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THE P-GLYCOPROTEIN-MEDIATED RELATIVE DECREASE IN CYTOSOLIC FREE DRUG CONCENTRATION IS SIMILAR FOR SEVERAL ANTHRACYCLINES WITH VARYING LIPOPHILICITY

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Abstract—We have used a new methodology to measure the activity of P-glycoprotein (P-gp) in multidrug-resistant (MDR) tumor cells. This activity leads to a lower cytosolic concentration and a lower cytotoxicity of the classical anthracyclines, daunorubicin (DNR), and doxorubicin (DOX). It has been reported that the anthracycline idarubicin (IDA), which is more lipophilic, has a higher clinical efficacy in acute myeloid leukemias (AML) than DNR and DOX. In our study, the aim was to determine for a series of anthracyclines how variations in the passive drug influx rate as well as the P-gp-mediated drug pumping rate affect their cytosolic free drug concentrations and how these parameters are related to drug cytotoxicity. We selected six anthracyclines: DOX, DNR, epidoxorubicin (EPI), IDA, cyano-morpholino-doxorubicin (CMD), and carminomycin (CAR), ordered according to their increasing octanol/PBS buffer concentration ratios, respectively. To measure the passive permeation coefficient, the P-gp-mediated drug pumping rate, and the cytosolic free drug concentration, we used a flow-through system in which cells were exposed to a flowing medium containing drugs. We used the MDR P-gp-containing cell line KB8-5. It was shown that the passive drug permeation coefficient as well as the drug pumping rate of P-gp increased with increasing lipophilicity in this series of anthracyclines. The cytosolic free drug concentration was lowered by P-gp to a similar extent in KB8-5 cells for all drugs tested (40–50% of the extracellular drug concentration). CMD, IDA, and CAR had lower IC_{50} values and lower resistance factors in comparison to DOX, DNR, and EPI. Verapamil reversed the resistance for all anthracyclines tested. In conclusion, for several anthracyclines the activity of P-gp leads to a similar relative decrease in the cytosolic free drug concentration; consequently, the reported lower resistance factor of IDA compared to that of DNR is not due to the inability of P-gp to export IDA from cells.

Key words: multidrug resistance; P-glycoprotein; doxorubicin; daunorubicin; idarubicin; fluorescence

Chemotherapy in the treatment of cancer patients is mainly limited by the presence of tumor cells resistant to cytotoxic agents. *In vitro* selected MDR† cell lines insensitive to a variety of cytotoxic agents [1] often revealed the presence of plasma membrane-associated drug transport proteins, such as P-glycoprotein (P-gp) [1–3]. The activity of P-gp is ATP-dependent, and leads to a lowered intracellular drug content [4]. In an earlier study, we provided evidence that P-gp pumps the cytotoxic anthracycline DNR out of the cell across the plasma membrane against a drug concentration gradient [5]. Clinical interest in P-gp stems from observations that in some malignancies, after relapse from chemotherapy, P-gp or P-gp-encoding *MDR*1 mRNA is present at higher levels, especially in hematologic malignancies such as AML and myeloma [6]. These observations have led to clinical trials attempting to increase the chemotherapeutic efficacy of some major P-gp substrates (DNR, etoposide, vincristine) by administering drug pump inhibitors such as verapamil and cyclosporins, to patients. When P-gp is active, the intracellular free drug concentration becomes a function of both the activity of the drug efflux pump and the passive membrane permeation coefficient [7]. In consequence, an alternative approach to reducing the effect of the drug efflux pump is to increase the passive permeation coefficient. Accordingly, more lipophilic drugs, such as IDA, have been implemented [8, 9]. Indeed, it has now been shown that IDA is active in AML [10]. Moreover, the resistance of MDR cell lines to IDA appeared to be lower than their resistance to DNR [11, 12]. One possible explanation for its high clinical efficacy is that the amount of cellular drug is only marginally controlled by P-gp, because of the rapid passive uptake of IDA across the cellular plasma membrane [13]. This explanation may be too simplistic. Earlier reports suggested that the affinity of drugs for P-gp also increased with increasing hydrophobicity of the substrate. Friche *et al.* established that the circumvention of anthracycline resistance increased with the lipid solubility of the analogs of the resistance modulator [14]. However, no specific correlation was found between the lipid solubility of these analogs and their ability to inhibit specific photolabeling of P-gp using azidopine [15]. Zamora *et al.* [16] and Facchetti *et al.* [13] report that lipid solubility is, indeed, an important physical property for modulators of MDR. Nogae *et al.* also report that several compounds that reverse drug resistance and inhibit P-gp-mediated vincristine efflux are relatively highly hydrophobic [17]. Accordingly, the P-gp-mediated pumping activity of IDA may also be

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† Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance(t); DOX, doxorubicin; DNR, daunorubicin; EPI, epidoxorubicin; IDA, idarubicin; CMD, cyanomorpholino-doxorubicin; CAR, carminomycin; PBS, phosphate-buffered saline; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazolyl blue); AML, acute myeloid leukemia.

increased. To date, the question whether the P-gp-mediated relative decrease in the cytosolic free drug concentration is lower for IDA than for DNR has not been adequately addressed.

In the present study, it was our aim to determine for a number of anthracyclines that differ in lipophilicity (DOX, DNR, and IDA) the extent to which variations in the P-gp-mediated drug pumping rate and the passive drug permeation rate affect the cytosolic free drug concentration. Cytosolic free drug is freely exchangeable between the cell and the extracellular medium. This cytosolic pharmacokinetic compartment is probably in rapid equilibrium with other cellular compartments, such as the nucleus, cytosolic vesicles, internal membranes, or a part of the plasma membrane. All these compartments contribute to the total cellular distribution volume of the drug, but at steady state they do not affect the cytosolic free drug concentration. In addition, the relation between these parameters and drug cytotoxicity was studied. We selected the anthracyclines DOX, EPI [18], DNR, CMD [19], IDA, and CAR [20] (in ascending order of lipophilicity, Fig. 1). To study the cellular pharmacokinetics, we used the human epidermoid MDR KB8-5 cell line [21], which contained P-gp and was derived from the drug-sensitive cell line KB3-1 by selection with colchicine.

The cellular pharmacokinetics was measured using a flow-through system in which cells, attached to a glass plate, were exposed to a flowing medium containing drugs [22]. The advantage of this system is that within one experiment all the pharmacokinetic parameters can be determined at once, and can be related to each other using the same cells. Moreover, for the first time a quantitative approach to the relation between the P-gp drug pumping rate and the lipophilicity of the drug could be established. Previously the flow-through system had been used:

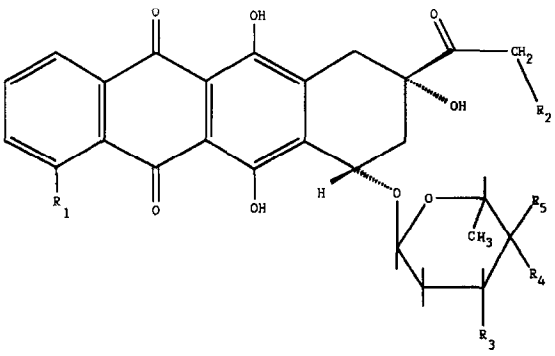

Fig. 1. Chemical structures of the anthracyclines doxorubicin (DOX), 4'-epidoxorubicin (EPI), daunorubicin (DNR), cyano-morpholino-doxorubicin (CMD), idarubicin (IDA, 4'-demethoxy-daunorubicin), and carminomycin (CAR).

	R_1	R_2	R_3	R_4	R_5
DOX	OCH_3	OH	NH_2	H	OH
EPI	OCH_3	OH	NH_2	OH	H
DNR	OCH_3	H	NH_2	H	OH
CMD	OCH_3	OH	C_4H_7OCN*	H	OH
IDA	H	H	NH_2	H	OH
CAR	OH	H	NH_2	H	OH

* Cyano-morpholino-group.

1. to demonstrate DNR efflux against a concentration gradient in both P-gp- and non-P-glycoprotein-mediated MDR cells [5, 23];
2. to demonstrate saturation of the P-gp-mediated and non-P-glycoprotein-mediated DNR efflux activity [23, 24];
3. to study the kinetics of DNR fluxes across the plasma membranes of P-gp-containing MDR cells [7]; and
4. to verify a model in which verapamil inhibits DNR pumping by P-gp in a non-competitive manner [25].

Here, the flow-through system was used to compare the pharmacokinetics of several anthracyclines in P-gp-containing cells. The data may be important for obtaining a rationale for the treatment of cancer patients with alternative anthracyclines, and to indicate the pharmacological manipulation of P-gp-mediated active drug efflux.

MATERIALS AND METHODS

Chemicals

Doxorubicin hydrochloride was obtained from Laboratoire Roger Bellon (France), 4'-epidoxorubicin from Farmitalia (Milan, Italy), and daunorubicin hydrochloride from Specia (Paris, France). Cyano-morpholino-doxorubicin was kindly provided by the Chemotherapeutic Agents Repository (Rockville, MD, U.S.A.). Idarubicin (4'-demethoxy-daunorubicin) was kindly provided by Dr. J. W. Scheeren (Catholic University of Nijmegen, the Netherlands). Carminomycin was obtained from Bristol Laboratories (Syracuse, NY, U.S.A.). Verapamil hydrochloride, colchicine, and MTT were purchased from Sigma (St. Louis, MO, U.S.A.). Cell culture media and supplements were obtained from Flow (Irvine, U.K.), and culture plastics from Nunc (Roskilde, Denmark). HEPES (4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid) was obtained from Serva (Heidelberg, Germany), phosphoric acid (H_3PO_4) and 1-octanol from Baker Chemicals b.v. (Deventer, the Netherlands), and acetonitril from Merck (Darmstadt, Germany).

Octanol/PBS drug concentration ratios

To determine the lipophilicity of the anthracyclines selected for this study (Fig. 1), we measured the 1-octanol/PBS concentration ratios. We added each drug to 3 mL 1-octanol (drug concentration 2 μM), and measured the fluorescence on a spectrofluorometer (FluoroMax™, SPEX Industries Inc., Edison, NJ, U.S.A.). The excitation and emission wavelengths were 480 and 560 nm, respectively. Then, an equal volume of PBS (pH 7.4) was added and, after 30 min of strong whirling, the mixtures were centrifuged at 14,000 g for 5 min. The fluorescence of the upper 1-octanol layer was measured again. From the difference in the fluorescence of the drug in 1-octanol before and after the addition of PBS, the 1-octanol/PBS drug concentration ratio was calculated.

Cells

The human epidermoid carcinoma cell line KB3-1 was obtained from Dr. I. B. Roninson (Department of Genetics, University of Illinois at Chicago, IL, U.S.A.). Its MDR P-gp-containing subline KB8-5 was obtained from the American Type Culture Collection (Rockville,

MD, U.S.A.). This cell line was cultured under the continuous presence of 25 nM colchicine [21]. The MDR cells were cultured in the presence of drug until 3–5 days prior to the experiment. Cells were cultured in (bicarbonate-buffered) Dulbecco's minimal essential medium (Eagle's modification) with 20 mM Hepes buffer and 10% heat-inactivated fetal calf serum (FCS; 30 min, 56°C) in a 5–6% carbon dioxide atmosphere, at pH 7.4. The cells were negative for mycoplasma, as verified with the mycoplasma T.C. rapid detection system with a ^{3}H-labeled DNA probe from Gen-Probe Inc. (San Diego, CA, U.S.A.). The viability was usually greater than 95%, as determined by Trypan Blue exclusion before and after the experiments.

The flow-through system

In this system [7, 22–25], cells (usually $10–15 \cdot 10^6$ cells, unless mentioned otherwise) were seeded on a glass plate within a silicone glue rim. By putting an upper glass plate on this bottom plate, a chamber was created with a volume of approximately 500 µL. The cells were exposed to a flowing medium of 37°C containing drugs, via an inlet and an outlet tube at both ends of the system (schematic representation of the flow-through system in Fig. 2). An HPLC pump (Gilson, Villiers, France, model 302, 5.S pump head) was used to pump the medium over the cells. The perfusion medium was usually growth medium containing HEPES (but not bicarbonate) with 10% FCS (medium A), and the flow rate was usually 100 or 200 µL/min. The fluorescence of the drugs was detected at the system's outlet by a fluorescence monitor (type 821-FP, Jasco, Hachioji City, Japan), and monitored in time using a recorder. For all the anthracyclines, the excitation and emission wavelengths were 480 and 560 nm, respectively.

Passive permeation coefficient (k)

The passive permeation coefficient (k) of the anthracyclines was determined as described earlier [7: method 4]. In short, the initial cellular drug uptake rate was determined just after the cells had been exposed to the drug. Before this exposure the cytosolic free drug concentration was zero. The drug-free medium above the cells caused an initial decline in fluorescence. After passage of the front of the medium-containing drug, a sharp bend in the increasing fluorescence signal was indicative of initial cellular drug uptake (illustrated in Fig. 3). The passive permeation coefficient was determined by the

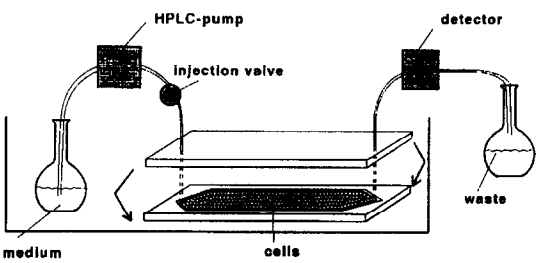

Fig. 2. Schematic representation of the flow-through system. Cells seeded on a glass plate are exposed to medium (37°C) containing drug, which is pumped over the glass plate by an HPLC pump. Putative MDR modulators can be injected into the system via an injection valve. The fluorescence of the drug is detected at the outlet of the system.

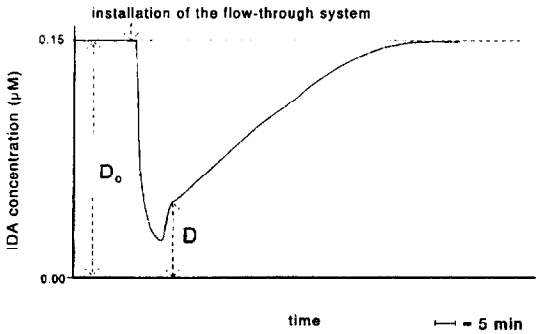

Fig. 3. Determination of the passive permeation coefficient of IDA in KB8-5 cells. $1.2 \cdot 10^6$ cells were exposed to a flowing medium containing 0.15 µM IDA. The initial decline in fluorescence was caused by medium without drug but with some phenol red of the Dulbecco's medium, which was above the cells before being exposed to the drug. Medium drug concentration was determined at the sharp bend in the curve, representing initial cellular drug intake. The passive permeation coefficient was determined using Eqn 6. The flow rate was 200 µL/min.

medium drug concentration at the outlet at the time of the bend in the signal. The equations used to calculate the passive permeation coefficient will be given below. To exclude an effect of the activity of P-gp on the measurement of initial drug uptake, the medium contained 50 µM verapamil for the first 5 min of drug exposure to inhibit the P-gp-mediated drug flux. This concentration of verapamil had no effect on the fluorescence of the anthracyclines, the intracellular pH, cellular viability, or the passive permeation coefficient, as measured under energy-depleting conditions.

Cytosolic free drug concentration (C_{in})

The cytosolic free drug concentration was determined when the steady state had been attained in the flow-through system. Verapamil, which inhibits P-gp-mediated drug pumping, was injected into the system via an HPLC injection valve at a concentration of 250 µM. Series of six pulse injections of 15 µL (dilution factor of 3–10 during migration over the cells) were given at time intervals of 30 sec at a medium flow rate of 200 µL/min and 60 sec at a medium flow rate of 100 µl/min. The resulting net cellular uptake of the drug was recorded as a dip in the fluorescence signal. The maximum dip depth corresponds to a maximum inhibition of drug pumping. An illustration of the determination of the cytosolic concentration of free IDA in KB8-5 cells is given in Fig. 4. The medium flows over the glass plate at a flow rate F (m^3/sec). If the cross section area within the silicone glue rim of the glass plate of the system is denoted by A (m^2), this implies that the medium flows over the cells at a velocity of F/A (m/sec). For a volume element that moves with this velocity over the cells in the presence of verapamil, the change in the medium drug concentration in time can be described by

$$dD/dt = P/h \cdot (D - C_{in}) \quad (1)$$

where P, h, D, and C_{in} represent the passive plasma membrane permeability of the drug (m/sec), the height of the medium column above the cells, the extracellular medium drug concentration, and the cytosolic free drug concentration. Here, it is assumed that the P-gp-mediated DNR efflux is completely inhibited by verapamil.

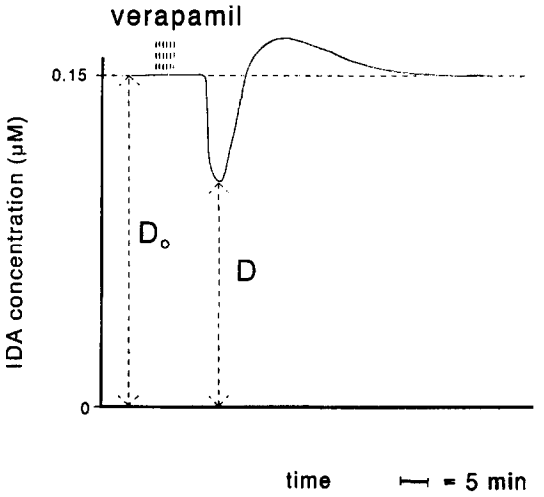

Fig. 4. Determination of the cytosolic concentration of free IDA in KB8-5 cells. When a steady state with 0.15 μM IDA and $1.2 \cdot 10^6$ cells was attained in the flow-through system, verapamil (250 μM) was injected into the flowing medium, leading to a net cellular uptake of the drug and a decrease in medium fluorescence. The cytosolic free drug concentration was determined at the maximum dip depth using Eqn 5. The medium flow rate was 200 μL/min.

At the front of the medium containing verapamil, the cytosolic free drug concentration is constant (C_{in}), due to the steady state attained before exposure to verapamil. For this front then, integration of Eqn 1 gives the drug concentration in the volume element as a function of time, $D(t)$, after verapamil began to travel over the cells:

$$D(t) = C_{in} + (D_o - C_{in}) \cdot e^{-P \cdot t/h} \quad (2)$$

where D_o represents the input concentration of the drug in the medium. The surface A of the flow-through chamber is $n \cdot s$, where n and s represent the number of cells on the glass plate and the surface per cell. Consequently, the volume of the flowing medium in the flow-through chamber is $n \cdot s \cdot h$, and the time spent by the medium in the flow-through chamber is $n \cdot s \cdot h/F$. The drug concentration at the front of the medium containing verapamil is therefore:

$$D(t) = C_{in} + (D_o - C_{in}) \cdot e^{-k/F} \quad (3)$$

where k represents the passive permeation coefficient of the drug:

$$k = s \cdot n \cdot P \quad (4)$$

Using Eqn 3, the steady-state cytosolic free drug concentration, which existed before the injection of verapamil, can be calculated:

$$C_{in} = (D - D_o \cdot e^{-k/F})/(1 - e^{-k/F}) \quad (5)$$

The passive permeation coefficient k was determined at the start of each experiment as described above. Here, the initial cytosolic free drug concentration C_{in} was 0. Using Eqn 5 it can be deduced that

$$k = -F \cdot \ln [D/D_o] \quad (6)$$

Equation 6 was used to determine the passive permeation coefficient k, which was used to determine the cytosolic free drug concentration (Eqn 5) in the same experiment. For some anthracyclines, especially the relatively lower lipophilic drugs such as DOX and DNR, the passive permeation coefficient k is much smaller than the medium flow rate F. Equation 5 can then be simplified to

$$C_{in} = D_o + (F \cdot (D - D_o)/k) \quad (7)$$

and at $C_{in} = 0$,

$$k = F \cdot (D_o - D)/D_o \quad (8)$$

Equations 7 and 8 have been used for DNR in previous studies [7, 23].

Since we used different extracellular concentrations for the drugs, the cytosolic free drug concentration was expressed as a percentage of the extracellular drug concentrations:

$$R = C_{in}/D_o \cdot 100\% \quad (9)$$

where R represents the relative cytosolic free drug concentration.

P-glycoprotein-mediated drug pumping rates (V)

At a maximum inhibition of P-gp-mediated drug pumping activity, the passive influx of the drug is determined by the drug concentration gradient across the cellular plasma membrane and the passive permeation coefficient. Since drug leakage is not affected by verapamil and because the cytosolic free drug concentration has not yet had the time to change, the influx rate just after inhibition of P-gp-mediated drug pumping equals the inhibited P-gp-mediated drug pumping rate (V):

$$V = k \cdot (D_o - C_{in}) = k \cdot (D_o - D)/(1 - e^{-k/F}) \quad (10)$$

To make a comparison between P-gp pumping rates for several drugs at equal cytosolic free drug concentrations, the drug pumping rates were divided by the cytosolic free drug concentrations:

$$V_{rel} = V/C_{in} \quad (11)$$

in which V_{rel} represents the relative P-gp drug pumping rate. V_{rel} also represents the ratio between the V_{MAX} and the K_M of P-gp, if we assume Michaelis-Menten kinetics, and that the cytosolic free drug concentration remained much lower than the K_M:

$$V_{rel} = V_{MAX}/K_M \quad (12)$$

Cellular steady-state drug content

The cellular steady-state drug content was determined by incubating trypsinized cells at a cell density of 0.15–$0.25 \cdot 10^6$ cells/mL in drug-containing medium A with and without 50 μM verapamil at 37°C for 90 min. The drug concentration was the same as that used for the flow-through experiment. The cells were then washed twice with ice-cold Hanks Buffered Salt Solution/medium A (1:1; v/v). Pellets were suspended in lysis buffer containing 15% acetonitril, 30% phosphoric acid, and 55% PBS [26]. The cellular fragments were largely dispersed by sonification for 30 min. The fluorescence of the suspensions was measured using a spectrofluorometer (FluoroMax™, SPEX Industries Inc.) at 37°C. Excitation and emission wavelengths were 480 and 560 nm, respectively. The drug fluorescence of drug-sensitive KB3-1 cells incubated with the same drug medium concentration as the MDR cell line KB8-5 was normalized to 1.0, and all measurements were related to that level.

Drug cytotoxicity

The cytotoxicity of the drugs was measured using an MTT method as described previously [27, 28]. In short, 1200–1500 cells per well were seeded in a 96-well microtiter plate (100 μL culture medium/well) on day 1. On day 2, drug solutions of 125 μL were added with or without verapamil (final medium concentration 6 μM). On day 5, the medium was removed and 50 μL MTT in PBS (0.4 mg/mL) was added. The plates were incubated with MTT at 37°C for 4 hr and then 150 μL DMSO was added to dissolve the formazan. The absorption at 540 nm was measured using a microtiter plate-reader (Titertek Multiskan MCC/340). Data are expressed as IC_{50} values (nM). All drug concentrations were tested in four replicate wells, and each experiment was performed three times.

RESULTS

1-Octanol/PBS drug concentration ratio

The 1-octanol/PBS drug concentration ratio as a measure for lipophilicity of the drugs was determined for the six anthracyclines selected for this study. The ratios varied between 2 and 23. The lipophilicity of the drugs increased in the following order: DOX, EPI, DNR, CMD, IDA, and CAR. This sequence was in line with data reported previously [13, 29].

Passive permeation coefficient

The passive permeation coefficient (k) of the anthracyclines in KB8-5 cells was calculated according to Eqn 6. Since the variation in k was approximately two orders of magnitude, the optimal concentration was used for each drug to enable us to record the bend in the medium fluorescence trace (Fig. 3). For DOX, DNR, and EPI we used extracellular drug concentrations of 0.5 μM. For DOX we decreased the flow rate from 200 μL/min to 50 μL/min. For the relatively highly lipophilic drugs CMD, IDA, and CAR, the cellular drug influx rate was too high to detect a bend in the fluorescence curve. Therefore, we decreased the number of cells to $\pm 1 \cdot 10^6$ cells. Moreover, for these drugs the extracellular concentration was decreased to 0.15 μM because of an increased verapamil-induced desorption of the drugs from the glass plates at extracellular drug concentrations of 0.5 μM and higher. The flow rate used for these drugs was 200 μL/min. For all drugs we verified that the initial cellular drug uptake rate was linear with the extracellular drug concentration by measuring this parameter at extracellular drug concentrations twice as high or twice as low as the optimal extracellular drug concentrations used. The measured passive permeation coefficient increased with the increasing lipophilicity of the drug (Fig. 5).

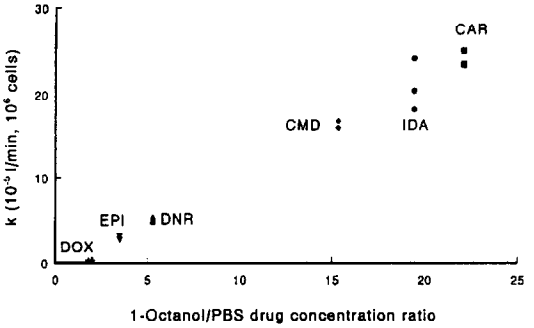

Fig. 5. Passive permeation coefficient (k) against the 1-octanol/PBS drug concentration ratio. Using the flow-through system, the passive permeation coefficient (L/min, 10^6 cells) of six anthracyclines was determined for KB8-5 cells and plotted against the 1-octanol/PBS drug concentration ratio of the anthracyclines. Each point represents the outcome of an independent experiment. The means of the k of DOX, DNR, and CAR are significantly different, $P < 0.05$ according to Student's t-test.

Cytosolic free drug concentration

The cytosolic free drug concentration was determined from the difference in medium drug concentration with and without P-gp-mediated drug pumping activity. When a steady state was attained in the flow-through system, injections of verapamil induced a fluorescence dip, which indicated a net cellular influx of the drug caused by an inhibition of the drug pumping of P-gp (illustrated in Fig. 4). After passage of the verapamil pulse, fluorescence increased above the steady state fluorescence signal, indicating an efflux of drugs against a drug concentration gradient. This was caused by the restored activity of P-gp. The area below the steady state concentration was of magnitude similar to the signal above the steady state concentration, indicating that the surplus influxed after inhibiting the drug pump was subsequently effluxed. At the end of each experiment cells were counted and the viability tested. Viability was usually greater than 95%.

The cytosolic free drug concentration was determined using Eqn 5. The passive permeation coefficient k in this equation had been measured in the same experiment by following the medium drug concentration after first exposure of the cells to the drug. The concentration of verapamil used (injection of 250 μM, dilution factor 3–10) induced a maximum inhibition of the P-gp-mediated drug flux, since higher concentrations of verapamil did not further amplify the fluorescence dips. This was verified for all drugs tested. Moreover, for all drugs a continuous infusion of 50 μM verapamil led to a similar maximum decrease in the drug fluorescence signal when compared to that of the maximum pulse-induced dip. For DOX we used a flow rate of 50 μL/min, and verapamil pulse injections at intervals of 2 min. For all other drugs we used a flow rate of 200 μL/min and verapamil pulse injections at intervals of 30 sec. The extracellular drug concentration used was the same as that employed for the determination of the passive permeation coefficient. The relative cytosolic free drug concentrations (R) in KB8-5 cells, set off against the passive permeation coefficients, are shown in Fig. 6. In this figure the data represent independent experiments. The cytosolic free drug concentration was lowered by P-gp by a similar factor for all drugs tested. From a linear fit of all data, an increase in the relative cytosolic free drug concentration with an increasing passive permeation coefficient was inferred from 40 to 50% of the extracellular drug concentration.

Cellular steady-state drug content

We determined the intracellular drug content of DOX, DNR, and IDA in drug-sensitive KB3-1 and MDR KB8-5 cells with or without 50 μM verapamil (complete inhibition of P-gp-mediated drug flux) at 37°C for 90 min. After incubation, the cells were lysed, and the rel-

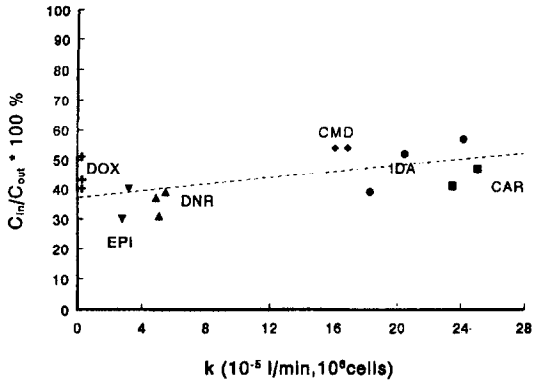

Fig. 6. Relative cytosolic free drug concentrations against the passive permeation coefficient. Using the flow-through system, the relative cytosolic free drug concentration of several anthracyclines was determined for KB8-5 cells using Eqn 5 and plotted against the passive permeation coefficient. Each point represents the outcome of an independent experiment. The dotted line represents a linear fit of the data.

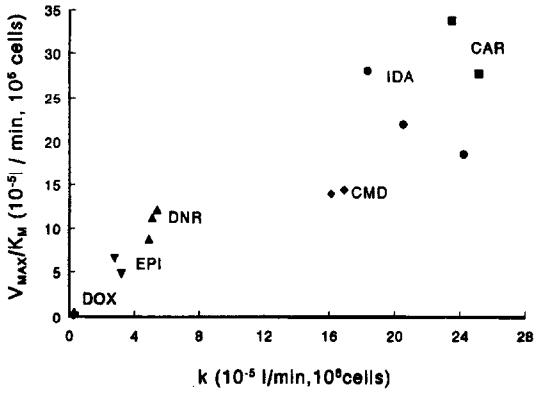

Fig. 7. V_{MAX}/K_M values representing the relative P-gp drug pumping rate (V_{rel}) against the passive permeation coefficient (k) of anthracyclines for KB8-5 cells. The data were calculated using Eqn 10. Each point represents the outcome of an independent experiment. The means of the V_{MAX}/K_M of DOX, DNR, and CAR are significantly different, $P < 0.05$ according to Student's t-test.

ative cellular drug content calculated from the fluorescence. The drug content of KB3-1 cells without verapamil was normalized to 1.0. For all drugs, the total amount of cellular drug in KB8-5 cells was 40–50% of that in KB3-1 cells (see Table 1). Incubation of KB8-5 cells with drugs in the presence of verapamil resulted in an increase of cellular drug. This increase almost reached the level of drug in drug-sensitive cells. The effect of verapamil on the amount of drug in drug-sensitive cells was less than 20%.

P-glycoprotein-mediated drug pumping rate

The P-gp pumping rate of the anthracyclines was calculated using Eqn 10. For each individual experiment we determined the passive permeation coefficient and the cytosolic free drug concentration, from which we could calculate the drug pumping rate. The P-gp-mediated drug efflux rate was linear with the extracellular drug concentration. This was determined by measuring this parameter at extracellular drug concentrations twice as high and twice as low as the optimal extracellular drug concentrations used. The V_{MAX}/K_M values (assuming $S \ll K_M$) representing the relative P-gp drug pumping rates (V_{rel}) and calculated using Eqns 11 and 12 are plotted against the passive permeation coefficient for all drugs tested in Fig. 7. It was apparent that the relative drug pumping rate increased with the drug permeation coefficient.

Table 1. Cellular drug steady-state contents in KB3-1 and KB8-5 cells

	DOX	DNR	IDA
KB3-1	1.0	1.0	1.0
+VP	1.1 ± 0.2	1.2 ± 0.1	1.0 ± 0.1
KB8-5	0.5 ± 0.1	0.5 ± 0.2	0.4 ± 0.1
+VP	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1

Cells were incubated with DOX (0.5 μM), DNR (0.5 μM), or IDA (0.15 μM) with or without 50 μM verapamil at 37°C for 90 min, then washed and suspended in a lysis buffer. The fluorescence of the suspensions was measured and normalized to 1.0 for the wild-type KB3-1 cells. Data represent the average of three independent experiments (±SD, $n = 3$).

Drug cytotoxicity

The cytotoxicity of the six anthracyclines for KB3-1 and KB8-5 cells was measured to compare them with the cytosolic free drug concentrations. The results are given in Table 2. IC_{50} values were somewhat lower for IDA, CMD, and CAR than for DOX, DNR, and EPI. Drug resistance factors varied between 2.0 and 6.3, and were lower for IDA, CMD, and CAR than for DOX, DNR, and EPI. Verapamil (6 μM) reversed the resistance in KB8-5 cells for all anthracyclines.

DISCUSSION

The aim of our study was to investigate the cellular pharmacokinetics of several clinically important anthracyclines in MDR tumor cells containing P-gp. In particular, the effect of P-gp on the cellular pharmacokinetics of the 'classical' anthracyclines, DOX and DNR, was compared with the presently advocated lipophilic anthracycline IDA. In addition, EPI, CMD, and CAR were studied, the latter two not being routinely in clinical use. Using the flow-through system, the intracellular free drug concentrations and P-gp pumping rates could be calculated for these drugs. It was found that the P-gp drug pumping rate, normalized with respect to differences in the cytosolic free drug concentration, increased with the lipophilicity and the passive permeation coefficient of the drug. The increased passive leak across the plasma membrane with increasing lipophilicity did not lead to a significant increase in the cytosolic free drug concentration. Apparently, the increased efflux largely compensates for the increased passive permeation coefficient. In fact, the relative decrease in the cytosolic free drug concentration in P-gp MDR KB8-5 cells was similar for all six compounds. The dotted line of Fig. 6, representing the relative cytosolic free drug concentration R (C_{in}/D_o) against the passive permeation coefficient k, was obtained with linear regression, and showed only a slight ascent from 40 to 50%. This increase was

Table 2. IC_{50} values (nM) of the anthracycline analogs on KB3-1 and KB8-5 cells

	IC_{50}					
	KB3-1		KB8-5			
Drug	−VP	+VP	−VP	+VP	RF	DMF
DOX	14 ± 2	9 ± 2	52 ± 4	15 ± 2	3.7	3.5
EPI	15 ± 3	10 ± 1	94 ± 6	18 ± 3	6.3	5.2
DNR	12 ± 1	11 ± 4	53 ± 3	14 ± 2	4.4	3.8
CMD	0.1 ± 0.02	0.1 ± 0.02	0.2 ± 0.01	0.1 ± 0.03	2.0	2.0
IDA	2.3 ± 0.3	2.0 ± 0.2	4.7 ± 0.5	2.2 ± 0.2	2.0	2.1
CAR	3.0 ± 0.5	2.0 ± 0.2	8.1 ± 0.9	4.0 ± 0.4	2.7	2.0

Cells were incubated with the drugs with or without 6 μM verapamil (− or + VP) for 72 hr. The resistance factor (RF) is defined by the ratio of the IC_{50} of the KB8-5 cells and the IC_{50} of the KB3-1 cells. The dose modifying factor (DMF) is defined by the ratio of the IC_{50} of the KB8-5 cells without verapamil and the IC_{50} of the KB8-5 cells with verapamil. Data are the means ± SD of three independent experiments.

insufficient to explain the observed differences in drug resistance (Table 2). The classical approach to measuring the effects of P-gp on cellular drug content is to measure the total amount of cellular drug in the cells at steady state. In this approach, the amount of drug in KB8-5 was also similar (40–50%) when related to the total amount of cellular drug in drug-sensitive cells at steady state. However, assuming the ratio of total cellular drug contents to be similar to the ratio of the cytosolic free drug concentrations (minus and plus verapamil) requires that verapamil not affect the intracellular drug distribution volume. We confirmed that verapamil did not have such an effect (data not shown) by measuring its effect on the ratio of the amount of drug bound to DNA to the amount of drug in the rest of the cell [30].

Previously, Berman and McBride also measured the effect of verapamil on the cellular content of IDA and DNR, using flow cytometry [11]. They found that verapamil had less effect on the content of IDA in MDR cells than on the DNR content. However, the authors did not consider that these findings could also be explained by a 40% decrease in extracellular IDA concentration during the incubation of the cells, leading to an apparently lesser effect of verapamil. The cytosolic free drug concentration most likely contributed to the drug resistance to the anthracyclines tested in this study. Both the IC_{50} values and drug resistance factors were lower for CMD, IDA, and CAR than for DOX, EPI, and DNR, in agreement with earlier reports. However, these drug resistance factors were always higher than 2, which corresponds to the decreasing effect of P-gp on the cytosolic free drug concentration. This indicates that the resistance to all the drugs can at least be explained by an effect of P-gp. Moreover, the resistance to all drugs could be reversed by verapamil (for IDA corresponding to the results from ref. [31]). The higher resistance factors for the less lipophilic drugs cannot be explained by an effect of P-gp on the cytosolic free drug concentration alone. Other mechanisms of drug resistance might play a role for the less lipophilic drugs. It is unclear why verapamil was able to reverse drug cytotoxicity completely. The limitations of our experimental set-up make it difficult to speculate on such mechanisms, since the concentrations of drug at IC_{50} levels are too low for sensitive kinetic measurements or for studies on mechanisms of drug resistance.

To explain the higher drug pumping activity of P-gp with respect to the more lipophilic drugs, we assume that a main characteristic of the substrate binding site on P-gp is a hydrophobic environment. The routing of the drug molecules from the cytosol to outside may be via *cytosolic* binding sites on the drug pump or via *plasma membrane* binding sites [32]. Presently, we cannot discriminate between these two possibilities. Indications for the second routing have been obtained with P-gp functioning as a hydrophobic 'vacuum cleaner' [33, 34]. For a non-P-glycoprotein-mediated MDR cell line, we found an example of a typical 'vacuum cleaner' [35].

In summary, we compared six anthracyclines having differing lipophilicity with respect to pharmacokinetics and cytotoxicity in P-gp-containing MDR cells. We found that the P-gp drug pumping rate increases with increasing lipophilicity of the drug. The P-gp-mediated relative decrease in the cytosolic free drug concentration is similar for the anthracyclines tested. At equal cytosolic free drug concentrations, drug cytotoxicity was higher and the drug resistance factors were lower for the more lipophilic drugs. We conclude that the lower resistance factor of IDA in comparison with that of DNR and DOX is not due to a lower effect of P-gp on the cytosolic free concentration of IDA.

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REFERENCES

1. Bradley G, Juranka PF and Ling V, Mechanisms of multidrug resistance. *Biochim Biophys Acta* **948:** 87–128, 1988.
2. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
3. Borst P, Genetic mechanisms of drug resistance. *Rev Oncol* **4:** 87–105, 1991.
4. Broxterman HJ and Pinedo HM, Energy metabolism in multidrug resistant tumor cells. *J Cell Pharmacol* **2:** 239–247, 1991.
5. Lankelma J, Spoelstra EC, Dekker H and Broxterman HJ, Evidence for daunomycin efflux from multidrug-resistant 2780^{AD} human ovarian carcinoma cells against a concentration gradient. *Biochim Biophys Acta* **1055:** 217–222, 1990.

6. Grogan TM, Spier CM, Salmon SE, Matzner M, Rybski J, Weinstein RS, Scheper RJ and Dalton WS, P-glycoprotein expression in human plasma cell myeloma: Correlation with prior chemotherapy. *Blood* **81:** 490–495, 1993.
7. Spoelstra EC, 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.
8. Tidefelt U, Sundman-Engberg B and Paul C, Comparison of the intracellular pharmacokinetics of daunorubicin and idarubicin in patients with acute leukemia. *Leukemia Res* **18:** 293–297, 1994.
9. Minderman H, Linssen P, Van der Lely N, Wessels J, Boezeman J, De Witte T and Haanen C, Toxicity of idarubicin and doxorubicin towards normal and leukemic human bone marrow progenitors in relation to their proliferative state. *Leukemia* **8:** 382–387, 1994.
10. Robert J, Clinical pharmacokinetics of idarubicin. *Clin Pharmacokinet* **24:** 275–288, 1993.
11. Berman E and McBride M, Comparative cellular pharmacology of daunorubicin and idarubicin in human multidrug-resistant leukemia cells. *Blood* **79:** 3267–3273, 1992.
12. Tsuruo T, Oh-Hara T, Sudo Y and Naito M, Antitumor activity of idarubicin, a derivative of daunorubicin, against drug sensitive and resistant P388 leukemia. *Anticanc Res* **13:** 357–362, 1993.
13. Facchetti I, Grandi M, Cucchi P, Geroni C, Penco S and Vigevani A, Influence of lipophilicity on cytotoxicity of anthracyclines in LoVo and LoVo/Dx human cell lines. *Anti-Cancer Drug Design* **6:** 385–397, 1991.
14. Friche E, Buhl Jensen P, Roed H, Skovsgaard T and Nissen NI, *In vitro* circumvention of anthracycline-resistance in Ehrlich ascites tumour by anthracycline analogues. *Biochem Pharmacol* **39:** 1721–1726, 1990.
15. Friche E, Demant EJF, Sehested M and Nissen NI, Effect of anthracycline analogs on photolabelling of P-glycoprotein by [^{125}I]iodomycin and [^{3}H]azidopine: Relation to lipophilicity and inhibition of daunorubicin transport in multidrug resistant cells. *Brit J Cancer* **67:** 226–231, 1993.
16. Zamora JM, Pearce HL and Beck WT, Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol Pharmacol* **33:** 454–462, 1988.
17. Nogae I, Kohno K, Kikuchi J, Kuwano M, Akiyama S, Kiue A, Suzuki K, Yoshida Y, Cornwell MM, Pastan I and Gottesman MM, Analysis of structural features of dihydropyridine analogs needed to reverse multidrug resistance and to inhibit photoaffinity labeling of P-glycoprotein. *Biochem Pharmacol* **38:** 519–527, 1989.
18. Weenen H, Van Maanen JMS, De Planque MM, Mcie JG and Pinedo HM, Metabolism of 4′-modified analogs of doxorubicin: Unique glucuronidation pathway for 4′-epidoxorubicin. *Eur J Cancer Clin Oncol* **20:** 919–926, 1984.
19. Begleiter A, Leith MK, and Johnston JB, Activity of 3′-(3-cyano-4-morpholinyl)-3′-deaminoadriamycin in sensitive and resistant L5178Y lymphoblasts *in vitro*. *Cancer Res* **54:** 482–486, 1994.
20. Comis RL, Issel BF, Pittman K, Ginsberg SJ, Rudolph A, Aust JC, DiFino SM, Tinsley RW, Poiesz BJ and Crooke ST, A phase I and clinical pharmacology study of intravenously administered carminomycin in cancer patients in the United States. *Cancer Res* **42:** 2944–2948, 1982.
21. Shen DW, Fojo A, Chin JE, Roninson IB, Richert N, Pastan I and Gottesman MM, Human multidrug-resistant cell lines: Increased *mdr*1 expression can precede gene amplification. *Science* **232:** 643–645, 1986.
22. Lankelma J, Laurensse E and Pinedo HM, A flow-through tissue culture system with a fast dynamic response. *Anal Biochem* **127:** 340–345, 1982.
23. Mülder HS, Lankelma J, Dekker H, Broxterman HJ and Pinedo HM, Daunorubicin efflux against a concentration gradient in non-P-glycoprotein multidrug-resistant lung-cancer cells. *Int J Cancer* **59:** 275–281, 1994.
24. Spoelstra EC, Dekker H, Schuurhuis GJ, Broxterman HJ and Lankelma J, P-glycoprotein drug efflux pump involved in the mechanisms of intrinsic drug resistance in various colon cancer cell lines. *Biochem Pharmacol* **41:** 349–359, 1991.
25. Spoelstra EC, Westerhoff HV, Pinedo HM, Dekker H and Lankelma J, The multidrug-resistance-reverser verapamil interferes with cellular P-glycoprotein-mediated pumping of daunorubicin as a non-competing substrate. *Eur J Biochem* **221:** 363–373, 1994.
26. De Jong J, Guérand WS, Schoofs PR, Bast A and Van der Vijgh WJF, Simple and sensitive quantification of anthracyclines in mouse atrial tissue using high performance liquid chromatography and fluorescence detection. *J Chromatogr* **570:** 209–216, 1991.
27. Broxterman HJ, Pinedo HM, Kuiper CM, Van der Hoeven JJM, De Lange P, Quak JJ, Scheper RJ, Keizer HG, Schuurhuis GJ and Lankelma J, Immunohistochemical detection of P-glycoprotein in human tumor cells with a low degree of drug resistance. *Int J Cancer* **43:** 340–343, 1989.
28. Mosmann T, Rapid colometric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Meth* **65:** 55–63, 1983.
29. Bachur NR, Steele M, Meriwether WD and Hildebrand RC, Cellular pharmacodynamics of several anthracycline antibiotics. *J Med Chemistry* **19:** 651–654, 1976.
30. Lankelma J, Mülder HS, Van Mourik F, Wong Fong Sang HW, Kraayenhof R and Van Grondelle R, Cellular daunomycin fluorescence in multidrug resistant 2780^{AD} cells and its relation to cellular drug localisation. *Biochim Biophys Acta* **1093:** 147–152, 1991.
31. Michieli M, Damiani D, Michelutti A, Melli C, Russo D, Fanin R and Baccarani M, P170-dependent multidrug resistance. Restoring full sensitivity to idarubicin with verapamil and cyclosporin A derivatives. *Haematologica* **79:** 119–126, 1994.
32. Roninson IB, *Molecular and cellular biology of multidrug resistance in tumor cells*, pp. 395–402. Plenum Press, New York and London, 1991.
33. Homolya L, Hollo Z, Germann UA, Pastan I, Gottesman MM and Sarkadi B, Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J Biol Chem* **29:** 21493–21496, 1993.
34. Raviv Y, Pollard HB, Bruggeman 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.
35. Mülder HS, Van Grondelle R, Westerhoff HV and Lankelma J, A plasma membrane 'vacuum cleaner' for daunorubicin in non-P-glycoprotein multidrug-resistant SW-1573 human non-small cell lung carcinoma cells. *Eur J Biochem* **218:** 871–882, 1993.