Pergamon

Biochemical Pharmacology, Vol. 50, No. 4, pp. 443–450, 1995.
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0006-2952/95 $9.50 + 0.00

0006-2952(95)00172-7

INFLUX OF DAUNORUBICIN IN MULTIDRUG RESISTANT EHRLICH ASCITES TUMOUR CELLS: CORRELATION TO EXPRESSION OF P-GLYCOPROTEIN AND EFFLUX. INFLUENCE OF VERAPAMIL

DORTE NIELSEN,* CHRISTIAN MAARE and TORBEN SKOVSGAARD

Department of Oncology R, Herlev Hospital, University of Copenhagen, Denmark

(*Received* 3 *November* 1994; *accepted* 10 *April* 1995)

Abstract—Classic multidrug resistance is characterized by a decrease in the intracellular concentration of drugs in resistant cells as compared to sensitive cells. This is correlated with the presence of P-glycoprotein in the membrane. P-glycoprotein is responsible for an active efflux of drug. In this study we investigated the correlation between P-glycoprotein and influx of daunorubicin. Four Ehrlich ascites tumour cell lines selected *in vivo* for resistance to daunorubicin were investigated. The sublines EHR2/0.1, EHR2/0.2, passage no. 12 of EHR2/0.8, EHR2/0.4, and passage no. 72 of EHR2/0.8 were 6-, 6-, 5-, 33-, and 35-fold resistant to daunorubicin, respectively. All sublines overexpressed P-glycoprotein as determined with Western blot. Influx was measured over 40 sec. In glucose-enriched medium influx was significantly decreased in all but one of the resistant sublines. A correlation between P-glycoprotein, degrees of resistance, and influx was demonstrated in four sublines. Comparing influx experiments with efflux experiments (Nielsen *et al.*, *Biochem Pharmacol* 1994, **47**, 2125–2135) we found a linear relationship between influx and efflux in the resistant sublines ($r = 0.97$). Verapamil (5.5 μM, 11.0 μM) increased influx significantly in all resistant sublines, whereas the drug had no effect on sensitive cells. Verapamil (3.3 μM) increased influx in the EHR2/0.8 (passage no. 72) subline to the level of sensitive cells. Comparing this result with efflux experiments, verapamil was found to increase influx preferentially. Depletion of energy (medium without glucose including Na^+-azide) increased influx in all resistant sublines. In EHR2/0.4 and EHR2/0.8 (passage no. 72) the influx, however, was still significantly decreased after depletion of energy. In these cells further addition of verapamil increased influx to the level of EHR2. These data were consistent with the hypothesis that P-glycoprotein effluxes drug directly from the plasma membrane.

Key words: P-glycoprotein; influx; daunorubicin; verapamil

Cellular resistance is a major problem in cancer chemotherapy. MDR† describes the phenomenon in which tumour cells exposed to one antitumour agent become resistant not only to the selecting agent but also to a variety of other chemically and structurally diverse chemotherapeutic agents. Several types of MDR have been described, classic MDR being the most well-characterized. The drugs most often involved in classic MDR are of fungal or plant origin, including the vinca alkaloids, anthracyclines, colchicine, actinomycin D, and epipodophyllotoxins. Resistance results from the expression of the *mdr1* gene which encodes a protein, termed P-glycoprotein [1, 2]. PGP belongs to a superfamily of transport systems including bacterial transporters, the cystic fibrosis transmembrane conductance regulator, and the *Plasmodium falciparum* drug resistance gene [3, 4]. PGP-positive cells are characterized by decreased drug accumulation. In 1973, Danø [5] proposed the existence of an active drug extrusion in tumour cells resistant to DNR. Studies on PGP led to a model which suggested that drugs bind directly to PGP, which then acts as a carrier that actively extrudes drugs through a pore or channel in the membrane formed by transmembrane domains of the molecules using energy derived from ATP hydrolysis [6].

Influx of drugs into PGP-positive cells has been proposed to take place by simple diffusion [7, 8]. In several MDR cell lines increased efflux, however, is accompanied by a decrease in influx [9]. Several studies have demonstrated that influx can be enhanced by addition of inhibitors of energy production [10–12]. In order to explain these findings a new model for the functioning of the molecule has been proposed [13, 14]. According to this model the two halves of PGP form a single channel for drug transport, and the major function of PGP is to act as a "vacuum-cleaner" which removes the drugs directly from the lipid bilayer of the plasma membrane. This means that the drug intercalates into the lipid bilayer and is subsequently recognized by PGP, which then extrudes it from the plasma membrane before it enters the cell. Assuming that PGP does indeed act as a "vacuum-cleaner", one would expect a close connection between PGP expression and influx. Furthermore, influx and efflux in MDR cells should be closely correlated.

The purpose of the present study was to investigate

* Correspondence to: Dr Dorte Nielsen M.D., Department of Oncology ONK, Finsen Center, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark. Tel. +45 35455074; FAX +45 35456966.

† Abbreviations: DNR, daunorubicin; MDR, multidrug resistance; PGP, P-glycoprotein; VER, verapamil.

Table 1. P-glycoprotein, degrees of resistance, efflux, and influx of daunorubicin in EHR2 and MDR sublines in glucose-enriched medium

Cell line	P-glycoprotein units* (mean ± SD)	Relative resistance	Efflux (k)† (min^{-1})	Influx ($pmol/10^6$ cells/sec)* (mean of slopes ± SD)
EHR2	12.0 ± 4.0 (20)	—	0.033	7.80 ± 1.74 (6)
EHR2/0.1	75.6 ± 15.8 (8)	6.2	0.381	6.36 ± 1.42 (6)‡
EHR2/0.2	38.4 ± 6.9 (8)	5.5	0.438	6.37 ± 0.94 (6)§
EHR2/0.4	210.6 ± 16.8 (8)	32.5	0.844	4.62 ± 0.54 (6)§
EHR2/0.8 p. 12	165.6 ± 14.8 (8)	5.1	0.436	6.41 ± 0.62 (6)§
EHR2/0.8 p. 72	255.0 ± 15.0 (8)	35.1	0.773	3.34 ± 0.42 (6)§

* The number of experiments are given in parentheses.
† From Ref. [17].
‡ Influx not significantly different from influx in EHR2.
§ Influx significantly different from influx in EHR2.

the relationship between PGP, influx and efflux of DNR, and effect of VER on influx in MDR cell lines with different expression of PGP.

MATERIALS AND METHODS

Chemicals. DNR as hydrochloride was obtained from Farmatalia Carlo Erba (Milan, Italy), sodium azide from Merck (Darmstadt, Germany), and VER as hydrochloride from Ercopharm Ltd (Copenhagen, Denmark).

Tumour cells lines. The sensitive Ehrlich ascites tumour cell line (EHR2) and four DNR-resistant sublines were used. All cell lines were maintained in first-generation hybrids of female NRMI and male inbred DBA/2 mice by weekly transplantation. The resistant sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and EHR2/0.8 were developed *in vivo* in mice 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. These sublines have previously been described in detail [15]. Cells from passage no. 72 of EHR2/0.1, passage no. 24 of EHR2/0.2, passage 54 of EHR2/0.4, and passages 12 and 72 of EHR2/0.8 were used for the experiments. The soft agar clonogenic assay was used for determination of degrees of resistance [16]. The sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and passage numbers 12 and 72 of EHR2/0.8 were 6.2-, 5.5-, 32.5-, 5.1-, and 35.1-fold resistant to DNR as compared to EHR2, respectively [17].

Quantitation of P-glycoprotein. Western blot analysis was performed for the quantitation of PGP as described in detail earlier [15]. The relative concentration of PGP was calculated using a curve composed of membrane preparations with a defined content of PGP (Table 1).

Efflux of daunorubicin. The method described by Skovsgaard [18] was applied. Several authors [19, 20] have found drug efflux from MDR cells to be made up of two main components: diffusion and active PGP-mediated efflux. Theoretically, efflux from MDR cells could be described by biexponential kinetics. Previously, we have described efflux from the resistant cell lines by the following equation:

$$C_t = A \times e^{-kt} + B \times e^{-qt}$$

where $A + B = C$; C is the total intracellular content of DNR; t is time; k and q are constants. The k values calculated from curve fitting by regression (Sigma plot) were found to be representative for PGP-mediated efflux [17].

Influx of daunorubicin. Influx of DNR in presence and absence of Na^+-azide was measured as previously described [18]. The phosphate-buffered medium was supplemented with DNR (2.5–25.0 mM) as well as either glucose (10 mM) or Na^+-azide (10 mM). The cell suspension was incubated at 37° for 10 min before the experiments were begun. Glucose and Na^+-azide were added to the cells 1 and 5 min before the experiments, respectively. The effect of VER on influx was investigated by addition of either 1.1 μM, 5.5 μM, or 11.0 μM VER 10 min before the start of experiments. Furthermore, dose–response of VER on influx was evaluated in EHR2/0.8 (passage no. 72) by addition of VER (0.1–11.0 μM). The experiments were started by addition of cells (2×10^6 cells/mL) to the medium. Serial samples were withdrawn at intervals of 3 sec, transferred to ice-cold phosphate buffer (pH 6.0), centrifugated, and washed twice. Extraction of drug and determination of fluorescence were performed as previously described.

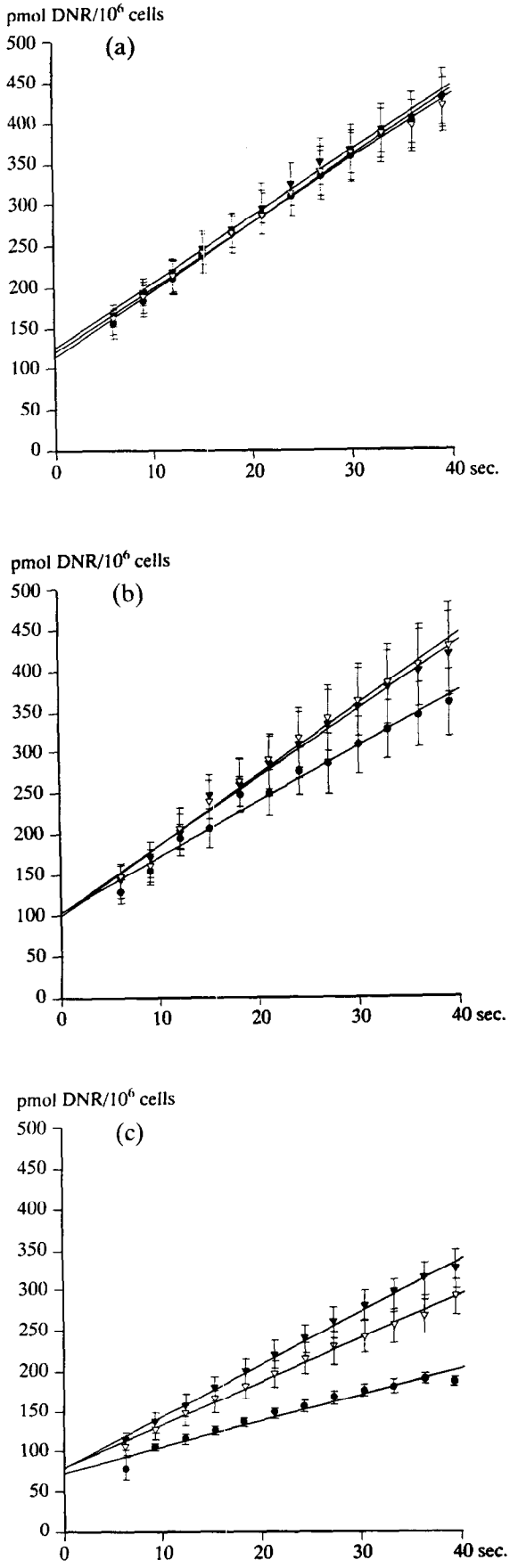
Statistics. Student's t-test was used at a significance level of 5%.

RESULTS

Influx of DNR in glucose-enriched medium

The influx of DNR (5.0 μM) in presence of glucose with and without VER (1.1 and 11.0 μM) is shown in Fig. 1. The curves obtained for EHR2/0.1 (Fig. 1b) are similar to those obtained for 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 similar to those obtained for EHR2/0.4.

Influx of DNR in glucose-enriched medium (mean of slopes ± SD; N = 6) and the relationship between influx, expression of PGP, degrees of resistance, and efflux (represented by the k-values calculated from the equation $C_t = A \times e^{-kt} + B \times e^{-qt}$ [17]) are given in Table 1. The influx of DNR in EHR2/0.1 was not significantly different from that in EHR2



(P = 0.17). In sublines EHR2/0.2, EHR2/0.4, EHR2/0.8 (passage no. 12), and EHR2/0.8 (passage no. 72) influx was significantly decreased (P = 0.026, 1.2×10^{-4}, 0.019, and 9.4×10^{-6}, respectively). Figure 2 shows influx capacity (mean of slopes; pmol/10^{-6} cells/sec) as a function of efflux capacity (k-values; min^{-1}). A linear relationship between efflux and influx was demonstrated (the correlation coefficient for the curve was 0.97). Thus, in all sublines influx of DNR was found to be inversely correlated with efflux. In four of five sublines a correlation between resistance, content of PGP, and influx of DNR was established. In passage no. 12 of EHR2/0.8, however, a high expression of PGP was found in spite of a low degree of resistance and a minimal decrease in influx.

The influx (mean of slopes ± SD; N = 6) of DNR (5.0 μM) in presence of glucose with and without VER (1.1, 5.5, and 11.0 μM) is given in Table 2. In sublines EHR2/0.1, EHR2/0.2, EHR2/0.4, and EHR2/0.8 (passage no. 72) VER (1.1 μM) significantly increased influx (P = 0.012, 0.012, 4.5×10^{-4}, and 2.0×10^{-5}, respectively), whereas influx in EHR2/0.8 (passage no. 12) was unchanged (P = 0.12). VER (5.5 and 11.0 μM) increased influx of DNR significantly in all sublines.

Influx of daunorubicin in medium without glucose including Na^+*-azide*

The influx of DNR (5.0 μM) in medium including Na^+-azide with and without VER (1.1 and 11.0 μM) is shown in Fig. 3. The curves obtained for EHR2/0.1 (Fig. 3b) are similar to those for sublines EHR2/0.2 and passage no. 12 of EHR2/0.8. The curves shown for passage no. 72 of EHR2/0.8 (Fig. 3c) are similar to those obtained for EHR2/0.4. The effect of VER on influx (mean of slopes ± SD; N = 6) of DNR is given in Table 3. When cells were depleted of energy (by inhibition of both glycolysis and oxidative phosphorylation), the influx of DNR in EHR2/0.1, EHR2/0.2, and EHR2/0.8 (passage no. 12) was not significantly different from that in EHR2 (P = 0.56, 0.29, and 0.49, respectively). Furthermore, in sublines EHR2/0.4 and EHR2/0.8 (passage no. 72) depletion of energy significantly increased influx (P = 0.032 and 1.6×10^{-5}). Compared to the sensitive cell line EHR2 the influx in these sublines was, however, still significantly decreased (P = 0.012 and 0.006). VER (11.0 μM) had no effect on influx of DNR in sensitive cells (P = 0.92). Furthermore, influx was not significantly increased in subline EHR2/0.2 (P = 0.16). In sublines EHR2/0.1, EHR2/0.4, EHR2/0.8 (passage no. 12),

Fig. 1. Influx of daunorubicin in sensitive and resistant EHR2 tumour cells in medium with glucose (●) or corresponding medium with glucose and different concentrations of verapamil (1.1 μM (▽) and 11.0 μM (▼)). The curves are the mean of six determinations. Bars represent SEM. (a) EHR2; (b) EHR2/0.1; (c) EHR2/0.8 (passage no. 72). The curves obtained for EHR2/0.1 are similar to those obtained for sublines EHR2/0.2 and passage no. 12 of EHR2/0.8. The curves shown for passage no. 72 of EHR2/0.8 are similar to those obtained for EHR2/0.4.

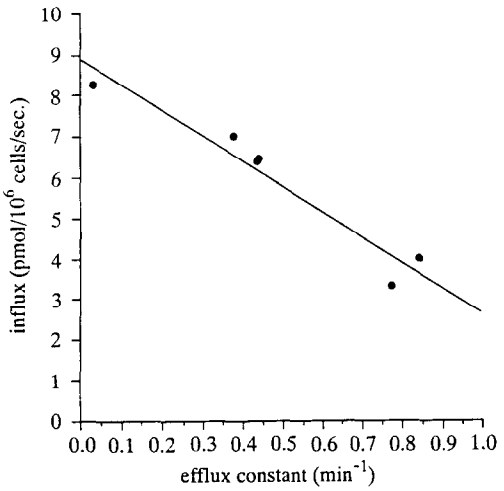


Fig. 2. The influx of daunorubicin (mean; pmol/10^6 cells/sec) as a function of the efflux capacity (k-values; min^{-1}) of the resistant cell lines calculated from the equation $C_t = A \times e^{-kt} + B \times e^{-qt}$ ($A + B = C$; where C is the total intracellular content of DNR; t is time; and k and q are constants).

and EHR2/0.8 (passage no. 72) addition of VER (11.0 μM), however, significantly increased the influx of DNR ($P = 5.2 \times 10^{-4}$, 0.0055, 0.026, and 2.5×10^{-5}).

Dose–response relationship for verapamil

The resistant subline EHR2/0.8 (passage no. 72) was used for investigation of the relationship between dose of VER and influx of DNR. Table 4 shows the relationship between influx of DNR (mean of slopes ± SD) and the concentration of VER (0.1 μM to 11.0 μM). A very steep dose–response relationship was established. Addition of VER ($\geq$3.3 μM) equalized influx of DNR in sensitive and resistant cells.

Saturation of influx

Saturation of influx was investigated in EHR2/0.8 (passage no. 72) by exposing the subline to DNR at concentrations of 2.5–25 μM. Figure 4 shows the Lineweaver–Burk (double reciprocal) plot of initial influx as a function of the extracellular concentration of DNR. The correlation coefficients (r) were 0.999 and 0.996 for the curves obtained in medium without and with VER (1.1 μM), respectively (Sigma plot). V_{max} for DNR in glucose-enriched medium was 50.8 pmol/10^6 cells/sec, K_m was 59.9 μM. V_{max} for DNR in glucose-enriched medium including VER (1.1 μM) was 75.2 pmol/10^6 cells/sec, while K_m was 54.6 μM.

Cell volume

In order to investigate the influence of cell volume on influx we determined the cytocrite of the cells. The cytocrite of a suspension of 4×10^7 cells/mL was 16.3% (SD 1.6), 11.7 (SD 1.7), 10.7 (SD 1.6), 13.5 (SD 1.2), 11.4 (SD 1.4), and 12.6 (SD 1.1) for cell lines EHR2, EHR2/0.1, EHR2/0.2, EHR2/0.4, EHR2/0.8 (passage no. 12), and EHR2/0.8 (passage no. 72), respectively. Thus, all the resistant cell lines had significantly decreased cell volume compared to EHR2. Variations in cell volume were, however, not correlated to variations in influx.

DISCUSSION

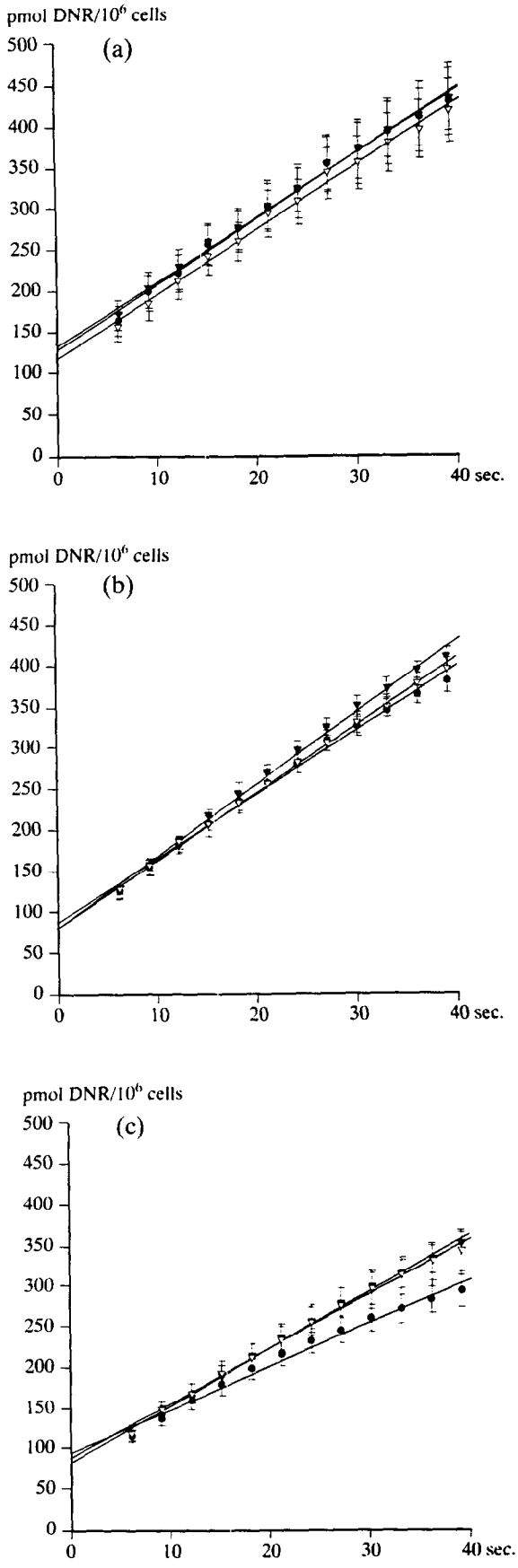
The influx of drug has only been studied in 11 single and one series (five sublines) of PGP-positive cell lines. The influx was found to be decreased in all resistant cell lines [10–12, 19–28]. In the present study, the influx was significantly decreased in all but one resistant subline. Thus, we were not able to demonstrate a significantly decreased influx in EHR2/0.1. This finding could possibly be explained by statistical variations in the influx experiments.

Theoretically, several cellular changes could account for the decreased influx. First, insertion of large quantities of PGP into the plasma membrane may have profound effects on the structural order

Table 2. Influx of daunorubicin into EHR2 and MDR sublines in glucose-enriched medium

Cell line	Influx* (pmol/10^6 cells/sec) (mean of slopes ± SD)	Influx* (pmol/10^6 cells/sec) (mean of slopes ± SD) Verapamil 1.1 μM	Influx* (pmol/10^6 cells/sec) (mean of slopes ± SD) Verapamil 5.5 μM	Influx* (pmol/10^6 cells/sec) (mean of slopes ± SD) Verapamil 11 μM
EHR2	7.80 ± 1.74 (6)	8.07 ± 1.56 (6)	8.81 ± 1.97 (6)	8.32 ± 1.77 (6)
EHR2/0.1	6.36 ± 1.42 (6)†	7.57 ± 1.53 (6)†§§	7.42 ± 1.26 (6)†	7.58 ± 1.40 (6)†§§
EHR2/0.2	6.37 ± 0.94 (6)‡	7.47 ± 0.64 (6)†§§	7.26 ± 1.36 (6)†	7.62 ± 0.70 (6)†§§
EHR2/0.4	4.62 ± 0.54 (6)‡	5.88 ± 0.76 (6)‡§§	6.46 ± 0.63 (6)†	6.81 ± 1.13 (6)†§§
EHR2/0.8 p. 12	6.41 ± 0.62 (6)‡	7.06 ± 0.54 (6)†§	7.41 ± 0.74 (6)†	7.49 ± 0.63 (6)†§§
EHR2/0.8 p. 72	3.34 ± 0.42 (6)‡	5.39 ± 0.98 (7)‡§§	6.63 ± 1.03 (7)†	6.56 ± 0.94 (8)†§§

* The number of experiments are given in parentheses.
† Influx not significantly different from influx in EHR2.
‡ Influx significantly different from influx in EHR2.
§ Influx not significantly different from influx in EHR2/0.8 (passage no. 12) in glucose-enriched medium without verapamil.
§§ Influx significantly different from influx in the respective cell lines in glucose-enriched medium without verapamil.



of the membrane and thereby on the diffusional permeability of drug. In the present study, it is, however, unlikely that this mechanism alone could account for the decreased influx, as the subline EHR2/0.8 (passage no. 12) had a minimal decrease in influx capacity in spite of a high expression of PGP.

Second, various changes in membrane lipids have been suggested to affect the permeability of drug [29]. In a recent study [30] we investigated the lipid composition of different passages of EHR2/0.8. Although significant variation was found during development of resistance, the composition of the lipids in the most resistant cell line was not significantly different from that in the wild-type cell line.

Third, changes in the fluidity of the plasma membrane may contribute to decreased permeability. In support of this argument, addition of VER (11.0 μM) increased influx significantly (to the level in sensitive cells) in energy-depleted EHR2/0.4 and EHR2/0.8 (passage no. 72) cells. VER has been shown to have a general ability to interact with and perturb membranes [31]. Changes in fluidity, however, are not a prerequisite for decreased influx, as Shalinsky *et al.* [12] have found significantly decreased influx in the KB-GRC1 cell line. This cell line has been transfected with the *mdr1* gene and differs theoretically from the sensitive cell line only by expression of PGP.

Finally, the decreased influx could represent increased PGP-mediated efflux. The following findings are consistent with this argument. (1) In all cell lines, a linear relationship between efflux and influx was demonstrated. Thus, in passage no. 12 of EHR2/0.8 the presence of PGP with low efflux capacity [17] was reflected in a minimal decrease in influx. (2) Addition of VER increased initial influx in all resistant cell lines whereas the influx in the sensitive cell line was unaffected. VER had previously been shown to bind to PGP [32, 33] and to be an effective inhibitor of efflux.

Recently, Raviv *et al.* [13] have reported that doxorubicin interacted with a site on PGP within the cellular membrane. The authors suggested a more prominent role for the membranally rather than the cytoplasmatically bound substrate (the "vacuum cleaner" model). In the present study, the following findings favour this hypothesis. First, the presence of PGP in the plasma membrane decreased influx measured over the first few seconds (first measurement after 6 sec). During this period the

Fig. 3. Influx of daunorubicin in sensitive and resistant EHR2 tumour cells in medium without glucose including Na^+-azide (●) or corresponding medium with different concentrations of verapamil (1.1 μM (▽) and 11.0 μM (▼)). The curves are the mean of six determinations. Bars represent SEM. (a) EHR2; (b) EHR2/0.1; (c) EHR2/0.8 (passage no. 72). The curves obtained for EHR2/0.1 are similar to those obtained for sublines EHR2/0.2 and passage no. 12 of EHR2/0.8. The curves shown for passage no. 72 of EHR2/0.8 are similar to those obtained for EHR2/0.4.

Table 3. Influx of daunorubicin into EHR2 and MDR sublines in medium including Na^+-azide (N = 6 for all experiments)

Cell line	Influx (pmol/10^6 cells/sec) (mean of slopes ± SD)	Influx (pmol/10^6 cells/sec) (mean of slopes ± SD) Verapamil 1.1 μM	Influx (pmol/10^6 cells/sec) (mean of slopes ± SD) Verapamil 5.5 μM	Influx (pmol/10^6 cells/sec) (mean of slopes ± SD) Verapamil 11 μM
EHR2	8.35 ± 2.19	8.45 ± 2.22	8.74 ± 1.83	8.74 ± 2.73‡
EHR2/0.1	7.77 ± 0.63*	8.15 ± 0.57	8.32 ± 0.60	8.59 ± 0.77§
EHR2/0.2	7.33 ± 0.78*	7.70 ± 0.78	7.92 ± 0.42	7.91 ± 0.92§
EHR2/0.4	5.50 ± 1.35†	5.94 ± 1.40	6.47 ± 1.46	6.58 ± 1.63§
EHR2/0.8 p. 12	7.68 ± 0.70*	7.66 ± 0.45	7.88 ± 0.73	8.19 ± 0.59‡
EHR2/0.8 p. 72	5.66 ± 0.79†	7.02 ± 1.07	7.15 ± 0.99	7.20 ± 0.85§

* Influx not significantly different from influx in EHR2.
† Influx significantly different from influx in EHR2.
‡ Influx not significantly different from influx in the respective cell lines in medium including Na^+-azide without verapamil.
§ Influx significantly different from influx in the respective cell lines in medium including Na^+-azide without verapamil.

Table 4. Influx of daunorubicin into the resistant subline EHR2/0.8 (passage no. 72) in glucose-enriched medium: influence of verapamil

Verapamil (μM)	Influx* (pmol/10^6 cells/sec) (mean of slopes ± SD)
—	3.34 ± 0.42 (7)
0.11	4.44 ± 0.40 (3)†
0.33	4.71 ± 1.12 (3)†
0.55	5.18 ± 0.67 (3)†
1.10	5.39 ± 0.98 (7)†
3.30	6.51 ± 1.08 (3)‡
5.50	6.63 ± 1.03 (7)‡
11.00	6.56 ± 0.94 (8)‡

* Number of experiments are given in parentheses.
† Influx of DNR significantly different from influx in EHR2.
‡ Influx of DNR not significantly different from influx in EHR2.

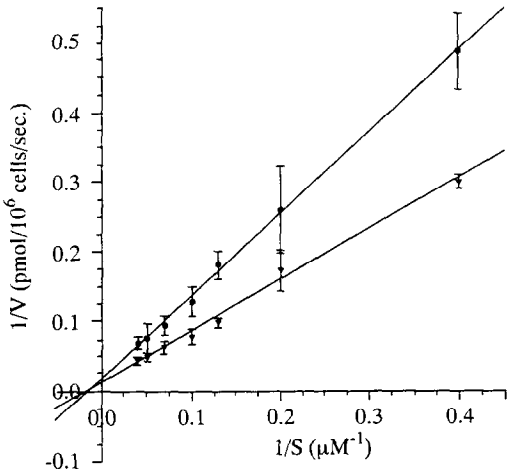


Fig. 4. The Lineweaver–Burk (double reciprocal) plot of influx of daunorubicin in medium with glucose and verapamil (1.1 μM) as a function of substrate concentration. ●, glucose-enriched medium; ▼, glucose-enriched medium including verapamil (1.1 μM).

cytosolic concentration of DNR was low. On the contrary the concentration of drug in the plasma membrane was probably high due to the hydrophobic nature of the drug. Thus, it was unlikely that efflux from the cytosol affected influx, whereas drug efflux directly from the membrane could be expected to affect influx. Second, addition of VER (≥3.3 μM) equalized initial influx of DNR in sensitive and resistant cells, whereas efflux was still significantly increased after addition of VER (≥5.5 μM) [17]. These findings are consistent with the results of Shalinsky *et al.* [12], who found decreased influx in the KB-GRC1 cell line transfected with the *mdr1* gene measured over the first few seconds and preferential effect of VER on influx. Kinetic studies by Stein *et al.* [28] are also consistent with this model. Using NIH 3T3 mouse cells transfected with the wild-type and a mutant strain of the human *mdr1* gene, these authors [28] found that: (1) influx was decreased in metabolically active cells; (2) stimulation of metabolism in energy-deprived cells increased efflux very rapidly whereas decreased accumulation appeared after a lag period; and (3) quinidine preferentially inhibited influx. Contrary to our study Stein *et al.* found VER to have an equal effect on influx and efflux whereas Fojo *et al.* [22] and Shalinsky *et al.* [12] did not. This discrepancy could be explained by differences in incubation conditions.

In EHR2/0.4 and EHR2/0.8 (passage no. 72) we found that energy depletion could not completely restore influx to the level in sensitive cells. Influx had been measured in energy-depleted medium in five previous studies [10–12, 20, 28]. Contrary to our result, these authors found that ATP depletion equalized influx in resistant and wild-type cells. One explanation could be that PGP was not completely inhibited due to traces of ATP or, as mentioned

above, that changes in membrane fluidity influenced influx.

The Lineweaver–Burk plot of influx in glucose-enriched medium confirmed the result of Skovsgaard [10] as a V_{max} for influx was demonstrated, indicating that influx is saturable. In other experimental systems drugs have been found to enter cells by simple diffusion [7, 8]. Thus, this finding further supports the theory that PGP actively effluxes drug directly from the membrane, as influx would then be expected to be saturable. Alternatively, this finding could be explained by self-association of the anthracycline molecules [34, 35]. The Lineweaver–Burk plot showed that VER changed the K_m value of the influx without changes in V_{max}. This finding is compatible with VER acting as a non-competitive inhibitor. The result is in contradiction with our previous results concerning the action of VER on efflux [17]. These results could suggest that both competitive and non-competitive inhibition are involved in the action of VER. The findings, however, could possibly be the result of artefacts. In studies of efflux the concentration of free cytosolic DNR was unknown [17]. Thus, intracellular binding and intercalation could have interfered with the efflux results, whereas the influx results could have been confounded by self-association of the DNR molecules [34, 35].

In conclusion the data presented in this paper support the hypothesis that PGP directly effluxes drug from the cellular membrane.

Acknowledgements—The authors are grateful to Marianne Fregil, Marianne Knudsen, and Bente Raatz for their excellent technical assistance. This work was supported by a grant from the Foundation of 1870 and from the Danish Cancer Society.

REFERENCES

1. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann Rev Biochem* **58**: 137–171, 1989.
2. Pastan I and Gottesman MM, Multidrug resistance. *Ann Rev Med* **42**: 277–286, 1991.
3. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE and Higgins CF, Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* **346**: 362–365, 1990.
4. Higgins CF, ABC transporters: from microorganism to man. *Ann Rev Cell Biol* **8**: 67–113, 1992.
5. Danø K, Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochim Biophys Acta* **323**: 466–483, 1973.
6. 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.
7. Dalmark M and Storm HH, A fickian diffusion transport process with features of transport catalysis. *J Gen Physiol* **78**: 349–364, 1981.
8. Siegfried JM, Burke TG and Tritton TR, Cellular transport of anthracyclines by passive diffusion. *Biochem Pharmacol* **34**: 593–598, 1985.
9. 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.
10. Skovsgaard T, Mechanism of cross-resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. *Cancer Res* **38**: 4722–4727, 1978.
11. Kessel D and Wilberding C, Anthracycline resistance in P388 murine leukemia and its circumvention by calcium antagonists. *Cancer Res* **45**: 1687–1691, 1985.
12. Shalinsky DR, Jekunen AP, Alcaraz JE, Christen RD, Kim S, Khatibi S and Howell SB, Regulation of initial vinblastine influx by P-glycoprotein. *Br J Cancer* **67**: 30–36, 1993.
13. Raviv Y, Pollard HB, Bruggemann EP, Pastan I and Gottesman MM, Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. *J Biol Chem* **265**: 3975–3980, 1990.
14. Gottesman MM, How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation award lecture. *Cancer Res* **53**: 747–754, 1993.
15. 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.
16. 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.
17. Nielsen D, Maare C and Skovsgaard T, Kinetics of daunorubicin transport in Ehrlich ascites tumor cells with different expression of P-glycoprotein. Influence of verapamil. *Biochem Pharmacol* **47**: 2125–2135, 1994.
18. Skovsgaard T, Transport and binding of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells. *Biochem Pharmacol* **26**: 215–222, 1977.
19. 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.
20. Frezard F and Garnier-Suillerot A, Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cell. *Eur J Biochem* **196**: 483–491, 1991.
21. Carlsen SA, Till JE and Ling V, Modulation of membrane drug permeability in Chinese hamster ovary cells. *Biochim Biophys Acta* **455**: 900–912, 1976.
22. Fojo A, Akiyama SI, Gottesman MM and Pastan I, Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. *Cancer Res* **45**: 3002–3007, 1985.
23. Sirotnak FM, Yang CH, Mines LS, Oribe E and Biedler JL, Markedly altered membrane transport and intracellular binding of vincristine in multidrug-resistant Chinese hamster cells selected for resistance to vinca alkaloids. *J Cell Physiol* **126**: 266–274, 1986.
24. Marsh W, Sicheri D and Center MS, Isolation and characterization of adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug. *Cancer Res* **46**: 4053–4057, 1986.
25. Ramu A, Pollard HB and Rosario LM, Doxorubicin resistance in P388 leukemia—evidence for reduced drug influx. *Int J Cancer* **44**: 539–547, 1989.
26. Soto F, Canaves JM, Gonzales-Ros JM and Ferragut JA, Rapid kinetics of the interaction between daunomycin and drug-sensitive or drug-resistant P388 leukemia cells. *FEBS Lett* **301**: 119–123, 1992.
27. Stow MW and Warr JR, Reduced influx is a factor in accounting for reduced vincristine accumulation in certain verapamil-hypersensitive multidrug-resistant CHO cell lines. *FEBS Lett* **320**: 87–91, 1993.
28. Stein WD, Cardarelli C, Pastan I and Gottesman MM, Kinetic evidence suggesting that the multidrug transporter differentially handles influx and efflux of its substrates. *Molec Pharmacol* **45**: 763–772, 1994.

29. Alon N, Busche R, Tummler B and Riordan JR, Membrane lipids of multidrug-resistant cells: chemical composition and physical state. In: *Molecular and Cellular Biology of Multidrug Resistant Tumor Cells* (Ed. Roninson IB), pp. 263–276. Plenum Press, New York, 1991.
30. Litman T, Nielsen D, Skovsgaard T and Bukhave K, Lipid composition of sensitive and multidrug resistant Ehrlich ascites tumour cells. *Cell Pharmacol* **2**: 9–14, 1995.
31. Cano-Gauci DF and Riordan JR, Action of calcium antagonists on multi-drug resistant cells. *Biochem Pharmacol* **36**: 2115–2123, 1987.
32. Cornwell MM, Pastan I and Gottesman MM, Certain calcium channel blockers bind specifically to multidrug resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* **262**: 2166–2170, 1987.
33. 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.
34. Eksborg S, Extraction of daunorubicin and doxorubicin and their hydroxyl metabolites: self-association in aqueous solution. *J Pharmacol Sci* **67**: 782–785, 1978.
35. Chaires JB, Dattagupta N and Crothers DM, Self-association of daunorubicin. *Biochemistry* **21**: 3927–3932, 1982.