflux (washout kinetics) of DNR

The efflux was investigated in standard medium including glucose. Prior to the eluation, the sensitive and resistant cells were preloaded with DNR (25 μM) in phosphate buffer for 30 min without glucose to reduce active efflux, if any. Within the individual cell lines, uptake of DNR (C_0) after loading showed a considerable inter-assay variation (mean uptake of DNR was: 2228, 2087, 2584, 2571, 2957, and 2475 pmol DNR/10^6 cells for EHR2, EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage Nos 12 and 72 of EHR2/0.8, respectively). This variation was probably a result of several factors: variations in the accumulation of DNR during preloading and variations in loss of drug or loss of cells during the washing procedure. However, within each subline the relative intracellular drug content $C_t/C_0 \times 100\%$ (C = intracellular drug content and t = time) remained constant between assay. Consequently, efflux data for the different cell lines were expressed by $C_t/C_0 \times 100\%$ as a function of time of washout (Fig. 1a–c). Only representative curves are shown. The curves obtained for EHR2/0.1 (Fig. 1b) are representative for the sublines EHR2/0.2 and passage No. 12 of EHR2/0.8. The curves shown for passage No. 72 of EHR2/0.8 (Fig. 1c) are representative for EHR2/0.4.

Drug efflux from P388 cells has been found to be

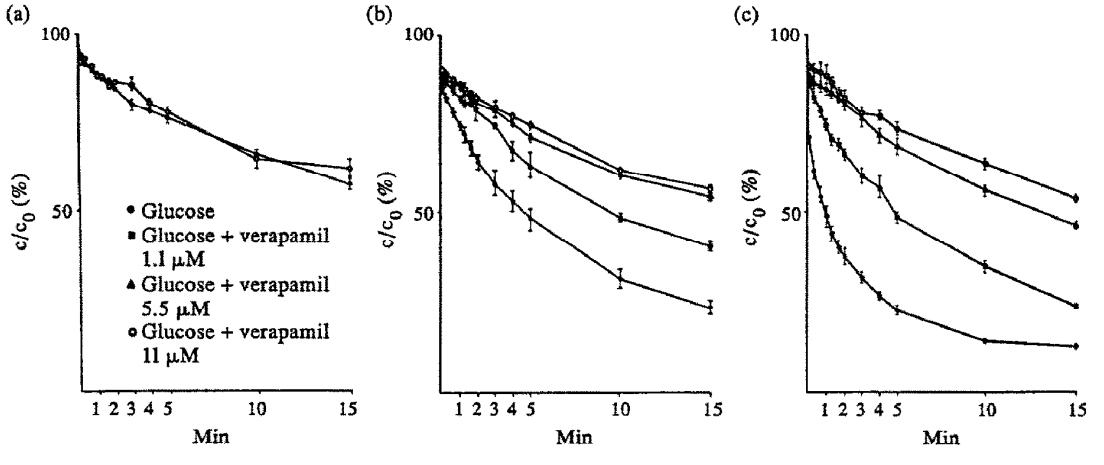

Fig. 1. Efflux of DNR from sensitive and resistant EHR2 tumor cells in drug-free medium with glucose or corresponding medium with glucose and different concentrations of VER (1.1, 5.5 and 11 μM). Each point represents the mean of six determinations and bars represent SEM values. (a) = EHR2; (b) = EHR2/0.1; and (c) = EHR2/0.8 (passage No. 72). The curves obtained for EHR2/0.1 are representative for the sublines EHR2/0.2 and passage No. 12 of EHR2/0.8. The curves shown for passage No. 72 of EHR2/0.8 are representative for EHR2/0.4.

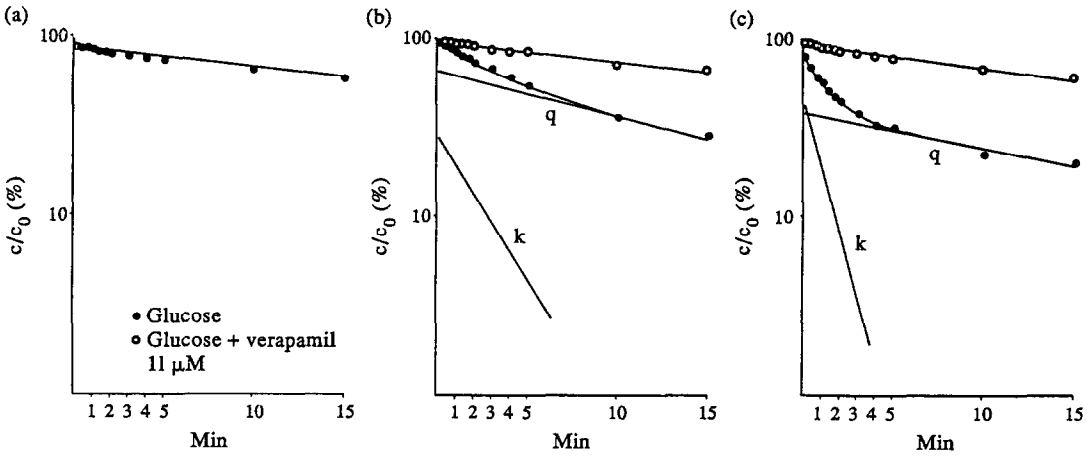

Fig. 2. The figures show semi-logarithmic plot of C_t/C_0 as a function of time. The washout curves for the sensitive cell line EHR2 and the resistant sublines in medium including VER (11 μM) were described by mono-exponential kinetics (appendix, equation 1). Efflux from the resistant sublines followed bi-exponential kinetics (appendix, equation 2). Furthermore, the figures illustrate the curves for the two functions $C = A \times e^{-Kt}$ and $C = B \times e^{-Qt}$. (a) = EHR2; (b) = EHR2/0.1; and (c) = EHR2/0.8 (passage No. 72). The curves obtained for EHR2/0.1 are representative for the sublines EHR2/0.2 and passage No. 12 of EHR2/0.8. The curves shown for passage No. 72 of EHR2/0.8 are representative for EHR2/0.4.

compatible with simple diffusion [12]. In our experiments efflux from wild-type cells followed mono-exponential kinetics (appendix; equation 1), that is, efflux from EHR2 cells is also compatible with simple diffusion [13]. Figure 2a shows a semi-logarithmic plot of $C_t/C_0 \times 100\%$ as a function of time of washout for the sensitive cell line. The K value was 0.033 min^{-1} in medium without VER and 0.030 min^{-1} in medium including VER (11 μM) (Table 2). Thus, VER had no effect on the flux of drug from sensitive cells ($P > 0.05$).

Several authors [14, 15 and reviewed in 6] have found drug efflux from MDR cells to be composed by two main components: diffusion and active P-gp mediated efflux. Theoretically, efflux from MDR cells could be described by bi-exponential kinetics (appendix; equation 2). Figure 2b and c illustrate semi-logarithmic plots of the total intracellular DNR content versus time of washout for EHR2/0.1 and passage No. 72 of EHR2/0.8, respectively. The curves obtained for EHR2/0.1 (Fig. 1b) are representative for the sublines EHR2/0.2 and passage No. 12 of EHR2/0.8. The curves shown for passage No. 72 of EHR2/0.8 (Fig. 1c) are representative for EHR2/0.4. Figure 2b and c also show the two functions $C_t = A \times e^{-Kt}$ and $C_t =$

Table 2. Kinetic parameters for efflux of DNR from EHR2 and the resistant sublines (N = 6)

Cell line	VER (μM)	K (min^{-1})	P-values	Q (min^{-1})	P-values
EHR2	—	0.033	—	—	—
EHR2/0.1	—	0.381	NS†	0.063	NS
EHR2/0.2	—	0.438	NS†	0.045	NS
EHR2/0.4	—	0.844	NS‡	0.047	NS
EHR2/0.8 (12)*	—	0.436	NS†	0.045	NS
EHR2/0.8 (72)*	—	0.773	NS‡	0.071	0.002
EHR2/0.1	1.1	0.057	NS	—	—
EHR2/0.2	1.1	0.048	NS	—	—
EHR2/0.4	1.1	0.077	0.04	—	—
EHR2/0.8 (12)*	1.1	0.056	NS	—	—
EHR2/0.8 (72)*	1.1	0.084	NS	—	—
EHR2	11.0	0.030	NS	—	—
EHR2/0.1	11.0	0.033	NS	—	—
EHR2/0.2	11.0	0.025	NS	—	—
EHR2/0.4	11.0	0.034	NS	—	—
EHR2/0.8 (12)*	11.0	0.030	NS	—	—
EHR2/0.8 (72)*	11.0	0.034	NS	—	—

* Passage No.

† The K values for EHR2/0.1, EHR2/0.2, and passage No. 12 of EHR2/0.8 were not significantly different ($P > 0.05$).

‡ The K values for EHR2/0.4, EHR2/0.2, and passage No. 72 of EHR2/0.8 were not significantly different ($P > 0.05$).

$B \times e^{-Qt}$ representing A, B, K, and Q which according to the curve fitting programme fit with the experimental data; K and Q are given in Table 2. The calculated K values for the cell lines EHR2/0.1, EHR2/0.2, and passage No. 12 of EHR2/0.8 were not significantly different ($P > 0.05$). In EHR2/0.4 and passage No. 72 of EHR2/0.8 the K values were about 2-fold higher. Thus, in the sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage No. 72 of EHR2/0.8 a reasonable correlation between P-gp expression, resistance, and efflux was established. In passage No. 12 of EHR2/0.8, however, a high expression of P-gp was found in spite of a low degree of resistance and a low efflux capacity.

In the resistant cells, it appeared that the efflux was composed of an initial fast component followed by a significantly slower component. Thus, the ratio between the velocity constants K and Q was 6, 10, 18, 10, and 11 for the cell lines EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage No. 12 and 72 of EHR2/0.8, respectively. In the resistant cell lines, Q varied from 0.045 to 0.071 min^{-1}, whereas the corresponding constant in the sensitive cell line was 0.033 min^{-1}. The Q values for the sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage No. 12 of EHR2/0.8 were not significantly different from the K value of EHR2 (P values 0.394, 0.818, 0.394, 0.065). The Q value for passage No. 72 of EHR2/0.8, however, was significantly different from the K value of EHR2 ($P = 0.002$).

Considering washout for $t = \infty$ (appendix; equation 4), the efflux component described by A, K constituted 31, 43, 53, 28 and 55% of the total drug efflux in the cell lines EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage Nos. 12 and 72 of EHR2/0.8, respectively.

In order to investigate the relation between intracellular content of DNR and efflux the sensitive EHR2 cell line and passage No. 72 of the resistant EHR2/0.8 subline were preloaded with a range of concentrations of DNR. The K values obtained for the wild-type cell line EHR2 were: 0.033, 0.035, and 0.028 min^{-1} corresponding to mean uptake of DNR of 1617, 3890, and 6761 pmol/10^6 cells, respectively ($N = 6$). The K values were not significantly different in spite of a more than 4-fold difference in mean uptake.

Figure 3 illustrates the amount of drug effluxed by the rapid efflux component in passage No. 72 of EHR2/0.8 (appendix; equation 3, $t = 1$ min) as a function of the total intracellular content of DNR (C_0). The initial rapid efflux phase seemed to saturate at an uptake of DNR above 4500 pmol/10^6 cells. The relationship between the capacity of the efflux pump and the effect of VER on efflux was investigated. Washout for the resistant cell lines obtained in medium including VER (1.1–11 μM) could be described by mono-exponential kinetics ($r > 0.98$). The kinetics parameters are given in Table 2. Representative curves for the resistant cell lines obtained in medium including VER (11 μM) are shown in Fig. 2b and c. The K values (slopes of the curves) for the resistant cell lines in medium including VER (11 μM) were not significantly different from the K value of the sensitive cell line ($P > 0.05$).

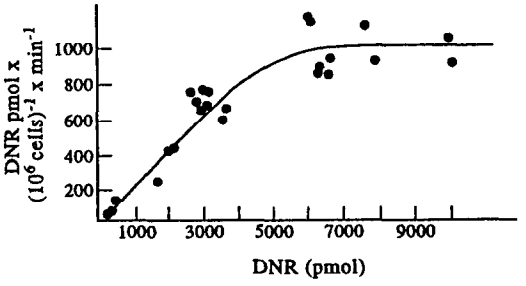

Fig. 3. The rapid component of efflux (see appendix, equation 3, $t = 1$ min) as a function of the total intracellular content of DNR (C_0).

In all resistant cell lines the efflux was decreased after application of VER (1.1 μM). Calculating the absolute amount of drug effluxed by the rapid component during the first minute (appendix; equation 3, $t = 1$ min), it appeared that addition of VER (1.1 μM) to the efflux medium decreased the efflux in the first min by 62, 114, 202, 59, and 183 pmol DNR/10^6, for the cell lines EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage Nos 12 and 72 of EHR2/0.8, respectively (mean of six experiments). Thus, the absolute inhibition by VER correlated to P-gp expression, degree of resistance, and efflux capacity in four of the sublines. In passage No. 12 of EHR2/0.8 the absolute inhibition only correlated to resistance and efflux. Comparing the relative inhibition, however, it appeared that VER (1.1 μM) blocked 85, 87 and 85% of the rapid efflux component in the cell lines EHR2/0.2, EHR2/0.4, and No. 72 of EHR2/0.8 ($P > 0.05$) and 72% in the sublines EHR2/0.1 and passage No. 12 of EHR2/0.8. A comparison between the individual cell lines in the two groups showed a significant difference ($P < 0.05$).

Passage No. 72 of the resistant subline EHR2/0.8 was used for investigation of the relationship between dose of VER and inhibition of efflux. Figure 4 illustrates $C_t/C_0 \times 100\%$ as a function of time of washout in presence of VER (0.1–11 μM). The relationship between K [calculated from equation 1 (VER $\geq$ 1.1 μM) and equation 2 (VER < 1.1 μM), respectively] and the concentration of VER is shown in Fig. 5. A very steep dose–response relationship for inhibition of the rapid component of drug efflux with VER was established.

The relation between mean uptake of DNR and effect of VER was investigated in passage No. 72 of the resistant EHR2/0.8 subline by preloading with a wide range of concentrations of DNR (5–150 μM). Washout was performed in medium without VER and with VER (1.1 μM). The method did not allow determination of the free concentration of DNR in the cytosol which is assumed to be the substrate (S) for P-gp. Previous experiments [11], however, have shown that the free intracellular concentration of DNR, within a wide range, is linearly correlated with total amount of DNR. Thus, the total uptake was used as an approximation of the actual substrate concentration (S). Figure 6 shows a Lineweaver–Burk plot (double reciprocal plot) of the initial rapid efflux (appendix; equation 3, $t = 1$ min) as a function

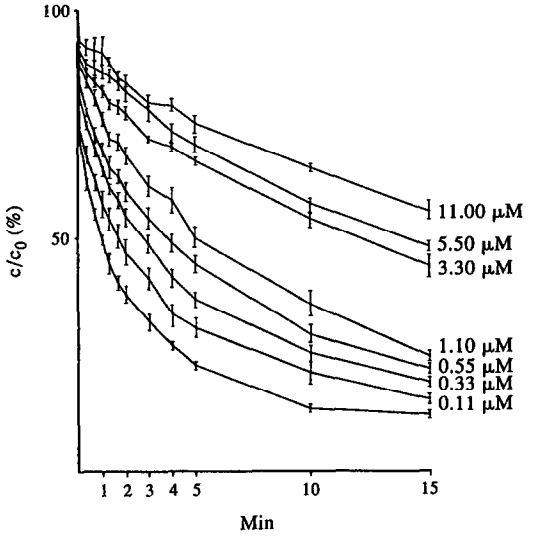

Fig. 4. Efflux of DNR from passage No. 72 of EHR2/0.8 in presence of glucose and different concentrations of VER. Each point of the curves represents the mean of six determinations and bars represent SEM values.

of total intracellular content of DNR. The correlation coefficients (r) were 0.93 and 0.98 for the curves obtained in medium without and with VER, respectively. It appeared that application of VER had no effect on "V_{max}", whereas "K_m" increased.

Release of DNR from sensitive and resistant nuclei

In order to investigate the kinetics of drug release from intracellular binding sites, washout experiments from isolated nuclei were performed. Whole cells were used in the loading and washing procedure.

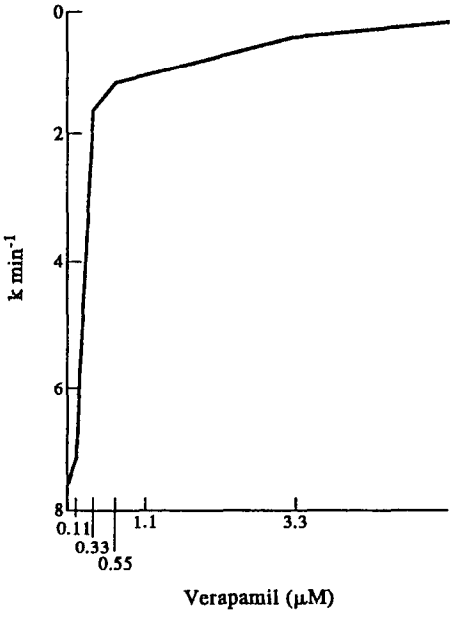

Fig. 5. The velocity constant K for EHR2/0.8 passage No. 72 as a function of the concentration of VER. K was calculated from the equation 2 (see Appendix).

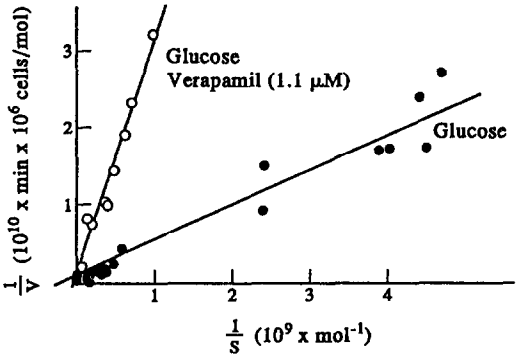

Fig. 6. Lineweaver–Burk plot (double reciprocal plot) of the rapid component of efflux of DNR (see appendix, equation 3) in medium with glucose and VER (1.1 μM) as a function of total intracellular drug content (C_o).

After resuspension in the washout medium a suspension of nuclei was formed by immediate lysis by Nonidet P40. Figure 7a and b show release of DNR from intact sensitive and resistant cells and nuclei, respectively. Figure 8a and b show the corresponding figures in a semi-logarithmic plot. In sensitive cells, the time constants (K) for whole cells and nuclei in efflux medium including sucrose were: -0.0115 and $-0.0108\ min^{-1}$ without significant difference. The correlation coefficients for the curves were 0.991 and 0.817, respectively. In resistant cells, the time constants (Q) for slow phase efflux of whole cells and nuclei were -0.0382 and $-0.0071\ min^{-1}$, respectively ($P = 0.0087$). The correlation coefficients of the curves were 0.998 and 0.972, respectively.

Effect of VER on cytotoxicity of DNR

Table 3 reports the IC_{50} values for the cell lines and the fold of sensitization after application of VER. The VER doses of 0.1 and 15.7 μM were non-toxic in dose–response assays. VER (3.1 μM and 15.7 μM) significantly increased the cytotoxicity of DNR in the sensitive cell line EHR2, the P values were 0.04 and 0.02, respectively. VER (15.7 μM) reversed resistance in all the sublines. VER (3.1 μM) reversed the resistance completely in the sublines EHR2/0.1, EHR2/0.2, and passage No. 12 of EHR2/0.8, whereas the sublines EHR2/0.4 and passage No. 72 of EHR2/0.8 were still 3- and 2-fold resistant to DNR. Thus, VER (3.1 μM) completely reversed resistance in the cell lines with low degree of resistance and low efflux capacity, whereas the cell lines with high degree of resistance and more pronounced active efflux were still resistant. As passage No. 12 of EHR2/0.8 had a high expression of P-gp, but a low efflux capacity, the effect of VER seemed to be determined by the degree of resistance and the active efflux capacity of P-gp and not the amount of P-gp.

DISCUSSION

The objective of the present study was to obtain a more detailed understanding of the relationship

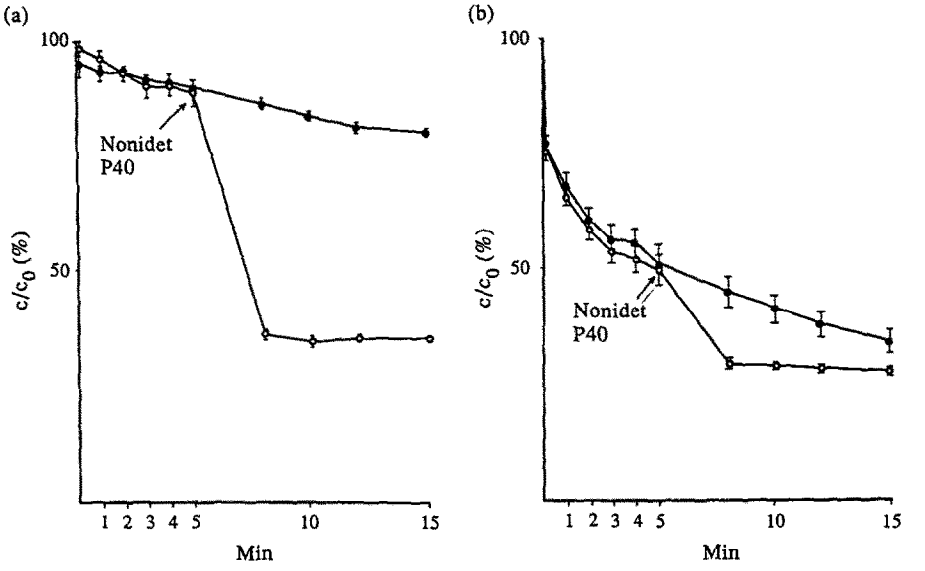

Fig. 7. Release of DNR from nuclei. Cells were preloaded with DNR (25 μM) for 30 min at 37°. After washing cells were suspended in glucose-enriched buffer. Nonidet P40 was applied after 5 min of incubation. Each point is mean of 6–8 determinations and bars represent SEM values. (a) = EHR2; and (b) = passage No. 72 of EHR2/0.8.

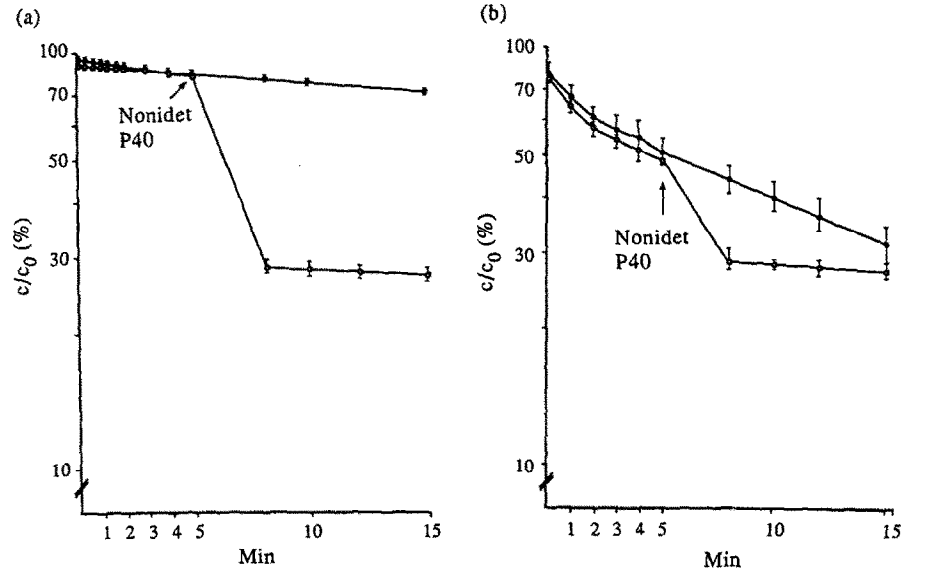

Fig. 8. Semi-logarithmic plot of release of DNR from nuclei. Nonidet P40 was applied after 5 min of incubation. Each point is mean of 6–8 determinations and bars represent SEM values. (a) = EHR2; and (b) = passage No. 72 of EHR2/0.8.

Table 3. Effect of VER on the drug sensitivity of DNR resistant EHR2 sublines

Cell line	IC_{50} mean ± SEM (μM DNR)	Fold reversal* VER (3.1 μM)	Fold reversal* VER (15.7 μM)
EHR2	0.019 ± 0.001	2.4	3.2
EHR2/0.1	0.117 ± 0.006 (6.2)†	5.3	11.7
EHR2/0.2	0.105 ± 0.023 (5.5)†	4.8	5.8
EHR2/0.4	0.617 ± 0.100 (32.5)†	15.8	34.3
EHR2/0.8 passage No. 12	0.097 ± 0.027 (5.1)†	16.2	8.8
EHR2/0.8 passage No. 72	0.667 ± 0.067 (35.1)†	13.6	37.1

* Fold reversal = ratio between IC_{50} for DNR in absence and presence of VER.
† Relative resistance.

between P-gp, resistance, and the mechanism of drug efflux in MDR cell lines developed *in vivo*. It appeared that the content of P-gp and the degree of resistance increased with increased selection pressure (dose of DNR used) and increased duration of exposure (No. of passages) [7]. Thus, in several but not all of the resistant sublines a reasonable correlation between expression of P-gp and degree of resistance was established.

In all cell lines efflux was measured after preloading the cells with DNR (25 μM) in phosphate buffer without glucose for 30 min. In other experimental systems, MDR drugs have been found to be released from wild-type cells by simple diffusion [12]. In the present study, washout data for sensitive cells could be described by mono-exponential kinetics [13]. Thus, efflux from EHR2 cells was also compatible with simple diffusion. In resistant cells, several authors [14, 15] have found the efflux to be composed of two main components (1) a component representing simple diffusion (2) P-gp mediated active efflux. Theoretically, drug efflux in MDR cells should follow bi-exponential kinetics (appendix; equation 2). The experimental data for the resistant cell lines in glucose-enriched medium fitted well to this equation. The rapid efflux component was completely blocked by VER. VER has been shown to act through interference with P-gp drug binding and transport [16]. Thus, it seemed reasonable to assume that this component represented P-gp mediated efflux. Supporting this assumption we found the component to be very rapid, accounting for 64–88% of the total efflux in the first 3 min. On the other hand, as VER has been shown to significantly inhibit exocytosis [17] and increased endosomal volume and membrane traffic has been described in MDR cells [18], the effect of VER did not exclude that exocytosis could contribute significantly to the efflux. However, Lankelma *et al.* [19] have found efflux to be independent of increased intravesicular pH. Demant *et al.* [20], who used numerical computer simulations to analyse some possible mechanisms controlling DNR fluxes in a kinetic model, have found the exocytotic model to be physically impossible. Furthermore, DNR is a lipophilic weak base and passive diffusion of the non-charged molecule across membranes has been shown to be much faster than of the protonated form [21]. Therefore, pH gradients across the membranes could interfere with DNR efflux.

In the present study, the slow component of drug efflux from all resistant cell lines but one was not significantly different from the release of drug from sensitive cells. This finding supported the assumption of diffusion as one of the components of efflux in MDR cells. However, this result could be explained by the limited number of assays and the experimental variation usually found in washout data [13].

In all the resistant cell lines, the active efflux proved to constitute a major route (28–55%) of total drug efflux ($t = \infty$). This finding is in contradiction to that of Spoelstra *et al.* [14], who measured DNR transport with a flow-through system. In contrast to the present findings, the passive efflux in this study (except for the cell lines with the highest amount of P-gp) was the main route of DNR efflux from MDR cells (approximately 75–90%). A possible explanation for the different conclusions could be differences in the amount of P-gp in the cell lines investigated.

In four of the resistant sublines a reasonable correlation between the expression of P-gp and the efflux capacity of the sublines was established. However, passage No. 12 of EHR2/0.8 had the same velocity constant K (active efflux) as the sublines EHR2/0.1, EHR2/0.2, whereas the P-gp content of this cell line was significantly higher. Thus, the content of P-gp and active efflux capacity did not correlate in a simple way for this subline. In other studies, the efflux of DNR has been found to correlate with the amount of P-gp [14, 15]. Correlation between expression of P-gp and resistance to vinca alkaloids has been reported in several studies [22, 23]. In MDR tranfectants the density of P-gp in the plasma membrane has been found to be sufficient to determine the level of resistance [24]. In other cases, no simple correlation between the P-gp expression and transport properties has been observed [reviewed in 25].

Several explanations could account for these discrepancies. First, the relevant relationship was between the number of P-gp molecules in the plasma membrane and the efflux of free intracellular drug. Our study measured total P-gp. Measuring total protein is not completely satisfactory as some of the protein could be inside the cell and not functional. However, by immunohistochemistry P-gp has been found to be located primarily in the plasma membrane [26]. Furthermore, the method used in the present study did not allow determination of the free concentration of DNR in the cytosol which is assumed to be the substrate (S) for P-gp. It has been shown previously [11] that the free concentration of DNR in the cytosol, within a wide range, is linearly correlated with the total amount of DNR. Changes in intracellular binding of drug and/or changes in intercalation of drug, however, could influence the results.

Second, the monoclonal antibody C219 applied for quantitation of P-gp was not specific for *mdr*1, as it detected *mdr*2 encoded P-gp too [27]. As *mdr*1 encodes for functional P-gp only [28], increased levels of P-gp due to *mdr*2 gene expression could confound the results [29]. Several studies, however, have shown correlations between *mdr*1 mRNA and P-gp detected with C219 [30, 31].

Another explanation could be modulation of the biological activity of P-gp. Recent evidence has indicated that phosphorylation is required for the function of P-gp [32].

The cell lines were developed by treatment *in vivo*. Thus, other resistance mechanisms mediated by anthracyclines (e.g. changes in glutathione transferase or topoisomerase II) could have been induced in the cell lines [33]. This possibility, however, was contradicted as addition of VER completely reversed the resistance in all the cell lines. Furthermore, *in vivo* metabolism and pharmacokinetics of DNR could influence drug level and resistance.

The efflux from resistant cells seemed to saturate at a total intracellular content of DNR above

4500 pmol/10^6 cells (the free cytoplasmatic concentration of DNR would be significantly lower than 4500 pmol/10^6 cells due to intercalation of drug in DNA and binding to intracellular binding sites). As we used very high DNR doses to achieve this saturation, the conclusion should, however, be interpreted with caution as self-association or unspecific toxicity (damage of the membrane) of the anthracycline molecules could confound the results [34]. On the other hand, the conclusion agrees with that of several investigators. Cazenave and Robert [35] reported saturation of doxorubicin efflux from rat glioma (C_6) cells. Saturation of vinca alkaloid transport has also been described [36]. Spoelstra *et al.* [37] have demonstrated saturation kinetics in several colon cancer cell lines with intrinsic resistance.

In medium including sucrose, the time constants (K) obtained for both the sensitive and resistant cell lines were significantly different from the time constants obtained in medium including glucose. This finding could be explained by differences in the pH gradient across the plasma membranes. The pK_a value of DNR is 8.2 [21]. Thus, near physiological pH an extreme pH dependence of efflux could be expected. Addition of sucrose to the efflux medium could change the pH gradient across the plasma membrane and thereby influence the efflux.

In the present study, the efflux from sensitive whole cells and nuclei were not significantly different. Although changes in pH could disturb the results, the data indicated that in sensitive cells the release of drug from the nuclei was the rate-limiting process. The even lower efflux found in resistant nuclei confirms this assumption. The linearity obtained in washout experiments indicates that drug bound to the nucleus represents the main intracellular compartment [13]. This supports previous results reported by Skovsgaard [38].

In medium including sucrose, the slow component of efflux from highly resistant whole cells was significantly faster than release from the corresponding nuclei. Changes due to addition of sucrose to the efflux medium could confound the results. It is, however, unlikely that the efflux in EHR2/0.8 (passage No. 72) cells reflected release of drug from the nuclei. The finding indicates that the drug must be bound to intracellular binding sites with weaker affinity than to the nuclei. Although the nuclear binding affinity in sensitive and resistant cells has been demonstrated to be nearly equal [8], the subcellular distribution of anthracyclines in MDR cells has been shown to shift from mainly nuclear to mainly cytoplasmic [39], which is in agreement with our data.

In the present study, a dose–response relationship for the effect of VER on the rapid drug efflux component was established. Previously, a similar dose–response effect of VER on drug accumulation and cytotoxicity has been described [40].

In four of five sublines, the effect of VER on drug efflux and cytotoxicity of DNR was correlated to expression of P-gp, degree of resistance, and efflux capacity. In passage No. 12 of EHR2/0.8, however, the effect of VER was independent of P-gp. The effect of VER on resistance or efflux has only been investigated in a limited number of cell lines with different expression of P-gp [23, 41, 42]. Among these, Huet *et al.* [41] investigated the effect of VER on doxorubicin efflux and cytotoxicity in three doxorubicin resistant rat glioblastoma cell lines. Twentyman *et al.* [42] investigated the effect of VER on cytotoxicity of different cytostatics in four MDR cell lines with different contents of P-gp. In both of these studies, the content of P-gp correlated to the degree of resistance, whereas the accumulation defects were the same in all investigated resistant sublines. In contrast to the present results, these authors found the degree of sensitization to correlate with the degree of P-gp in all investigated cell lines. An explanation for these conflicting results could be the missing correlation between P-gp and drug accumulation found in both of these studies. Thus, in our study and both of these studies, the degree of resistance was determining for the effect of VER. The discrepancy could be explained by the existence of other resistance mechanisms, e.g. quantitative/qualitative changes in topoisomerase II [41]. At last, the effect of VER on cytotoxicity and efflux of vinca alkaloids have been carefully investigated by Shalinsky *et al.* [23]. These authors used a MDR transfectant and a vincristine resistant cell line for the experiments and demonstrated a close correlation between P-gp, resistance, drug efflux, and effect of VER.

In all the resistant cell lines, application of VER (1.1 μM) resulted in approximately the same relative inhibition of the rapid efflux component (72–87%). This relative inhibition appeared to be independent of the amount of P-gp and of the effectiveness of the efflux pump (value of K). Assuming that the small variations in inhibition between the different cell lines reflected variations in intracellular concentrations of DNR, we suggest that the ratio between inhibitor and anthracycline determines the relative inhibition of the efflux, i.e. the probability that P-gp is occupied by either VER or anthracycline depends on the relative substrate concentrations solely. This finding supports that VER acts as a competitive inhibitor [43].

Lineweaver–Burk plot (double reciprocal plot of initial rapid efflux (appendix; equation 3, $t = 1$ min) as a function of total intracellular content of DNR) showed that VER changed the apparent "K_m" value of the rapid efflux component without changes in apparent "V_{max}". The result, however, should be taken with reservation, though, as the linearity between substrate concentration for P-gp (free drug concentration) and total intracellular drug content could be influenced by changes in drug binding and/or intercalation. Our findings are in agreement with the proposal that VER acts as a competitive inhibitor [16].

In the present study, addition of VER to the efflux medium had no effect on the efflux from sensitive cells. In contrast, Friche *et al.* [44] reported previously a 15% enhancement of the steady state accumulation after addition of VER (10 μM) to sensitive EHR2 cells. In agreement with the present results, Bellamy *et al.* [40] found no effect of VER on the efflux of MDR compounds in sensitive cells. In the present study, however, VER significantly increased cytotoxicity of DNR in sensitive cells. These findings are

supported by Tsuruo *et al.* [45], but the responsiveness to MDR drugs of most sensitive cell lines is not significantly affected by VER in non-cytotoxic doses [4].

In conclusion, the results confirm that P-gp acts as a drug efflux pump. As no simple correlation between P-gp and drug efflux could be established, it is suggested that other cellular factors influence transmembranous transport of DNR, possibly by regulation of the function of P-gp. Active efflux seems to be saturable and to constitute a major route of drug efflux in MDR cells. Our data are compatible with the action of VER as a competitive inhibitor of P-gp.

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REFERENCES

1. Bradley G, Juranka PF and Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948**: 87–128, 1988.
2. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58**: 137–171, 1989.
3. van der Bliek AM and Borst P, Multidrug resistance. *Adv Cancer Res* **52**: 165–203, 1989.
4. Ford JM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42**: 155–199, 1990.
5. Higgins CF, ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**: 67–113, 1992.
6. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**: 385–427, 1993.
7. Nielsen D, Maare C, Poulsen F, Lauridsen ST and Skovsgaard T, The relationship between resistance, P-glycoprotein content, and steady state accumulation in five series of Ehrlich ascites tumor cell lines selected for resistance to daunorubicin. *Cell Pharmacol* **1**: 127–135, 1994.
8. Skovsgaard T, Mechanisms of resistance to daunorubicin in Ehrlich ascites tumor cells. *Cancer Res* **38**: 1785–1791, 1978.
9. Sehested M, Bindslev N, Demant EJF, Skovsgaard T and Jensen PB, Daunorubicin and vincristine binding to plasma membrane vesicles from daunorubicin-resistant and wild type Ehrlich ascites tumor cells. *Biochem Pharmacol* **38**: 3017–3027, 1989.
10. Roed H, Christensen IJ, Vindeløv LL, Spang-Thomsen M and Hansen HH, Interexperiment variation and dependence on culture conditions in assaying the chemosensitivity of human small cell lung cancer cell lines. *Eur J Cancer Clin Oncol* **23**: 177–188, 1987.
11. Skovsgaard T, Transport and binding of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells. *Biochem Pharmacol* **26**: 215–222, 1977.
12. Siegfried JM, Burke TG and Tritton TR, Cellular transport of anthracyclines by passive diffusion. *Biochem Pharmacol* **34**: 593–598, 1985.
13. Schafer JA and Barfus DW, Mechanisms of transmembrane transport in isolated cells and their experimental study. In: *Membrane Transport of Antineoplastic Agents* (Ed. Goldman ID), pp. 1–37. Pergamon Press, New York, 1986.
14. Spoelstra E, Westerhoff HV, Dekker H and Lankelma J, Kinetics of daunorubicin transport by P-glycoprotein of intact cancer cells. *Eur J Biochem* **207**: 567–579, 1992.
15. Frezard F and Garnier-Suillerot A, Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **196**: 483–491, 1991.
16. Yusa K and Tsuruo T, Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* **49**: 5002–5006, 1989.
17. Cano-Gauci DF and Riordan JR, Action of calcium antagonists on multidrug resistant cells. *Biochem Pharmacol* **36**: 2115–2123, 1987.
18. Sehested M, Skovsgaard T, van Deurs B and Winther-Nielsen H, Increase in non-specific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant Ehrlich ascites tumor cell lines. *J Natl Cancer Inst* **78**: 171–179, 1987.
19. Lankelma J, Spoelstra EC, Dekker H and Broxterman HJ, Evidence for daunorubicin efflux from multidrug-resistant 2780^{AD} human ovarian carcinoma cells against a concentration gradient. *Biochim Biophys Acta* **1055**: 217–222, 1990.
20. Demant EJF, Sehested M and Jensen PB, A model for computer simulation of P-glycoprotein and transmembrane pH-mediated anthracycline transport in multidrug-resistant tumor cells. *Biochim Biophys Acta* **1055**: 117–125, 1990.
21. Skovsgaard T, Drug transport in multidrug-resistant tumor cells. A revision of the pump-hypothesis. In: *Anticancer Drugs* (Eds. Tapiero H, Robert J and Lampidis TJ), Vol. 191, pp. 233–242. Colloque INSERM/John Lippey Eurotext Ltd, 1989.
22. Gerlach JH, Structure and function of P-glycoprotein. In: *Drug Resistance in Cancer Therapy* (Ed. Ozols RF), pp. 37–53. Kluwer Academic Publishers, Boston, 1989.
23. Shalinsky DR, Andreeff M and Howell SB, Modulation of drug sensitivity by dipyridamole in multidrug resistant tumor cells *in vitro*. *Cancer Res* **50**: 7537–7543, 1990.
24. Choi K, Frommel TO, Stern RK, Perez CF, Kriegler M, Tsuruo T and Roninson IB, Multidrug resistance after retroviral transfer of the human MDR1 gene correlates with P-glycoprotein density in the plasma membrane and is not affected by cytotoxic selection. *Proc Natl Acad Sci USA* **88**: 7386–7390, 1991.
25. Nielsen D and Skovsgaard T, P-glycoprotein as multidrug transporter—a critical review of current multidrug resistant cell lines. *Biochim Biophys Acta* **1139**: 169–183, 1992.
26. Willingham MC, Richert ND, Cornwell MM, Tsuruo T, Hamada H, Gottesman MM and Pastan IH, Immunocytochemical localization of P170 at the plasma membrane of multidrug resistant human cells. *J Histochem Cytochem* **35**: 1451–1456, 1987.
27. Kartner N, Evernden-Porelle D, Bradley G and Ling V, Detection of P-glycoprotein in multidrug resistant cell lines by monoclonal antibodies. *Nature* **316**: 820–823, 1985.
28. Raymond M, Rose E, Housman DE and Gros P, Physical mapping, amplification, and overexpression of the mouse *mdr* gene family in multidrug-resistant cells. *Mol Cell Biol* **10**: 1642–1651, 1990.
29. van der Bliek AM, van der Velde-Koerts T, Ling V and Borst P, Overexpression and amplification of five genes in a multidrug-resistant Chinese hamster ovary cell line. *Mol Cell Biol* **6**: 1671–1678, 1986.
30. Ramachandran C, Sauerteig A, Sridhar KS, Thurer RJ and Krishan A, MDR-1 gene expression, anthracycline retention and cytotoxicity in human lung-tumor cells from refractory patients. *Cancer Chemother Pharmacol* **31**: 431–441, 1993.

31. Ueda K, Cornwell MM, Gottesman MM, Pastan I, Roninson IB, Ling V and Riordan JR, The *mdr*1 gene, responsible for multidrug-resistance, codes for P-glycoprotein. *Biochem Biophys Res Commun* **141**: 956–962, 1986.
32. Bates SE, Currier SJ, Alvares M and Fojo AT, Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. *Biochemistry* **31**: 6366–6372, 1992.
33. Friche E, Danks MK, Schmidt CA and Beck WT, Decreased DNA topoisomerase II in daunorubicin-resistant Ehrlich ascites tumor cells. *Cancer Res* **51**: 4213–4218, 1991.
34. Chaires JB, Dattagupta N and Crothers DM, Self-association of daunorubicin. *Biochemistry* **21**: 3927–3932, 1982.
35. Cazenave M and Robert J, A model for the pharmacology of doxorubicin. *J Cell Pharmacol* **2**: 293–299, 1991.
36. Watanabe T, Inaba M and Sugiyama Y, Saturable process involved in active efflux of vincristine as a mechanism of multidrug resistance in P388 leukemia cells. *Pharm Res* **6**: 690–696, 1989.
37. Spoelstra EC, Dekker H, Schuurhuis GJ, Broxterman HJ and Lankelma J, P-glycoprotein drug efflux pump involved in the mechanisms of intrinsic resistance in various colon cancer cell lines; evidence for a saturation of active daunorubicin transport. *Biochem Pharmacol* **41**: 349–359, 1991.
38. Skovsgaard T, Carrier-mediated transport of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells. *Biochem Pharmacol* **27**: 1221–1227, 1978.
39. Hindenburg AA, Baker MA, Gleyzer E, Stewart VJ, Case N and Taub RN, Effect of verapamil and other agents on the distribution of anthracyclines and on reversal of drug resistance. *Cancer Res* **47**: 1421–1425, 1987.
40. Bellamy WT, Dalton WS, Kailey JM, Gleason MC, McCloskey TM, Dorr RT and Alberts DS, Verapamil reversal of doxorubicin resistance in multidrug-resistant human myeloma cells and association with drug accumulation and DNA damage. *Cancer Res* **48**: 6303–6308, 1988.
41. Huet S, Schott B and Robert J, P-glycoprotein overexpression cannot explain the complete doxorubicin-resistance phenotype in rat glioblastoma cell lines. *Br J Cancer* **65**: 538–544, 1992.
42. Twentyman PR, Reeve JG, Koch G and Wright KA, Chemosensitisation by verapamil and cyclosporin A in mouse tumour cells expressing different levels of P-glycoprotein and CP22 (sorcin). *Br J Cancer* **62**: 89–95, 1990.
43. Stein WD (Ed.), *Transport and Diffusion Across Cell Membranes*. Academic Press, Orlando, 1986.
44. Friche E, Skovsgaard T and Nissen NI, Effect of verapamil on daunorubicin accumulation in Ehrlich ascites tumor cells. *Cancer Chemother Pharmacol* **19**: 35–39, 1987.
45. Tsuruo T, Iida H, Tsukagoshi S and Sakurai Y, Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* **41**: 1967–1972, 1981.

APPENDIX

Calculations of drug efflux

Sensitive cells (mono-exponential kinetics)

$$C_t = C_0 \times e^{-Kt},$$

where C = intracellular content of DNR, t = time and K = constant. (1)

Resistant cells (bi-exponential kinetics)

$$C_t = A \times e^{-Kt} + B \times e^{-Qt},$$

where $A + B = C$, C = total intracellular content of DNR, t = time, K and Q = constants. (2)

Amount of drug effluxed by the component described by K *and* A (*time* = t)

$$A_{\text{effluxed}} = A_0 - A_0 \times e^{-Kt} \tag{3}$$

Efflux described by K *and* A (*time* = ∞)

$$\frac{A_0 - A_0 \times e^{-Kt}}{A_0 - A \times e^{-Kt} + B_0 - B_0 \times e^{-Qt}} \text{ time} = \infty$$

$$\frac{A_0 - A_0 \times e^{-K\infty}}{A_0 - A \times e^{-K\infty} + B_0 - B_0 \times e^{-Q\infty}}$$

$$\frac{A_0}{A_0 + B_0} \tag{4}$$