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Cooperative P-glycoprotein mediated daunorubicin transport into DNA-loaded plasma membrane vesicles

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Abstract

Most of the multidrug resistant human tumor cell lines overexpress the *MDR*1 gene product P-glycoprotein (P-gp) which is believed to function as an energy-dependent drug efflux pump. Here we describe a novel method that allows the kinetic characterization of P-gp-mediated active drug transport. This method is based on the fluorescence quenching of anthracyclines transported into DNA-loaded plasma membrane vesicles. The uptake of daunorubicin (DNR) into the plasma membrane vesicles was saturable in terms of the extravesicular DNR concentration with a K_m of 1.5 ± 0.1 μM. This transport occured by a cooperative process with a Hill coefficient close to 2 for DNR. A model is discussed in which P-gp pumps two molecules of drug per turnover.

Key words: Multidrug resistance; P-Glycoprotein; P-gp; Plasma membrane vesicle; Daunorubicin

1. Introduction

The MDR gene product P-glycoprotein (P-gp) is involved in the resistance to a wide range of structurally and functionally unrelated drugs used in cancer chemotherapy [1]. The resistance mechanism associated with P-gp overexpression has been shown to be due to an increase of drug efflux rate [2], subsequent to drug efflux against the concentration gradient [3]. Horio et al. [4], showed that an ATPase activity was associated with drug transport. P-gp is a plasma membrane protein and a member of the ATP-binding cassette superfamily [5]. The mechanism by which P-gp transports drugs and the basis for its limited specificity are not yet clear.

A kinetic study of the pump activity of P-gp should lead to functional information with implications for the mechanism of the active transport. However the in situ substrate concentration cannot easily be determined. Only recently, methods have been developed through which kinetic information concerning P-gp could be derived from results obtained with intact cells [6]. For a further characterization of P-gp transport kinetics, experiments with inside-out plasma membrane vesicles are required.

Towards this aim, we prepared DNA-containing plasma membrane vesicles from the human ovarian carcinoma cell line A2780 and its drug-resistant derivative 2780^{AD} which contains a high level of P-gp [6]. Previous measurements using plasma membrane preparations have shown an accumulation of drug [7]. However, this method used radioactive drugs which may present a number of limitations. One is the high non-specific binding of these hydrophobic drugs to the lipid bilayer, which prevents measurements at low drug concentration including concentrations which give half-stimulation of the ATPase activity [8]. A second limitation concerns the maximal accumulation of drugs, which is reached too rapidly to measure accurately initial transport rates. Thirdly, the earlier method is noncontinuous with time. Furthermore, drug efflux during vesicle filtration may compromise the results. We have developed an experimental model system for P-gp which lacks these three limitations. This system was adapted from previous work performed with DNA containing large unilamellar vesicles [9]. We have used the system to examine the P-gp-mediated transport of DNR kinetically.

2. Materials and methods

2.1. Chemicals

Daunorubicin hydrochloride was obtained from Specia (France) and doxorubicin hydrochloride from Farmitalia (Italy). ATP, reduced Triton X-100, purified calf thymus DNA, PMSF and DNase I were purchased from Sigma Chemical Company (St. Louis, MO, USA). Culture plastics were from Nunc (Roskilde, Denmark). Cell culture media and supplements were obtained from Flow Laboratories (Irvine, UK).

2.2. Cells

The multidrug-resistant 2780^{AD} human ovarian carcinoma cell line and its drug-sensitive A2780 parent cell line were obtained from Dr. R.F. Ozols (National Cancer Institute, Bethesda, MD, USA). Cells were maintained in the continuous presence of 2 μM doxorubicin until

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Abbreviations: MDR, multidrug-resistant/resistance; P-gp, P-glycoprotein; DNR, daunorubicin; Dox, doxorubicin; PBS, phosphate-buffered saline; PMSF, paramethyl sulfonyl fluoride.

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two weeks before the experiments. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified essential medium supplemented with 7.5% heat-inactivated fetal calf serum. The cell lines were negative for mycoplasma as checked with the mycoplasma T.C. kit (Gen-Probe Inc., San Diego, CA, USA).

2.3. DNA containing plasma membrane vesicles

Crude membrane vesicles were prepared from drug-sensitive A2780 and MDR 2780^{AD} cell lines as follows. Cells ($40 \cdot 10^7$ for A2780 and $30 \cdot 10^7$ for 2780^{AD}) were harvested, homogenized by pottering in 100 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.4) and 1 mM PMSF was present to inhibit protease activity. The homogenate was centrifuged at 4,000 rpm for 15 min at 4°C to spin down nuclei and mitochondria. The supernatant was layered onto a 20%/30%/50% discontinuous sucrose gradient in 10 mM Tris-HCl (pH 7.4), and then centrifuged for 7 h at 35,000 rpm, at 4°C using a 70.1 Ti rotor. The plasma membrane vesicle fraction at the 30%/50% interface was collected, diluted in PBS buffer at pH 7.4 and then centrifuged for 1 h at 35,000 rpm using a 70.1 Ti rotor. The freshly prepared vesicle pellet was diluted in 0.33 ml PBS (pH 7.4) containing 10% glycerol and stored at −70°C prior to use. The plasma membrane preparation was thawed and resuspended in a DNA solution 10 mM previously ultrasonicated at maximal power (Soniprep type 150, MSE, Scientific Instruments, Loughborough, UK). Prior to resuspension in a DNA solution, the plasma membrane protein concentrations were determined by using a Biorad assay. This DNA vesicle suspension was sonicated in an ultrasonication bath (Branson type 5200) at 4°C during 5 min. The external DNA was hydrolysed in the presence of 100 units/ml of DNase I and 1.5 mM $MgCl_2$ at 37°C for 1.5 h. The reaction was stopped by addition of 2 mM EDTA and the suspension was centrifuged at 4,000 rpm, 4°C during 10 min. The DNA-containing vesicle pellet was resuspended in 2 ml and 1.4 ml PBS, pH 7.4, for vesicles from 2780^{AD} and A2780 cells, respectively.

2.4. Determination of the amount of DNA encapsulated in plasma membrane vesicles

A total of 0.5 ml PBS (pH 7.4) containing 0.8 μM Dox was put into a cuvette in a spectrofluorometer (FluoroMax, SPEX Industries Inc.). The temperature was kept at 37°C. Aliquots of DNA were added stepwise resulting in a quenching of the fluorescence emission intensity of the drug at 590 nm when excited at 490 nm. The slit widths were 1 mm. A calibration curve was obtained by plotting the fluorescence intensity at 590 nm as a function of the DNA concentration in the presence of 0.1% reduced Triton X-100. Under the same conditions, an aliquot of DNA-vesicle suspension was added to 0.8 μM Dox in the presence of 0.1% reduced Triton X-100.

The addition of 0.1% reduced Triton X-100 by itself did not modify the fluorescence emission of the Dox and DNR. DNA appeared to be well entrapped in the vesicles because rapid quenching of Dox fluorescence by added vesicles occurred only after addition of reduced Triton X-100 which means that DNA was not accessible to the drug present in the cuvette.

2.5. Measurement of the initial rate of uptake of DNR in DNA-containing vesicles

The kinetics of DNR uptake by DNA-containing vesicles was studied using a fluorometric approach developed by Frezard and Garnier-Suillerot and based on DNR fluorescence quenching upon intercalation between the base pairs of DNA [10]. In such systems the fluorescence of anthracyclines is not affected by their interaction with the lipid bilayer [11]. All experiments were conducted in a 1 cm quartz cuvette at 37°C, containing 0.5 ml of PBS plus 1 mM of ATP and 0.9 mM $MgCl_2$ at pH 7.4 and different DNR concentrations. The various solutions of ATP, $MgCl_2$ and DNR were preincubated at 37°C for at least 30 min before adding the vesicles because DNR fluorescence quenched somewhat upon addition of ATP. Immediately after adding 6 μl of DNA–vesicle suspension (containing 1.6 μg or 3 μg protein for 2780^{AD} and A2780, respectively), the initial rate of uptake of the drugs was measured as the rate at which the fluorescence of DNR decreased. That rate was calculated from Eq. (1),

$$v_i = (dF/dt)_{t=0} \cdot [\text{drug}]/\{F_0 \cdot [\text{vesicle}]\} \quad (1)$$

where (dF/dt) is the initial rate of change of fluorescence (see Fig. 1); v_i, the initial rate of drug uptake, F_0, the total drug fluorescence at $t = 0$ and F the fluorescence of the drug at any time. The uptake was followed as a function of time, and the rate of uptake was essentially constant for more than 60 s. The initial rate of drug uptake was evaluated from the change in fluorescence immediately after adding the vesicles to the change 60 s later. Medium background fluorescence was less than 10% of the drug fluorescence for each DNR concentration used in the uptake measurements. Moreover, we have determined that the change of rate of DNR fluorescence observed was due to the quenching of DNR fluorescence upon intercalation with DNA present in the vesicles and not to the interaction with ATP. We so calculated the rate of change of DNR fluorescence in the presence of 1 mM ATP which was less than 1.5%/min at the lowest DNR concentration used.

3. Results

3.1. ATP-dependent transport of DNR into DNA-containing plasma-membrane vesicles

To examine the kinetic properties of P-glycoprotein, we isolated plasma membrane vesicles from the multidrug resistant cell line 2780^{AD}. Proper kinetic assessment of transport is greatly facilitated by maintaining the concentration of the transported substance low in the compartment into which it is transported (*zero trans* uptake). To achieve this aim, we loaded plasma membrane vesicles with sufficient DNA to trap the imported drug. Because binding of DNR to DNA quenches the fluorescence of the drug, transport of this drug into vesicles containing DNA should be accompanied by a decrease of fluorescence. Indeed the fluorescence of DNR preincubated in the presence of Mg^{2+} and ATP decreased upon addition of plasma membrane vesicles prepared from MDR cells (Fig. 1, trace 3). When ATP was absent (not shown) or verapamil was present (Fig. 1, trace 2) this rate was significantly reduced. The rate obtained with vesicles from drug-sensitive cells (Fig. 1, trace 1) was even lower. Verapamil is a known inhibitor of P-gp-mediated DNR transport [11].

The rate of decrease fluorescence should reflect the rate at which the drug is taken up into the vesicles. The rate of fluorescence decrease was virtually constant for 1 min, allowing accurate assessment of the initial rate of drug uptake into vesicles.

3.2. Substrate cooperativity of drug transport

We examined the kinetics of the ATP-dependent DNR uptake. Fig. 2 shows that the uptake rate depended on the concentration of DNR both in vesicles from the MDR and the drug-sensitive cell line. The difference between the two curves should represent the contribution of the MDR-related pumping to the uptake. That difference was a saturating function of the DNR concentration (not shown). A Terrell Hill plot [12] of the MDR-dependent transport confirmed the saturability and estimated a K_m of 1.5 ± 0.1 μM (Fig. 3). In addition, it is suggested that the MDR-related transport is cooperative in DNR concentration with an index of cooperatively slightly higher than 2.

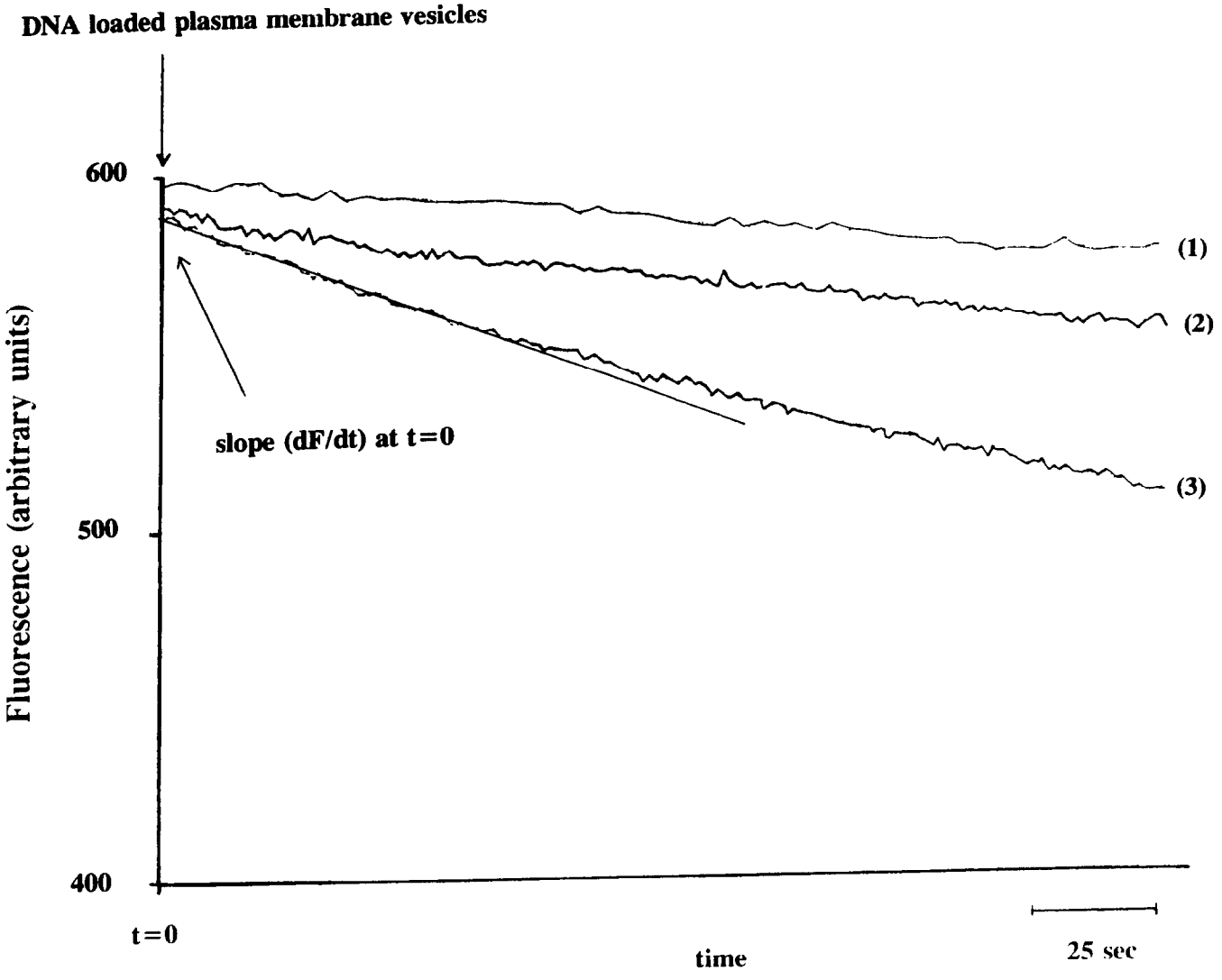

Fig. 1. Fluorometric determination of the initial rate of uptake of DNR into DNA-containing plasma membrane vesicles. The fluorescence intensity was recorded at a wavelength of 590 nm; the excitation wavelength was 490 nm. To a total of 0.5 ml, 1.45 μM DNR solution and 1 mM MgATP was added, and the decrease of DNR fluorescence was recorded as a function of time at 37°C. Trace 1 and trace 3: DNA-loaded vesicles derived from drug sensitive A2780 and drug resistant 2780^{AD} cells, respectively. Trace 2: vesicles from 2780^{AD} cells in the presence of 25 μM verapamil.

4. Discussion

A new method was developed for measurement of the kinetics of anthracycline transport mediated by P-gp. Although earlier methods allowed the demonstration of such transport, they were not suitable for kinetic studies owing to the difficulty of assaying truly initial transport rates because the intravesicular drug concentration increased significantly already when the first data points were collected. This essential problem has been circumvented in the approach presented here by trapping transported anthracyclines into DNA included in the plasma membrane vesicles. Moreover, because binding to the DNA affects DNR fluorescence, this introduced the possibility to assay drug transport through fluorescence measurement. Importantly, the new method does not

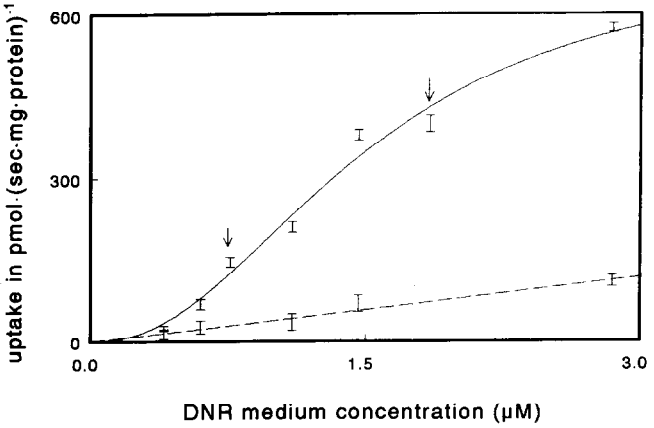

Fig. 2. Initial uptake of DNR by DNA containing vesicles as a function of DNR concentration. The DNA-containing vesicle suspension derived from the drug-resistant 2780^{AD} (upper trace) or the drug-sensitive A2780 (lower trace) cell line.

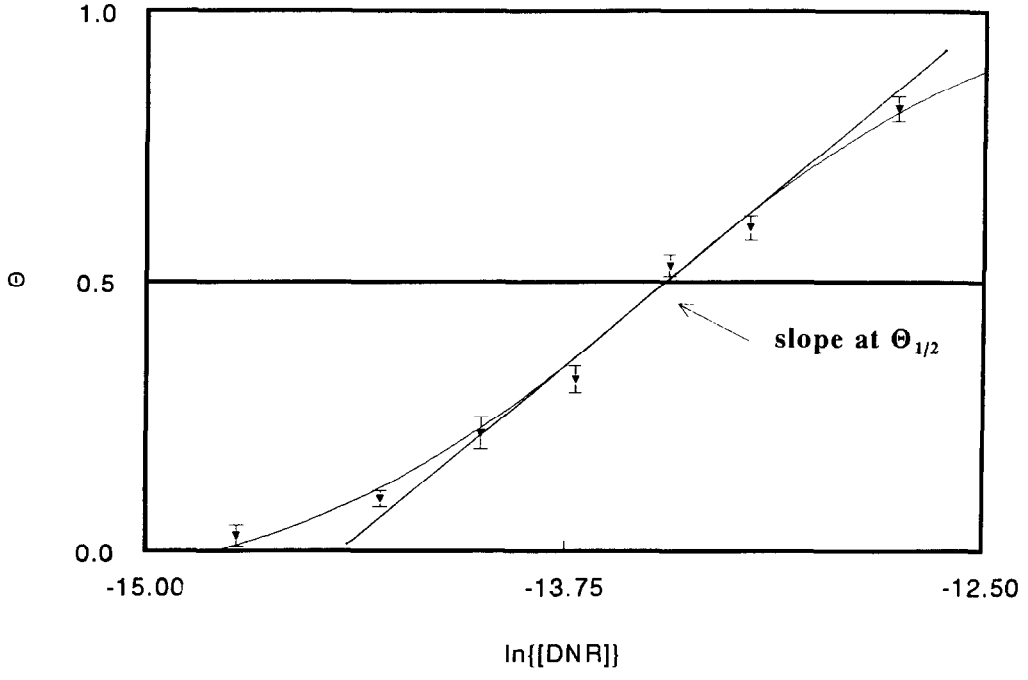

Fig. 3. T. Hill plot of the MDR-related transport data. The difference in transport rate between the vesicles form the 2780^{AD} and the A2780 cell line is called v and plotted here. For the two transport data indicated by an arrow, we used the drawn line (—) in order to calculate v from Fig. 2. The maximum rate V was estimated by fitting the data to an A.V. Hill equation (not shown). $\Theta = v/V$. The Hill coefficient is given by the slope of the curve at $\Theta_{1/2}$ multiplied by 4. The curve has been obtained by fitting the data with to an A.V. Hill equation with a cooperativity index of 2.2.

require the filtration of the plasma membrane vesicles required in the earlier method, and is therefore insensitive to efflux during that separation step. As ATP cannot cross the plasma membrane to any significant extent, only the inverted vesicles can actively accumulate DNR. Consequently, the right-side-out subpopulation should remain silent in this active drug transport process but will contribute to non-specific binding of drugs and to passive transport.

DNA molecules were well encapsulated into the vesicles: addition of vesicles to a suspension containing 0.8 μM Dox did not immediately quench the Dox fluorescence. A subsequent addition of 0.1% reduced Triton X-100 did lead to quenching of about 50% of the fluorescence (not shown). The encapsulated DNA concentration in the assays was about 5 μM base pairs (on the basis of the total cuvette volume). These results suggest that our experimental system could be used in initial anthracycline uptake measurements.

We measured the initial uptake rate of DNR as a function of increasing concentrations of this drug. We observed that the dependence of drug pumping on drug concentration was not simply hyperbolic, but positively cooperative. In order to determine the Hill coefficient, we plotted the curve $\Theta = f(\ln[DNR])$ where Θ is v/V and [DNR] the medium DNR concentration [13]. The resulting slope at $\Theta_{1/2}$ multiplied by 4 gives a Hill coefficient close to 2, which suggests a cooperative transport of two daunorubicin molecules via the P-gp which has also been described by Spoelstra et al. [13,14].

In the view of this result we propose the existence of two binding sites on the P-gp. Only after two drug molecules have bound, the pump may allow a conformation compatible with an active drug transport. After releasing both molecules of drug outside the cells, the unloaded P-gp may recover its native form.

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