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Relation among the Resistance Factor, Kinetics of Uptake, and Kinetics of the P-Glycoprotein-Mediated Efflux of Doxorubicin, Daunorubicin, 8-(*S*)-Fluoroidarubicin, and Idarubicin in Multidrug-Resistant K562 Cells

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SUMMARY

Multidrug resistance (MDR) is frequently associated with decreased cellular drug accumulation resulting from enhanced drug efflux. This is correlated with the presence of a membrane protein, the P-glycoprotein, which pumps a wide variety of drugs out of cells, reducing their intracellular concentration and thus their toxicity. The influx and efflux of drugs across the cell membrane are in large part responsible for their intracellular concentrations, and in the search for new compounds able to overcome MDR, it is of prime importance to determine the molecular parameters whose modification would lead to an increase in the kinetics of uptake and/or to a decrease in the P-glycoprotein-mediated efflux. Four anthracycline derivatives, doxorubicin, daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin, which have the same amino sugar, were used to analyze the respective contribution of the kinetics of uptake and the P-glycoprotein-mediated efflux in their impaired accumulation in MDR cells. The kinetics of uptake of the four drugs vary over a very large range: the kinetics of uptake of daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin are 16, 200, and 400 times higher than that of doxorubicin, respectively. However, the four drugs are extruded by P-glycoprotein at comparable rates. The apparent K_m values for P-glycoprotein-mediated transport, the intracellular free cytosolic drug concentrations at half-maximal velocity for the cell lines used, were ~2.2 μM for daunorubicin and ~1 μM for idarubicin and 8-(*S*)-fluoroidarubicin.

The only possible method of curing systemic cancers, such as leukemia, lymphoma, and unifocal tumors that have spread by metastasis, has been systemic treatments such as chemotherapy and immunotherapy. Unfortunately, the appearance of cell populations resistant to multidrug-based chemotherapy constitutes the major obstacle to a cure.

MDR is a well-characterized phenomenon (1, 2), frequently associated with decreased drug accumulation resulting from enhanced drug efflux (3–7). This is correlated with the presence of a membrane protein, the P-glycoprotein, which pumps a wide variety of drugs out of cells, thus reducing their toxicity. P-glycoprotein has been shown to be encoded by the *mdr* gene in both rodents and humans (8–10).

The search for new compounds able to overcome MDR is of prime clinical importance, and for this reason, it is important to know the mechanism of the P-glycoprotein-mediated efflux of drug. However, despite the multitude of articles on the MDR phenomenon, the exact mechanism of the P-glycoprotein-mediated drug efflux remains unknown. Current strategies to reverse MDR are based on (i) the identification of selective and potent MDR-reversing agents, i.e., the chemosensitization of resistant cells, leading to increased intracellular accumulation of drug and the restoration of cytotoxicity (11–13), and (ii) the design and synthesis of non-cross-resistant analogues of MDR drugs (14–17). We focus on this second point.

Because the activity of a drug depends on its concentration in the compartment where its cellular target is located and because the influx and efflux of drugs across the cell membrane are in large part responsible for their intracellular concentration, the mechanism of transport of these agents into the cell and, in particular, the kinetics of this transport are of crucial importance.

Numerous researchers have focused on new analogues of antitumor drugs whose uptake would not be affected by P-glycoprotein and on identifying chemical modifications that would decrease drug efflux (17). The criteria proposed by

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ABBREVIATIONS: MDR, multidrug resistance or multidrug-resistant; RF, resistance factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Beck *et al.* (18) for this research are that a substrate for P-glycoprotein must have two planar aromatic groups and a tertiary nitrogen. However, numerous data show that molecules without these characteristics can be substrates for P-glycoprotein; one of the best known examples is cyclosporin (19).

We studied the respective contributions of the kinetics of uptake and of P-glycoprotein-mediated efflux of drugs to their impaired accumulation in MDR cells and their relation with the RF. We used four anthracyclines [doxorubicin, daunorubicin, idarubicin, and 8-(*S*)-fluoroidarubicin] and studied their uptake and release in MDR K562 cells. Our data show that the kinetics of uptake of these four molecules vary over a very large scale. A good negative correlation occurs between the kinetics of uptake and the RF, with the RF low when the kinetics of uptake are high. In addition, our data clearly establish that the kinetics of the P-glycoprotein-mediated efflux of these four drugs are comparable.

Materials and Methods

Cell culture and cytotoxicity assay. K562 is a human leukemia cell line established from a patient with a chronic myelogeneous leukemia in blast transformation (20). K562 cells resistant to doxorubicin were obtained through continuous exposure to increasing doxorubicin concentrations and were maintained in medium containing doxorubicin (4 nM). This subline expresses a unique membrane glycoprotein with a molecular mass of 180,000 Da (21). Doxorubicin-sensitive and -resistant erythroleukemia K562 cells were grown in suspension in RPMI 1640 (Sigma Chemical Co.) medium supplemented with L-glutamine and 10% fetal calf serum at 37° in a humidified atmosphere of 95% air/5% CO_2. Cultures, initiated at a density of 10^5 cells/ml, grew exponentially to 8–10 $\times 10^5$ cells/ml in 3 days. To have cells in the exponential growth phase for the spectrofluorometric assays, culture was initiated at 5 $\times 10^5$ cells/ml and cells were used 24 hr later, when the culture had grown to ~8–10 $\times 10^5$ cells/ml. Cell viability was assessed with Trypan blue exclusion. The cell number was determined by Coulter counter analysis.

An RF was defined as the IC_{50} of resistant cells divided by the IC_{50} of the corresponding sensitive cells. The IC_{50} was determined as follow: cells (10^5/ml) were incubated in the presence of various anthracycline concentrations, and the IC_{50} was determined by plotting the percentage of cell growth inhibition versus the logarithm of the antitumor dug concentration (IC_{50} is the drug concentration that inhibits cell growth by 50% when measured at 72 hr).

Total RNA was prepared from frozen cells according to a CsCl/guanidinium isothiocyanate method proposed by Maniatis *et al.* (22) and modified by Ferrandis *et al.* (23). Transcript level of the *mdr1* gene was measured comparatively to that of the KB-8–5 cell line, which shows an arbitrary expression of 30 arbitrary units (24). Our K562 resistant cells exhibited an *mdr1* gene transcript level of 800 arbitrary units.[1]

Drugs and chemicals. Purified doxorubicin, daunorubicin, idarubicin, and pirarubicin were kindly provided by Pharmacia and Roger Bellon. 8-(*S*)-Fluoroidarubicin was provided by Menarini Ricerche Sud, the group that originally synthesized it (25). Concentrations were determined by diluting stock solutions to ~10^{-5} M and using ϵ_{480} = 11,500 M^{-1} cm^{-1}. Stock solutions were prepared just before use. All other reagents were of the highest quality available. Deionized double-distilled water was used throughout the experiments. Experiments were performed in HEPES/Na^+ buffer solutions containing 20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.25, in either the presence or the absence of 5 mM glucose. For some experiments, Na^+ was replaced by K^+, and vice versa.

[1] J. Benard and A. Garnier-Suillerot, unpublished observations.

Absorption spectra were recorded on a Cary 219 spectrophotometer, and fluorescence spectra were recorded on a Perkin Elmer LS 50 B spectrofluorometer.

Cellular drug accumulation. The uptake of anthracycline in cells was followed by monitoring the decrease of the fluorescence signal at 590 nm (λ_{ex} = 480 nm). This spectrofluorometric method has been previously described (26–28). The incubation of the cells with the drug proceeds without compromising cell viability. It is thus possible, with this method, to accurately quantify the kinetics of uptake of the drug by the cells as well as the amount of anthracycline intercalated between the base-pairs in the nucleus and the free drug concentration in the cytosol in the steady state. All experiments were conducted in 1-cm quartz cuvettes containing 2 ml of buffer at 37°. The net influx detected may include the rate of three reactions: passive influx, active efflux through the MDR pump, and intercalation into DNA. We have previously demonstrated that the rate of anthracycline intercalation into DNA is very fast compared with the rate of passage of the drug through the plasma membrane and that the transport of the drug across the cell membrane is the rate-limiting step of the entire process (26, 27). This is supported by the observation that the time required to obtain 50% of drug intercalation inside the nucleus decreases to <30 sec when the plasma membrane is permeabilized with Triton X-100. On the other hand, as it is the initial rate of uptake that is measured, the drug concentration in the compartment toward which net flow is proceeding is close to zero, and therefore the active efflux through the MDR pump is undetectable.

The initial rate of uptake V_+ can thus be written as $(V_+)_{t=0} = k_+ \cdot n \cdot C_e$, where k_+ is the mean influx coefficient for the drug, n is the number of cells/ml, and C_e is the extracellular free drug concentration (27).

Determination of the P-glycoprotein-mediated efflux of anthracycline derivatives. The following method, which has been previously described (29, 30), has been used to determine the kinetics of P-glycoprotein-mediated efflux of anthracycline. Cells (1×10^6/ml) are incubated for 30 min in HEPES buffer in the presence of 10 mM NaN_3 and in the absence of glucose. The incorporation of anthracycline in these energy-depleted resistant cells compares with that observed in sensitive cells. At the steady state, concentration of the neutral form of the free drug must be the same in the extracellular medium and in the cytosol. Where $pH_i = pH_e$, this means that C_e, the extracellular free drug concentration, is equal to C_i, the cytosolic free drug concentration. The addition at the steady state of 5 mM glucose gives rise to ATP synthesis via the glycolysis pathway and, after ~30 sec, to an increase in the fluorescent signal due to the release of drug from the cells. The rate of efflux is determined from the slope of the tangent to the curve F = f(t), where F is the fluorescence intensity at 590 nm, at the time point corresponding to the time (t_{glu}) of the addition of glucose (29, 30).

The net efflux may include the rate of three reactions: (i) the rate of drug release from DNA; however, as stated above, this rate is very fast compared with the rate of passage of the drug through the plasma membrane; (ii) the rate of passive efflux; and (iii) the rate of active efflux through the MDR pump. Under our experimental conditions, i.e., in the absence of energy, the free drug concentration is the same in the cytosol and in the extracellular medium and the rate of passive efflux is equal to that of passive influx. Thus, when glucose is added, yielding the ATP synthesis and reactivation of the MDR pump, the net efflux that is measured is the efflux through the MDR pump only.

The kinetics of active efflux V_a can be written as $(V_a)_{tglu} = k_a \cdot n \cdot Ci$, where k_a is the mean active efflux coefficient and n is the number of cells/ml.

With daunorubicin, idarubicin, and 8-(*S*)-fluoroidarubicin, the steady state was reached within 90 min for daunorubicin and within 30 min for the other two, and when glucose was added at the steady state, C_i was equal to C_e. However, the steady state could not be reached with doxorubicin because it would have required more than

5–6 hr in the presence of the high doxorubicin concentration needed to saturate the efflux. Therefore, the following procedure was used: cells were incubated in the absence of glucose and in the presence of N_3^- and various doxorubicin concentrations for 3 hr. Despite the fact that the steady state was not reached and taking into account that $pH_e = pH_i$, it was possible to calculate C_i using the following equation: $C_i = (C_E/C_N)/C_n$, where C_E is the free drug concentration in the extracellular medium after permeabilization of the cell membrane, and C_n and C_N are the overall concentrations of drug bound to the nucleus before and after permeabilization of the cell membrane, respectively (28). V_a was determined after the addition of glucose. As the steady state was not reached, an important transmembrane gradient of concentration was present (e.g., at 5 μM doxorubicin, after 5 hr, C_e = 4.3 μM and C_i = 0.65 μM). However, the kinetics of passive diffusion of doxorubicin through the plasmic membrane were so slow that a correction of the V_a value taking into account the passive diffusion of the molecules was not necessary.

Results

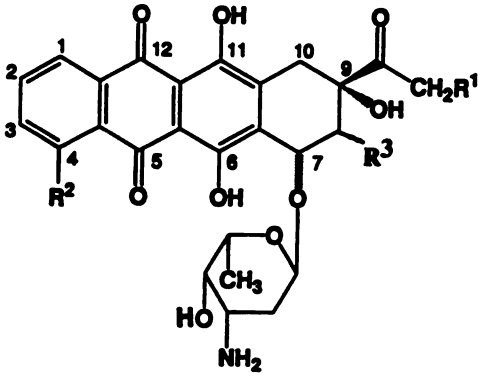
The structures of the four anthracycline derivatives used in the present study are shown in Fig. 1. The pK_a values of deprotonation of the amino group, daunosamine, of doxorubicin and daunorubicin have been previously determined. At 37° in KCl 0.1 M, p*Ka* = 8.4 (31). We measured pK_a of idarubicin and 8-(*S*)-fluoroidarubicin under the same experimental conditions (data not shown); as expected, pK_a = 8.4. For the four molecules, the hydroxyl groups present in positions C6 and C11 undergo deprotonation at pH >10, and therefore they will not be deprotonated at pH 7.2, the pH used in the following study. At pH 7.2, 94% of the molecules of either doxorubicin, daunorubicin, idarubicin, or 8-(*S*)-fluoroidarubicin will bear a single positive charge at the level of the amino group on the sugar.

The spectrofluorometric method that we used is based on the observation that the quenching of the anthracycline fluorescence, which occurs when these molecules are incubated with cells, is due essentially to their intercalation between the base-pairs in the nucleus, their strongest binding site within the cell. Interactions with other cellular components, which might modify the fluorescence signal, can be neglected (26). This is correct if the intracellular drug concentration is not too high. Under our experimental conditions, this is the case when 10^6 cells/ml are incubated with anthracycline at concentrations <3 μM. However, at higher concentrations, the binding sites for anthracycline in the nucleus become saturated, and non-negligible amounts of drug accumulate in compartments other than the nucleus. This can led to an additional fluorescence quenching, where our method must be used with caution.

One aim of the present study was to obtain saturation of the efflux and therefore determination of the Michaelis constant. For this purpose, the use of high drug concentrations (up to 12 μM) was required. The following experiments were thus designed to determine the amount of drug that could be accumulated in non-nuclear compartments and to find experimental conditions under which such accumulation would be eliminated.

Accumulation of doxorubicin, daunorubicin, idarubicin, and 8-(*S*)-fluoroidarubicin in isolated cell nuclei. In the following discussion, C_N represents the overall molar concentration of anthracycline intercalated between the base-pairs either in isolated cell nuclei or in the nuclei of membrane-permeabilized cells, and C_n represents the overall molar concentration of anthracycline bound to the living cells at the steady state.

We determined the amount of drug bound to nuclei isolated from sensitive and resistant cells, as a function of the drug concentrations. Here, the fluorescence quenching can only be due to intercalation of drug between the base-pairs in the nucleus. In Fig. 2, the overall concentration of idarubicin or daunorubicin intercalated between the base-pairs in the nucleus (C_N) is plotted as a function of the drug concentration added to the nuclei (10^6/ml at pH 7.25, 37°). The data for the two drugs are very similar, indicating that their affinities for



	R^1	R^2	R^3
Doxorubicin	OH	OCH_3	H
Daunorubicin	H	OCH_3	H
F-Idarubicin	H	H	F
Idarubicin	H	H	H

Fig. 1. Schema of the structures of the four anthracycline derivatives used in the study.

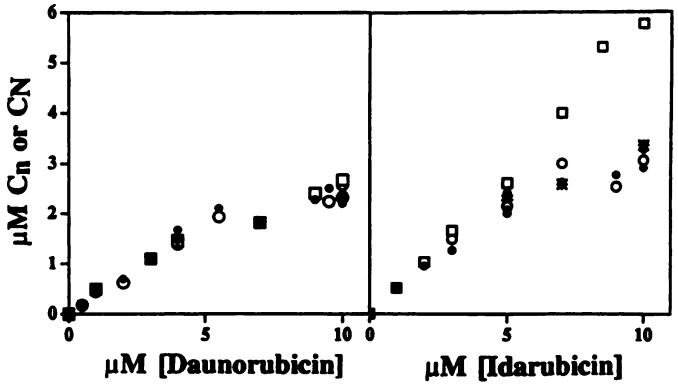


Fig. 2. Accumulation of daunorubicin (*left*) and idarubicin (*right*) in isolated cell nuclei and in drug-sensitive cells. C_N (●), the overall molar concentration of drug bound to isolated nuclei or to nuclei at the equilibrium state (after permeabilization of the cell membranes), and C_n, the overall molar concentrations of drug bound to the cells at the steady state, are plotted as a function of the drug concentration (□, ○, and ∗). The incubation of living cells was performed in the presence of glucose (□), in the absence of glucose and presence of N_3^- (energy-depleted cells) (○), and in the presence of glucose and of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (∗). The experimental conditions are described under Materials and Methods.

DNA in the nucleus are comparable. Similar data (not shown) were obtained for doxorubicin and 8-(*S*)-fluoroidarubicin. Under our experimental conditions, saturation of the binding sites in the nucleus is observed at anthracycline concentrations of >5 μM.

Accumulation of the four anthracyclines in membrane-permeabilized cells. Strictly analogous experiments were performed with cells whose membranes were permeabilized with Triton X-100 (0.05%). Under these conditions, the amount of drug intercalated between the base-pairs (C_N) was the same as in the isolated nuclei (Fig. 2 shows the data obtained for daunorubicin and idarubicin). We can thus infer that under these conditions, only the binding of the drug to the nuclei yields quenching of the fluorescence signal.

Accumulation of daunorubicin and idarubicin in sensitive cells. When living sensitive cells are incubated with anthracyclines, the decay of the fluorescence signal observed at the steady state is not modified by membrane permeabilization when the following conditions are fulfilled: (i) intracellular and extracellular pH values are the same and (ii) there is no significant non-nuclear localization of the drug that could led to additional fluorescence quenching. The aim of the following experiments was to determine the conditions required to avoid non-nuclear accumulation of the anthracyclines. pH of the HEPES buffer used to follow the uptake of anthracycline by cells was equal to intracellular pH (i.e., 7.25) (28).

In a first set of experiments, the decay of the fluorescence signal of daunorubicin (10 μM) was followed as a function of the time of incubation with cells (10^6/ml). Glucose-containing buffer was used, and the steady state was reached within 90 min. The addition of Triton X-100, which permeabilized the membrane, did not lead to a signal modification (Fig. 3), and this allows us to conclude that daunorubicin accumulation in non-nuclear compartment can be neglected in comparison with its accumulation in the nucleus.

In a second set of experiments, the decay of the fluorescence signal of idarubicin (10 μM) was followed as a function of the time of incubation with cells (10^6/ml). The experiments are described for idarubicin, but similar data were obtained with 8-(*S*)-fluoroidarubicin. The steady state was reached within 30 min. When glucose-containing buffer was used, the decay of the fluorescence signal was very important, and the addition of Triton X-100 yielded an increase in this signal (Fig. 3). This effect was greater when the total drug concentration added to the cells increased (Fig. 2). However, when idarubicin incorporation was measured in energy-depleted cells (30-min incubation in the presence of N_3^- and absence of glucose), the addition of Triton X-100 at the steady state did not yield modification of the fluorescence signal. The same result was obtained when idarubicin was added to cells incubated in K^+-containing buffer in the presence of a protonophore (1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) (Fig. 2). We can infer that in these two cases, the drug accumulation occurred mainly in the nucleus.

The energy-dependent accumulation of anthracycline into subcellular compartments of cells has been observed (32), and we do not discuss this point in more detail. For the current study, we note that because the non-nuclear accumulation of idarubicin and 8-(*S*)-fluoroidarubicin is energy dependent, it can be neglected under the experimental conditions where the cells are energy deprived.

Comparison of the initial rates of uptake (V+) of doxorubicin, daunorubicin, idarubicin, and 8-(*S*)-fluoroidarubicin in resistant K562 cells. Cells (10^6/ml) were incubated at 37° in the presence of various concentrations of glucose, ranging from 0.25 to 12 μM. Fig. 4 shows three typical experiments with 1 μM doxorubicin, daunorubicin, idarubicin, and 8-(*S*)-fluoroidarubicin, respectively. Similar data were obtained with energy-deprived cells.

For daunorubicin, idarubicin, and 8-(*S*)-fluoroidarubicin, V_+ can be easily determined from the slope of the tangent to the curve F(t) as indicated above. However, with doxorubicin,

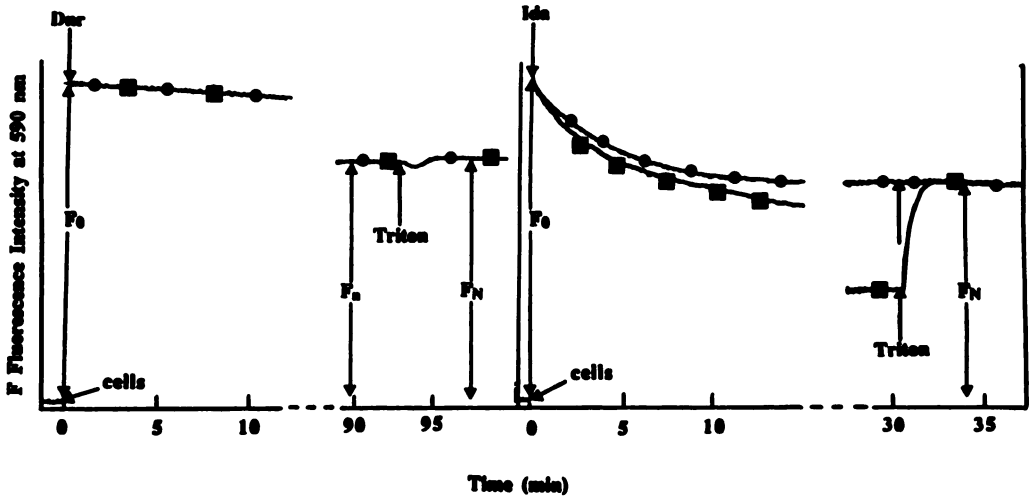


Fig. 3. Uptake of daunorubicin (*Dnr*) (*left*) and idarubicin (*Ida*) (*right*) by drug-sensitive cells. F, the fluorescence intensity at 590 nm (λ_{ex} = 480 nm) is recorded as a function of time. Cells (2 ×10^6/ml) were suspended in a cuvette filled with 2 ml buffer at pH_e 7.25 and 37° under vigorous stirring. At t = 0, 50 μl of a stock anthracycline solution was added to the cells, yielding an anthracycline concentration of C_T = 10 μM; the fluorescence intensity was then F_0. Once the steady state was reached, the fluorescence intensity was F_n, and the intracellular concentration of drug was $C_n = C_T (F_0 - F_n)/F_0$. The addition of Triton X-100 yielded the equilibrium state. The fluorescence intensity was then F_N, and the concentration of drug intercalated between the base-pairs $C_N = C_T (F_0 - F_N)/F_0$. The experiments were performed either in the presence of glucose (■) or in the absence of glucose and presence of N_3^- (●).

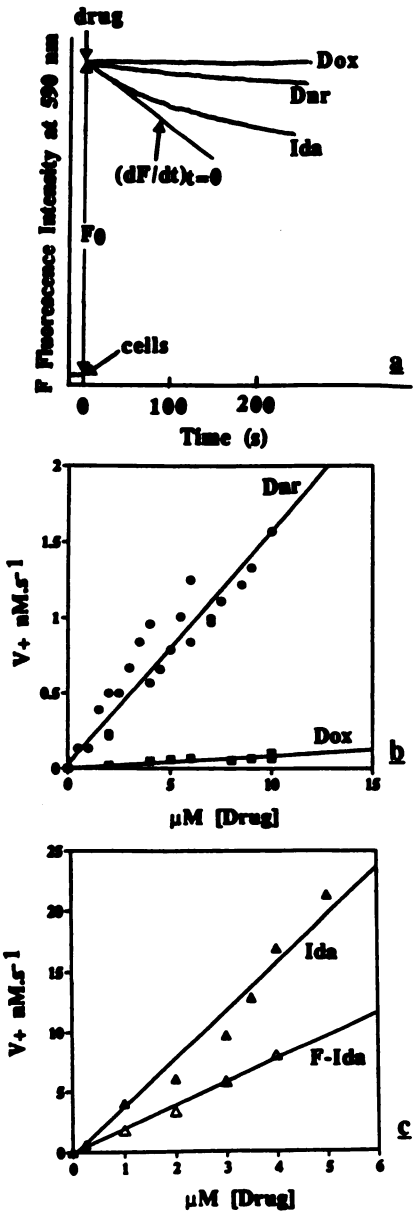


Fig. 4. Kinetics of uptake of doxorubicin (*Dox*), daunorubicin (*Dnr*), 8-(*S*)-fluoroidarubicin (*F-Ida*), and idarubicin (*Ida*) by drug-resistant cells. F, the fluorescence intensity is recorded as a function of time. Cells (2 $\times 10^6$/ml) were suspended in a cuvette filled with 2 ml buffer at pH_e 7.25 and 37° under vigorous stirring. At t = 0, 20 μl of a stock anthracycline solution was added to the cells, yielding a C_T anthracycline concentration. The slope to the tangent of the curve F = f(t) at t = 0 was $(dF/dt)_{t=0}$ and the initial rate of uptake $V_+ = (dF/dt)_{t=0}(C_T/F_0)$. a, The record of typical experiments performed with 1 μM doxorubicin (Dox), daunorubicin (Dnr), or idarubicin (Ida). The kinetics of uptake have been plotted as a function of the doxorubicin or daunorubicin concentration (b) or of the 8-(*S*)-fluoroidarubicin or idarubicin concentration (c). The fitted lines have been calculated by linear regression. The correlation coefficients were >0.92.

the uptake was so slow that we determined the rate from the decrease of the fluorescent signal after 2 hr of incubation of energy-deprived cells with doxorubicin. In that case, the net influx detected mainly includes the rate of passive influx: in the absence of energy, the MDR pump does not work and the free cytosolic drug concentration is so low that the passive efflux can be neglected compared with the passive influx.

V_+ is plotted as a function of the extracellular concentration (C_T) of drug added to the cells (at t = 0, $C_T = C_e$) in Fig. 4, b and c. As can be seen, in the four cases, V_+ was approximately proportional to C_T. The mean influx coefficients, determined from the slopes of the tangents to the curve as shown in Fig. 4, were 0.10 ± 0.03, 1.6 ± 0.3, 20 ± 4, and 40 ± 8 $\times 10^{-10}$ sec^{-1} for doxorubicin, daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin, respectively. When similar experiments were performed with sensitive cells, the values for the mean influx coefficients were, within the limits of experimental errors, the same as for resistant cells.

Kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin. We first consider the data obtained with idarubicin and 8-(*S*)-fluoroidarubicin. The kinetics of active efflux (V_a) are plotted as a function of C_i, and a clear saturation of the active efflux was observed (Fig. 5c). We compared the kinetics of efflux for these two drugs and for each obtained V_M = 1.8 ± 0.2 nM sec^{-1}. The apparent K_m values for P-glycoprotein-mediated transport, the intracellular free cytosolic drug concentration yielding half-maximal velocity, were ~0.9 ± 0.2 μM for each drug. The mean coefficient for the active efflux k_a, determined from the slope of the linear part of the curve, was equal to 9.5 ± 0.8 $\times 10^{-10}$ sec^{-1}.

The kinetics of the P-glycoprotein-mediated efflux of daunorubicin were determined under the same experimental conditions, and Fig. 5b shows the plot of V_a as a function of C_i. We obtained V_M = 3 ± 0.3 μM, K_m = 2.1 ± 0.3 μM, and k_a = 6.1 ± 0.6 $\times 10^{-10}$ sec^{-1}.

For these three drugs, we observed that the dependence of Va on drug concentration was not simply hyperbolic but rather positively cooperative. This was expressed quantitatively with the use of the Hill equation (33):

$$V_a = V_M(C_i)^{nH}/(K_m{}^{nH} + C_i{}^{nH})$$

where n_H, the Hill coefficient, represents the cooperativity constant. n_H can be determined in two ways: (i) the plot of log $V_a/(V_M - V_a)$ versus log C_i should yield a straight line with a slope is equal to n_H; and (ii) the plot of $\Theta = V_a/V_M$ versus ln C_i should yield a symmetrical curve, and the slope at $\Theta_{1/2}$ multiplied by 4 gives n_H. These two plots were drawn for the three drugs (data not shown). n_H was equal to 2 ± 0.2 for idarubicin and 8-(*S*)-fluoroidarubicin and to 2 ± 0.1, suggesting a cooperative transport of two molecules via P-glycoprotein. This was already suggested by Lankelma and colleagues for daunorubicin (34, 35). V_a was calculated the use of the above equation and n_H = 2 with V_M = 3 nM sec^{-1} and K_m = 2.1 μM for daunorubicin and V_M = 1.8 nM sec^{-1} and K_m = 0.9 μM for idarubicin and 8-(*S*)-fluoroidarubicin (Fig. 5, b and c).

Fig. 5a shows the plot of V_a as a function of C_i, for doxorubicin. The mean coefficient for the active efflux was obtained as k_a = 2.9 ± 0.4 $\times 10^{-10}$ sec^{-1}. As it was not possible to reach high cytosolic free drug concentrations, it was impossible to observe a saturation of the efflux. The K_m appears to be higher than 2 μM.

Cell-growth inhibition. The IC_{50} values obtained for the four drugs for sensitive cells are shown in Table 1 with the RF. The cytotoxicities of the four drugs toward sensitive cells are similar. Idarubicin is approximately twice as cytotoxic as the others. However, their cytotoxicities toward resistant

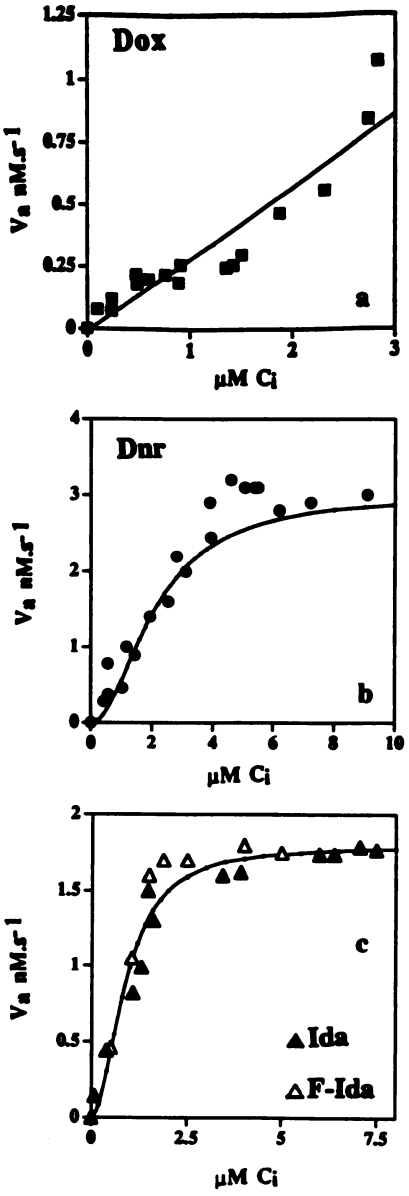


Fig. 5. Kinetics of the P-glycoprotein-mediated efflux of doxorubicin (*Dox*), daunorubicin (*Dnr*), 8-(*S*)-fluoroidarubicin (*F-Ida*), and idarubicin (*Ida*) plotted as a function of C_i, the cytosol free drug concentration. Resistant cells (2 $\times 10^6$/ml) were incubated in the presence of various concentrations of drug ranging from 0 to 12 µM. V_a and C_i were determined as described. The fitted curves were calculated using the equation and parameters given in Results.

cells are far different, with idarubicin being ~15 times more potent than doxorubicin.

Discussion

Structural modifications of the doxorubicin and daunorubicin molecules represent an effective approach for overcoming MDR, and anthracycline analogues have been designed to circumvent MDR and thereby enhance chemotherapeutic efficacy against drug-resistant neoplastic cells (14–17). Thus, idarubicin is a new daunorubicin analogue that is being used to treat a variety of human malignancies. *In vitro* studies indicate that it is more effective than daunorubicin and doxorubicin in tumor cell lines that display the MDR phenotype

TABLE 1

Cross-resistance pattern of doxorubicin-resistant K562 cells

IC_{50} (S) is the drug concentration required to inhibit 50% of sensitive cell growth. Resistance factor value was calculated as resistant cell IC_{50}/sensitive cell IC_{50}. The values represent mean ± standard deviation of triplicate determinations.

	IC_{50} (S)	RF
	nM	
Doxorubicin	10 ± 2	30
Daunorubicin	12 ± 3	20
8-(*S*)-Fluoroidarubicin	12 ± 3	6
Idarubicin	6 ± 2	3

(36). It has been supposed that this might depend on the high lipophilicity of idarubicin, which enhances intracellular accumulation of this drug (37–39). It has also been proposed that idarubicin could be less affected by P-glycoprotein activity (36). 8-(*S*)-Fluoroidarubicin is a newly synthesized idarubicin derivative (25) and is more cytotoxic against MDR cells than is daunorubicin.

The ability to overcome MDR appears to be related to the increased lipophilicity of the compounds and consequently to their ability to enter cells rapidly to reach cytotoxic intracellular levels (37). However, it is not known how these different molecules are handled by P-glycoprotein. Conceivably, the kinetics of P-glycoprotein-mediated efflux of drug are the same for molecules such are idarubicin and doxorubicin that have very different lipophilicity. In MDR cells, resistance is due to outward drug transport by P-glycoprotein. Thus, the lack of cross-resistance has often been taken as an indication of reduced transport by P-glycoprotein (40). The design of new antitumor drugs able to overcome MDR has focused on new analogues whose uptake would not be affected by P-glycoprotein and on the identification of chemical modifications that would led to minimal drug efflux (14–17). However, both the kinetics of influx and of P-glycoprotein-mediated efflux of the drugs across the cell membrane are, in large part, responsible for the intracellular concentration.

Our aim was to identify the respective contributions of the kinetics of uptake and efflux in the intracellular drug concentration and to provide quantitative data that could help to elucidate the mechanism of the P-glycoprotein-mediated efflux of drug. The four drugs that we studied were chosen because they have the same amino sugar and the same amino pK_a of deprotonation. Consequently, under similar experimental conditions, the same percentage of the neutral form and of the singly positively charged form will be present for the four drugs. This is important because although anthracycline uptake occurs through passive diffusion of the neutral form of the molecule, the molecular form that is pumped out by P-glycoprotein is not yet clear (27). It has been shown that hydroxyrubicin, a fully neutral derivative of doxorubicin, can be pumped out by P-glycoprotein (30). Also, Skovsgaard (41) has observed that *N*-acetyl-daunorubicin, an analogue that does not possess the base property of daunorubicin, is extruded as effectively as daunorubicin. However, it has not yet been demonstrated that the positively charged form of anthracycline could be effluxed, although it has been shown that lipophilic cations can be P-glycoprotein substrates (42). (It should be noted that in these cases, the positive charge is delocalized over the entire molecule, which does not occur with anthracyclines.) We have not formed a hypothesis on the molecular form or forms that are P-glycoprotein sub-

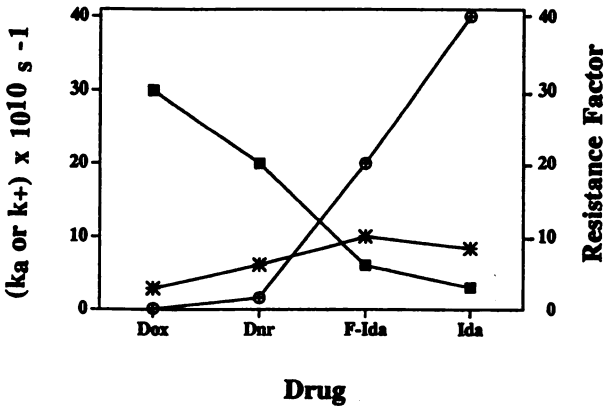


Fig. 6. Kinetics parameters and RF for the four anthracycline derivatives: doxorubicin (*Dox*), daunorubicin (*Dnr*), 8-(*S*)-fluoroidarubicin (*F-Ida*), and idarubicin (*Ida*). Shown are k_+ (⊕, mean influx coefficient), k_a (*, mean active efflux coefficient), and RF (■).

strate and have merely determined a mean active efflux coefficient (k_a).

Our data clearly show that the kinetics of uptake of the four drugs vary over a very large range: the kinetics of uptake of daunorubicin, 8-(*S*)-fluoroidarubicin, are idarubicin are 16, 200, and 400 times higher than that of doxorubicin, respectively. As can be expected, the kinetics of the drug uptake increase as the lipophilicity increases. In contrast, the four molecules are extruded at comparable rates, although the k_a values for idarubicin and 8-(*S*)-fluoroidarubicin, which are the two more lipophilic compounds, are slightly (3 times) higher than that for doxorubicin. Also, the K_m values compare for the four drugs, within a 3-fold range, indicating that the four molecules have comparable affinity for P-glycoprotein. Spoelstra *et al.* (34) reported a comparable K_m value (1.5 μM) for the P-glycoprotein transport of daunorubicin. With the efficiency of P-glycoprotein in pumping out the four drugs being almost the same, it follows that the different intracellular free cytosolic drug concentrations found mainly depend on their kinetics of uptake. Therefore, the different values of the RF obtained for the four drugs are mainly due to the different kinetics of their uptake and not to any difference in the P-glycoprotein-mediated efflux of these drugs. This is clearly seen in Fig. 6.

Priebe *et al.* (17) hypothesized that the amino group in the sugar portion of doxorubicin might be a key functionality for substrate recognition by the P-glycoprotein. Our observation that these four anthracyclines, which have the same sugar portion, are extruded at comparable rates by P-glycoprotein is in good agreement with this hypothesis.

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