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The ability of verapamil to restore intracellular accumulation of anthracyclines in multidrug resistant cells depends on the kinetics of their uptake

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Abstract

The basic distinguishing feature of all cells expressing functional P-glycoprotein-multidrug resistance is a decrease of steady state drug levels as compared to those in drug-sensitive controls. A variety of small molecules, such as verapamil and cyclosporin A, bind to P-glycoprotein and inhibit its ability to pump out antitumor drugs. The kinetics of P-glycoprotein-mediated efflux of various anthracycline derivatives was measured in multidrug-resistant (MDR) K562 cells in the presence of verapamil. Used for the purpose were daunorubicin, idarubicin and 8-*S*-fluoro-idarubicin which have the same pK_a of deprotonation equal to 8.4, but different lipophilicity, 4′-epi-2′-bromo-daunorubicin which has a lipophilicity which is comparable to that of daunorubicin but a pK_a equal to 6.3, pirarubicin with pK_a equal to 7.7 and lipophilicity different from that of these derivatives were used. Our data show (1) that verapamil is unable to completely block the P-glycoprotein-mediated efflux of anthracyclines and that 10% of its functionality remains even with high verapamil concentrations, (2) that the ability of verapamil to restore intracellular accumulation of anthracyclines in MDR cells depends on the kinetics of their uptake. With fast kinetics uptake, as is the case for idarubicin, 8-*S*-fluoro-idarubicin, 4′-epi-2′-bromo-daunorubicin and pirarubicin (which have either a low pK_a and/or high lipophilicity), verapamil can restore in multidrug resistant cells an intracellular drug level which is comparable to that observed in sensitive cells. This is not possible when the kinetics of uptake is low as is the case for daunorubicin. Cyclosporin A is a more potent modulator and is able to fully restore daunorubicin accumulation in multidrug resistant cells. © 1998 Elsevier Science B.V.

Keywords: Multidrug resistance; P-glycoprotein; Verapamil; Daunorubicin; Anthracycline; Kinetics; Cyclosporin

1. Introduction

Development of drug resistance in tumor cells is a significant obstacle to long-term, sustained patient response to chemotherapy. There is accumulating evidence that active export of anticancer drugs from cells is one of the major mechanisms of drug resistance and a clinically significant class of drug resistance correlates with the presence of a 170 kDa glycoprotein in the cell membrane (Bradley et al., 1988; Gottesman and Pastan, 1993).

Thus, P-glycoprotein, the product of the human *mdr1* gene, is responsible for an ATP-dependent extrusion of numerous cytotoxic drugs from a wide variety of cancer cells. Its overexpression has been associated with a poor outcome in the treatment of some cancers (Arceci, 1993). A variety of small molecules, such as verapamil, dihydropyridines, forskolin, and cyclosporin A, bind to P-glycoprotein and inhibit its ability to pump out antitumor drugs (Beck and Qian, 1992; Twentyman, 1992). Therefore, the clinical utility of non-toxic derivatives of these and other molecules as chemosensitizing agents has been extensively studied (Dalton, 1993; Sikic, 1993). However, the molecular basis of the interaction of anticancer drugs and chemosensitizers with P-glycoprotein is poorly understood.

Verapamil was the first reverting agent proposed by Tsuruo et al. (1981, 1982) and has received considerable attention as an agent which can reverse multidrug resistance in certain animal and human cells. It has thus been the subject of numerous studies which have shown that verapamil is extruded from multidrug resistant cells, that

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photoactive analogues of verapamil bind to P-glycoprotein, that verapamil inhibits the P-glycoprotein-mediated efflux of various antitumor drugs such as anthracyclines and vinca alkaloids (Beck et al., 1986; Safa, 1988; Yusa and Tsuruo, 1989; Horio et al., 1989; Qian and Beck, 1990; Spoelstra et al., 1994; Pereira et al., 1994). Two groups, at least, have reported that, in terms of its influence on resistance to anthracyclines, the interaction of verapamil with multidrug resistant cells is not the same as for vinca alkaloids. Tsuruo et al. (1983) have reported that verapamil had a greater effect on vincristine toxicity than on anthracycline toxicity in MDR P388 cells and Beck et al. (1986) found that verapamil was able to reverse the resistance to vinca alkaloids (defined as enhanced toxicity), but not the resistance to anthracyclines in human leukaemia cells. Interestingly, vinblastine was the most effective inhibitor of binding of a verapamil photoactive analogue to P-glycoprotein, while doxorubicin only partially inhibited verapamil binding to this protein (Safa, 1988). It has also been observed that even a relatively high concentration of the modulator usually does not completely reverse multidrug resistance; in particular, such concentrations do not restore the accumulation of the multidrug resistance-related drugs daunorubicin and doxorubicin (Ford and Hait, 1990).

Despite the fact that verapamil is one of the most studied inhibitor of the P-glycoprotein-mediated efflux of various multidrug resistance drugs, its precise mechanism of action is still unknown and, during the last years, conflicting data have appeared in the literature. For instance, some authors have claimed that there was only one common site for all the drugs and chemosensitizers transported by P-glycoprotein (Borgnia et al., 1996) whereas others have reported non-competitive inhibition of drug efflux by verapamil and others chemosensitizers (Spoelstra et al., 1994; Pereira et al., 1994) and also co-operative, competitive and non-competitive interactions between modulators (Ayesh et al., 1996).

In an attempt to gain an insight into the mechanism of interaction of verapamil with multidrug resistant cells we have studied its ability to inhibit the P-glycoprotein-mediated efflux of various anthracycline derivatives. As far as anthracyclines are concerned most of the literature data were obtained using doxorubicin and daunorubicin and in most of cases the data are poorly quantitative. We now addressed more particularly the following questions: (1) Is verapamil able to totally inhibit the P-glycoprotein-mediated efflux of anthracyclines? (2) Does the concentration required to inhibit this efflux depend on characteristics of the anthracycline such as lipophilicity and charge, and therefore on its kinetics of uptake by the cells? To answer these questions the following derivatives were used: daunorubicin, idarubicin and 8-*S*-fluoro-idarubicin which have the same pK_a of deprotonation equal to 8.4, but different lipophilicity, 4′-epi-2′-bromo-daunorubicin which has a lipophilicity which is comparable to that of daunorubicin but a lower pK_a value equal to 6.3 (Garnier-Suillerot and Priebe, unpublished observation). Pirarubicin, a doxorubicin derivative, which has pK_a and lipophilicity different from those of these derivatives was also used. Our data showed that verapamil inhibits the P-glycoprotein efflux of the different anthracyclines to the same extent (about 90%), but that the accompanying increase in drug incorporation depends on their kinetics of uptake by the cells.

2. Material and methods

2.1. Cell culture and cytotoxicity assay

K562 is a human leukemia cell line, established from a patient with a chronic myelogeneous leukemia in blast transformation (Lozio and Lozzio, 1975). K562 cells resistant to doxorubicin were obtained by continuous exposure to increasing doxorubicin concentrations, and were maintained in medium containing doxorubicin (40 nM). This subline expresses a unique membrane glycoprotein with a molecular mass of 170 kDa (Tsuruo et al., 1986). Doxorubicin-sensitive and -resistant erythroleukemia K562 cells were grown in suspension in RPMI 1640 (Sigma) medium supplemented with L-glutamine and 10% foetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cultures, initiated at a density of 10^5 cells/ml, grew exponentially to $8–10 \times 10^5$ cells/ml in three days. For the spectrofluorometric assays, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/ml, and cells were used 24 h later, when the culture had grown to about $8–10 \times 10^5$ cells/ml. Cell viability was assessed by trypan blue exclusion. The cell number was determined by Coulter counter analysis.

A resistance factor 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 h. The resistance factors obtained were 20, 6, 3, 7 and 3 for daunorubicin, 8-*S*-fluoro-idarubicin, idarubicin, pirarubicin and 4′-epi-2′-bromo-daunorubicin, respectively.

2.2. Drugs and chemicals

Purified doxorubicin, daunorubicin, idarubicin and pirarubicin were kindly provided by Pharmacia-Upjohn and Roger Bellon. 8-(*S*)-fluoro-idarubicin was provided by Menarini Ricerche Sud, the group that originally synthesized it (Guidi et al., 1994). 4′-epi-2′-bromo-daunorubicin was provided by W. Priebe (Priebe et al., 1992). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11500\ M^{-1}\ cm^{-1}$.

Stock solutions were prepared just before use. Verapamil was from Sigma and cyclosporin A was kindly provided by Sandoz. 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 $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose at pH 7.25.

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

2.3. Cellular drug accumulation

The rationale and validation of our experimental set-up for measuring the kinetics of active transport of anthracyclines from tumor cells have been extensively described and discussed before (Frezard and Garnier-Suillerot, 1991a,b; Pereira et al., 1994; Borrel et al., 1994, 1995). The setup is based on continuous spectrofluorometric monitoring of the decrease of the fluorescence signal of anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) during incubation with cells in a 1-cm quartz cuvette. A typical experiment is shown in Fig. 1. The decrease of fluorescence occurring during incubation with cells is due to quenching of the fluorescence after intercalation of anthracycline between the base-pairs of DNA. We have previously shown that this methodology allows accurate measurement of the free cytosolic concentration of anthracyclines at steady state, their initial rates of uptake and kinetics of active efflux. With idarubicin, 8-(*S*)-fluoro-idarubicin, 4′-epi-2′-bromo-daunorubicin and pirarubicin, steady state was reached within 30 min, while 90 min was required for daunorubicin. Verapamil was added either at steady state or at the beginning of the incubation of the anthracycline with cells. In both cases, this yielded a strictly analogous new steady state.

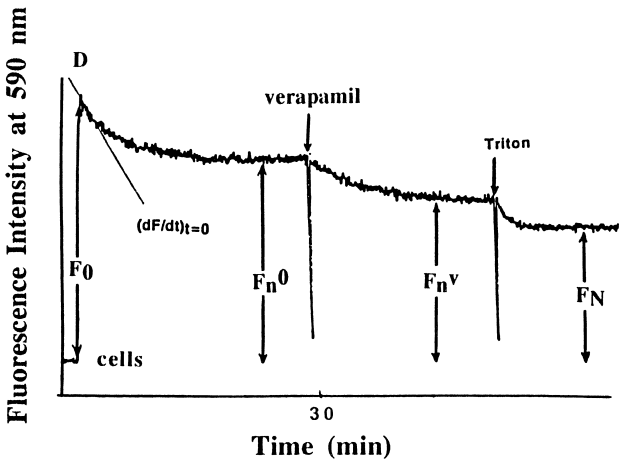
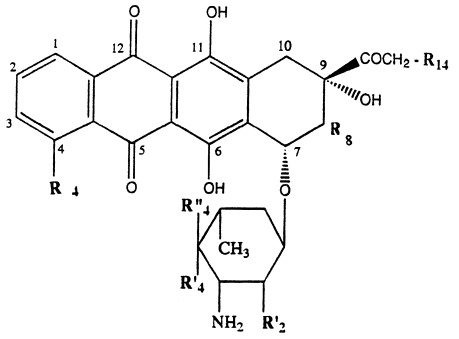


Fig. 1. Typical kinetics of uptake of an anthracycline derivative by drug-resistant K562 cells. *F*, fluorescence intensity at 590 nm ($\lambda_{ex} = 480$ nm) was recorded as a function of time. Cells, 2×10^6/ml were suspended in a cuvette filled with 2 ml buffer at pH = 7.25 under vigorous stirring. At $t = 0$ a small volume of a stock anthracycline (D) solution was added to the cells, yielding a C_T μM anthracycline solution. The fluorescence intensity was then F_0. The slope of the tangent to the curve $F = f(t)$ was $(dF/dt)_{t=0}$ and the initial rate of uptake $V_+ = (dF/dt)_{t=0}\cdot(C_T/F_0)$. Once steady state was reached, the fluorescence was F_n^0 and the concentration of drug intercalated between the base pairs in the nucleus was $C_n = C_T(F_0 - F_n)/F_0$. When steady state was reached, verapamil was added. At the new steady state, the fluorescence intensity was F_n^V. The addition of 0.05% Triton X-100 yielded an equilibrium state. Fluorescence was then F_N. As a typical example the plot is shown for pirarubicin.



Anthracycline	R_4	R_8	R_{14}	R'_2	R'_4	R''_4	Σfi	pK_a
DNR	OCH_3	H	H	H	OH	H	0	8.4
Br-DNR	OCH_3	H	H	Br	H	OH	0.09	6.3
PIRA	OCH_3	H	OH	H		H	-1.1	7.7
IDA	H	H	H	H	OH	H	1.05	8.4
F-IDA	H	F	H	H	OH	H	0.41	8.4

Fig. 2. Structures, pK_a and hydrophobicity values of the anthracyclines used. Variation of hydrophobicity evaluated as sum of fragmental values f_i, refers to daunorubicin.

All experiments were conducted in 1-cm quartz cuvettes containing 2 ml of buffer at 37°C. At the end of the experiment, cell viability was assessed using trypan blue exclusion. Unless otherwise stated the experiments were performed at pH = 7.25 ± 0.05.

The initial rate of uptake $(V_+)_{t=0}$ can be determined from the curve in Fig. 1: $V_+ = (dF/dt)\cdot(C_T/F_0)$ where C_T is the total drug concentration added to the cells and F_0 the anthracycline fluorescence at $t = 0$.

The initial rate of uptake can be written as

$$(V_+)_{t=0} = k_+ \cdot n \cdot (C_e)_{t=0} \quad (1)$$

where k_+ is the mean influx coefficient for the drug, n the number of cells per ml and $(C_e)_{t=0}$ the extracellular free drug concentration at $t = 0$ is equal to the total drug concentration C_T added to the cells.

The overall intracellular anthracycline concentration, C_n, is calculated from the decrease of the fluorescent signal when anthracycline is incubated with these drugs: in

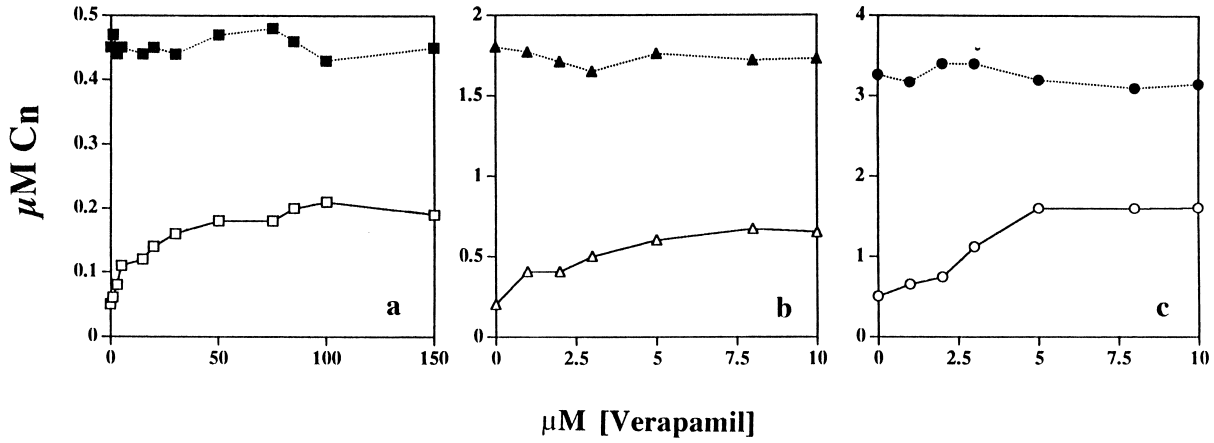


Fig. 3. Accumulation of daunorubicin in drug-resistant and -sensitive cells in the presence of verapamil. C_n, the overall molar concentrations of drug bound to the nuclei of resistant cells (open symbols) and of sensitive cells (full symbols) at steady state plotted as a function of the concentration of verapamil added. The daunorubicin concentration was 1 μM (a), 5 μM (b) or 10 μM (c). The experimental conditions are described in Section 2.

the absence of verapamil, $C_n^0 = C_T \cdot (F_0 - F_n^0)/F_0$ and in the presence of verapamil.

$C_n^v = C_T \cdot (F_0 - F_n^v)/F_0$ where F_n^0 and F_n^v are the fluorescence signal at steady state before and after the addition of verapamil, respectively.

At steady state, 0.05% Triton X-100 was added to permeabilize the plasmic membrane. This yielded the equilibrium state, the fluorescence signal was F_N and the overall concentration of drug bound to the nucleus was $C_N = C_T \cdot (F_0 - F_N)/F_0$. The C_N value was the same for sensitive and resistant cells, indicating that the DNA content was similar in both cell lines (Frezard and Garnier-Suillerot, 1991b).

2.4. Determination of the P-glycoprotein-mediated efflux of anthracycline derivatives

The following method, which has been described previously, has been used to determine the kinetics of P-glycoprotein-mediated efflux of anthracycline (Frezard and Garnier-Suillerot, 1991a,b). Briefly, at steady state the kinetics of the P-glycoprotein-mediated efflux of drug can be written as

$$V_a = V_+ - V_- \quad (2)$$

where V_+ and V_- are the kinetics of passive influx and efflux of the drug, respectively. In this expression, $V_+ = k_+ \cdot n \cdot C_e$ and $V_- = k_- \cdot n \cdot C_i$, where C_e and C_i are the extra and intracellular free drug concentrations, respectively, k_+ and k_-, the passive coefficients of influx and efflux, respectively. Making the reasonable assumption that $k_+ = k_-$, it follows that

$$V_a = k_+ \cdot n \cdot (C_e - C_i) \quad (3)$$

On the other hand, one can write that, in a first approximation

$$V_a = k_a \cdot n \cdot C_i \quad (4)$$

k_a being the active, or P-glycoprotein-mediated, efflux coefficient for the drug. Using Eqs. (3) and (4), k_a can be written as

$$k_a = k_+ \cdot (C_e - C_i)/C_i \quad (5)$$

where $C_e = C_T - C_n$ and $C_i = C_E/C_N \cdot (C_n)$.

This equation is valid when the efflux is not saturated, i.e. when the kinetics of active efflux is linearly dependent on the intracellular free drug concentration. According to our previous work the present study fulfilled this condition (Mankhetkorn et al., 1996a).

In the following, k_a^0 and k_a^v stand for the P-glycoprotein-mediated active efflux coefficient of anthracycline in the absence and in the presence of verapamil, respectively. The ability of verapamil to inhibit the P-glycoprotein-mediated efflux of drug can be calculated using the ratio $r = k_a^v/k_a^0$ which is equal to 1 when there is no inhibition of the active efflux and to 0 when the P-glycoprotein-mediated active efflux is completely blocked.

We have checked that the passive influx coefficient, k_+, that characterizes the uptake of the drug does not depend on the presence of verapamil.

2.5. Hydrophobicity of the anthracycline derivatives

An estimation of the hydrophobicity of a compound is given by log P, where P is the partition coefficient in an *n*-octanol/water system. In a first approximation, the log P value of a compound can be estimated by adding the f_i values of its fragments (Rekker, 1977). We have determined the variation of log P of compounds to be tested

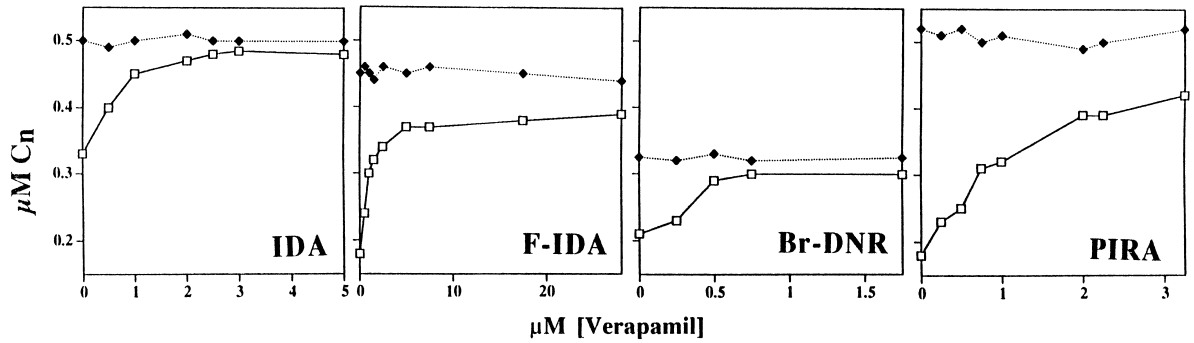


Fig. 4. Accumulation of idarubicin (IDA), 8-(*S*)-fluoro-idarubicin (F-IDA), 4′-epi-2′-bromo-daunorubicin (Br-DNR) and pirarubicin (PIRA) in drug-resistant and -sensitive cells in the presence of verapamil. C_n, the overall molar concentrations of drug bound to the nuclei of resistant cells (open symbols) and of sensitive cells (full symbols) at steady state are plotted as a function of the concentration of verapamil added. The anthracycline concentration was 1 μM. The experimental conditions are described in Section 2.

using daunorubicin as a reference compound and equation

$$\log P = \log P_0 + \Sigma f_i$$

where P_0 is the partition coefficient for daunorubicin in *n*-octanol/water and f_i is the *n*-octanol/water fragmental constant of the fragments of a compound that differentiate it from daunorubicin (Van de Waterbeemd and Testa, 1987). The values are reported in Fig. 2.

3. Results

The structures of the five anthracylines used are shown in Fig. 2 their pK_a values and hydrophobicity parameters are also indicated.

3.1. Accumulation of daunorubicin in resistant cells in the presence of various verapamil concentrations

Fig. 3 shows the data from experiments in which the amount of daunorubicin incorporated in the nucleus of resistant cells was measured at steady state in the presence of increasing concentrations of verapamil. In a first set of experiments the daunorubicin concentration was 1 μM and the verapamil concentration was varied from 1 to 150 μM (Fig. 3a). As can be seen C_n^v, the overall concentration of daunorubicin incorporated between the base pairs in the nucleus increased from $C_n^0 = 0.05 \pm 0.01$ μM, in the absence of verapamil, to 0.20 ± 0.02 μM and did not exceed this value. C_n^v, obtained under similar conditions using sensitive cells, was also plotted in this figure. Its value did not depend on the verapamil concentration and was equal to 0.45 ± 0.03 μM. This first experiment clearly showed that even the addition of very high concentrations of verapamil did not restore, in resistant cells, a daunorubicin accumulation comparable to that observed with sensitive cells.

Three other sets of experiments were performed in the presence of daunorubicin concentrations equal to 5, 10 and 0.2 μM and similar results were obtained. At 5 μM daunorubicin, in the absence of verapamil, C_n^0 was equal to 0.28 ± 0.03 μM. In the presence of verapamil C_n^v plateaued at 0.65 ± 0.04 μM while C_n^v was equal to 1.80 ± 0.1 μM in sensitive cells (Fig. 3b). At 10 μM daunorubicin concentration, C_n^0 was equal to 0.43 ± 0.03 μM and increased up to 1.4 ± 0.1 μM in the presence of high concentrations of verapamil. C_n^v was equal to 3.1 ± 0.2 μM in sensitive cells (Fig. 3c). At 0.2 μM daunorubicin, C_n^0 was equal to 0.010 ± 0.002 μM and C_n^v to 0.031 ± 0.006 μM and 0.072 ± 0.015 μM in sensitive and resistant cells, respectively. So, whatever the daunorubicin concentration added to the cells, the addition of verapamil, even at concentrations as high as 150 μM, did not restore, in resistant cells, an intracellular daunorubicin concentration comparable to that observed in sensitive cells.

Table 1
Pirarubicin accumulation in drug-resistant and-sensitive K562 cells

[pirarubicin] M	C_n (S)	C_n^0 (R)	C_n^v (R)	k_a^v/k_a^0
10^{-6}	$5.2 \pm 0.2 \times 10^{-7}$	$1.8 \pm 0.2 \times 10^{-7}$	$4.2 \pm 0.3 \times 10^{-7}$	0.13 ± 0.05
10^{-7}	$5.8 \pm 0.3 \times x10^{-8}$	$2.6 \pm 0.3 \times 10^{-8}$	$4.7 \pm 0.4 \times 10^{-8}$	0.19 ± 0.05
10^{-8}	$6.4 \pm 0.4 \times x10^{-9}$	$3.0 \pm 0.3 \times 10^{-9}$	$5.8 \pm 0.4 \times 10^{-9}$	0.10 ± 0.05

The overall concentration of pirarubicin accumulated at steady state in sensitive, C_n(S), and resistant cells C_n (R) was determined in the absence, C_n^0 (R), and in the presence, C_n^v(R), of 10 μM verapamil. The ratio $r = k_a^v/k_a^0$ which characterizes the inhibition by verapamil of the P-gp-mediated efflux of anthracyclines was calculated as indicated in Section 2.

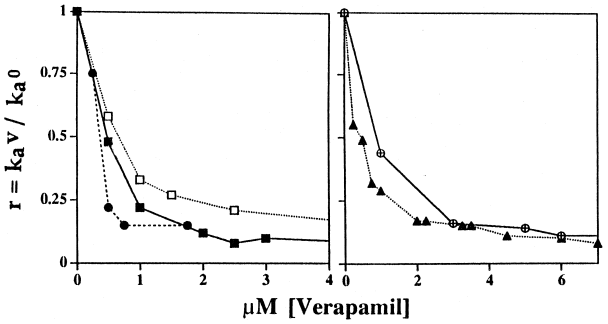


Fig. 5. Inhibition by verapamil of the anthracycline derivatives efflux in MDR cells. The ratio $r = k_a^v / k_a^0$, which characterizes the inhibition by verapamil of the P-gp-mediated efflux of anthracycline derivatives, was calculated for pirarubicin (▲), daunorubicin (⊕), 4′-epi-2′-bromo-daunorubicin (●), idarubicin (■) and 8-(*S*)-fluoro-idarubicin (□) as indicated in Section 2.

3.2. Incorporation of idarubicin, 8-(S)-fluoro-idarubicin, 4′-epi-2′-bromo-daunorubicin and pirarubicin in resistant cells in the presence of various verapamil concentrations

The overall concentrations of idarubicin, 8-(*S*)-fluoro-idarubicin, 4′-epi-2′-bromo-daunorubicin and pirarubicin incorporated in the nucleus of resistant cells at steady state in the presence of various amounts of verapamil are shown in Fig. 4. In these experiments, 1 μM anthracycline was added to the cells. For comparison, the amounts of drug incorporated into sensitive cells are also shown. As can be seen in the case of these four drugs the ability of verapamil to increase the intracellular anthracycline concentration was more efficient than in the case of daunorubicin and a level of intracellular drug concentration close to that observed in sensitive cells was obtained.

To clearly demonstrate that the verapamil concentration required to inhibit the active efflux of anthracycline does not depend on the anthracycline concentration, experiments were performed using 10^6 cells/ml and pirarubicin at concentrations equal to 10^{-6}, 10^{-7} and 10^{-8} M. The C_n values determined in the absence and in the presence of 10 μM verapamil are reported in Table 1.

3.3. Calculation of the active efflux inhibition coefficient r for the various anthracycline derivatives

The values of the coefficient $r = k_a^v/k_a^0$ were calculated for the five anthracycline derivatives and its variations, as a function of the concentrations of verapamil added, are shown in Fig. 5. The verapamil concentrations required to obtained fifty percent inhibition of the P-glycoprotein-mediated efflux of the drugs ($r = 0.5$) were 1.1 ± 0.5, 0.5 ± 0.2, 0.7 ± 0.2, 0.4 ± 0.2 and 0.5 ± 0.2 μM for daunorubicin, idarubicin, 8-(*S*)-fluoro-idarubicin, 4′-epi-2′-bromodaunorubicin and pirarubicin, respectively. In addition, the r values were never lower than 0.13 ± 0.05, even in the presence of large verapamil concentrations.

In the case of pirarubicin, whose concentration was varied by a factor 100, the same phenomenon was observed, i.e., at 10 μM verapamil, the r value was, in the three cases, within the range of 0.14 ± 0.05 (Table 1).

3.4. Calculation of the overall intracellular drug concentrations as a function of r, the active efflux inhibition coefficient

Using Eqs. (1)–(5), C_n^v can be written as

$$C_n^v = C_T/\left[1 + C_E/C_N + (C_E/C_N)\cdot(k_a^v/k_+)\right] \quad (6)$$

as $k_a^v/k_+ = r\cdot(k_a^0/k_+)$, it follows that

$$C_n^v = C_T/\left[1 + C_E/C_N + (C_E/C_N)\cdot r\cdot\left(k_a^0/k_+\right)\right] \quad (7)$$

where r is equal to 1 in the absence of inhibitor and to 0 if P-glycoprotein functionality is completely blocked.

C_T was equal to 1 μM and the values of the parameters C_N, C_E and k_a^0/k_+ used for this calculations were equal to, respectively, 0.45 μM, 0.55 μM and 13.8 for daunoru-

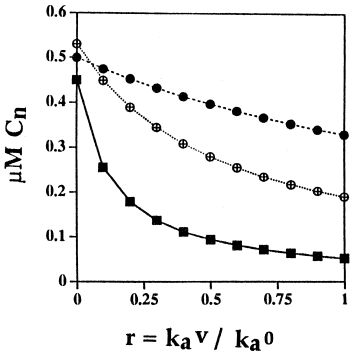


Fig. 6. Overall concentration of anthracycline derivatives expected at steady state in multidrug resistant K562 cells, as a function of the P-glycoprotein functionality. C_n, calculated according to Eq. (7) was plotted as a function of $r = k_a^v/k_a^0$, which characterizes P-glycoprotein functionality, for daunorubicin (●), pirarubicin (⊕) and idarubicin (■).

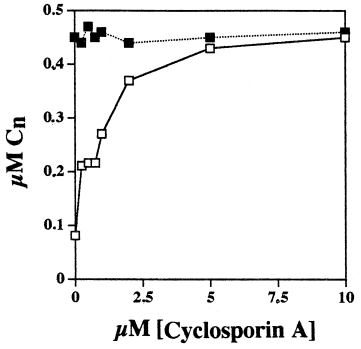


Fig. 7. Accumulation of daunorubicin in drug-resistant and -sensitive cells in the presence of cyclosporin A. C_n, the overall molar concentrations of drug bound to the nuclei of resistant cells (open symbols) and of sensitive cells (full symbols) at steady state are plotted as a function of the concentration of cyclosporin A added. The daunorubicin concentration was 1 μM. The experimental conditions are described in Section 2.

bicin, 0.50 μM, 0.50 μM and 1.03 for idarubicin, 0.53 μM, 0.47 μM and 3.8 for pirarubicin. The C_n^v values thus calculated are shown in Fig. 6. We have observed that when the cells were incubated with 1 μM daunorubicin, the overall intracellular concentration cannot exceed 0.2 μM. From the curve in Fig. 6 we can see that this corresponds to $r = 0.15 \pm 0.06$. The curve shows that, however, at this r value, the C_n^v values calculated for idarubicin and pirarubicin are very close to that observed for sensitive cells.

3.5. Incorporation of daunorubicin in resistant cells in the presence of various cyclosporin A concentrations

To clearly demonstrate that the inability of verapamil to restore the intracellular daunorubicin concentration to a level comparable to that observed in sensitive cells was related to verapamil itself, we checked the ability of cyclosporin A to increase the uptake of daunorubicin by resistant cells. Experiments strictly analogous to those described above were performed but with verapamil replaced by cyclosporin A, and daunorubicin 1 μM was used. Cyclosporin A 1 μM was able to increase the uptake of daunorubicin by fifty percent and, in the presence of 3 μM cyclosporin, the intracellular concentration of daunorubicin was the same in both resistant and sensitive cells (Fig. 7).

4. Discussion

Two questions were posed in the present study: (1) Is verapamil able to completely block the P-glycoprotein-mediated efflux of anthracycline? (2) Does the ability of verapamil to restore the intracellular accumulation of anthracycline derivatives in multidrug resistant cells depend on the nature of the anthracycline? Our findings lead us to conclude that the answer to the first question is 'no' and this is because the answer to the second question is 'yes'.

Although several mechanisms have been proposed for the decreased drug accumulation observed in many multidrug resistant cell lines, it is generally accepted that it is the result of P-glycoprotein activity removing drugs from the cells in an ATP-dependent manner. However, little is known about the mechanism underlying drug efflux and the way P-glycoprotein handles drugs that have totally different structures. One main approach for modulation of P-glycoprotein-related multidrug resistance is the use of chemosensitizing agents. Verapamil, a Ca^{2+} channel blocker, and cyclosporin A are the most often mentioned and have been shown to effectively reverse MDR in vitro (Yusa and Tsuruo, 1989; Sonneveld et al., 1992; Merlin et al., 1994). However, the clinical use of chemosensitizers remains questionable and the identification of more active, but less toxic, compounds is needed. Also, thorough knowledge of their molecular mechanism of action would be of the utmost importance for clinical intervention application.

The property of chemosensitizing agents to reverse multidrug resistance has been explained in terms of an improved cellular accumulation of antitumor drug in resistant cells. The role of P-glycoprotein in this modified uptake can only be assessed by a kinetic study of the uptake and efflux of drugs in resistant cells and the rational design of new drugs requires an understanding of the mechanisms of P-glycoprotein-mediated efflux of drug and of its inhibition by different compounds such as verapamil.

Recently, Didier et al. (1995) have measured the restoration of daunorubicin retention in multidrug resistant human T leukemia (CEM) cells in the presence of a variety of chemosensitizers, among which verapamil and cyclosporin A. In the absence of chemosensitizer agent treatment, the daunorubicin retention levels by multidrug resistant-CEM cells microcultures were about 12% of those in the parent cell microculture. Complete restoration (95% or above) of daunorubicin retention was obtained with 30 μg/ml cyclosporin A, but verapamil was less active and could at best restore 65% of the daunorubicin retention. Urasaki et al. (1996) have recently established a daunorubicin-resistant cell line derived from the human leukemia cell line K562 (K562/D1-9) which also shows multidrug resistance and expresses P-glycoprotein. These cells were found to be 28 times more resistant to daunorubicin than the parent cells and the intracellular accumulation of daunorubicin was less than in the wild type. Both daunorubicin resistance and its intracellular accumulation were only partially reversed by addition of verapamil. On the other hand, Michieli et al. (1994) have observed that in high-resistant cells, D-verapamil was unable to restore the intracellular daunorubicin concentration to the value for non-resistant cells, but it did so with idarubicin.

Our present results are in agreement with these previous observations. In addition the study showed that if one reasons in terms of drug accumulation, it is obvious that in the case of daunorubicin it is impossible to restore, in resistant cells, a drug concentration comparable to that observed in sensitive cells, whatever the concentration of verapamil used. However, restoration is possible, at least to 90%, in the case of the other derivatives. Nevertheless, cyclosporin A is able to fully restore daunorubicin accumulation in resistant cells.

These observations do not mean that daunorubicin has a behavior different from that of the other anthracycline derivatives in terms of efflux by P-glycoprotein and, more precisely this does not mean that there is competition between daunorubicin and verapamil for some common sites on P-glycoprotein that would not be observed for the other derivatives. The data do emphasizes the importance to reason in terms of kinetics and to always take into consideration not only the kinetics of P-glycoprotein-mediated efflux of the drug but also the kinetics of its

uptake by the cells because its intracellular concentration depends on both parameters. The calculation of the overall intracellular drug concentration that could be expected in the cells, at the steady state, as a function of the percentage of P-glycoprotein inhibition emphasizes this point.

We have previously studied P-glycoprotein functionality in living cells after its photolabeling with verapamil derivatives and shown that this is not sufficient to completely inhibit the efflux of pirarubicin (Mankhetkorn et al., 1996b). Only about 80% of the P-glycoprotein functionality was inhibited.

The answer to the second question is that the ability of verapamil to increase the intracellular anthracycline concentration in MDR cells depends on the nature of anthracycline, i.e. hydrophobicity and pK_a, and therefore on its kinetics of uptake. Anthracycline uptake occurs through passive diffusion of the neutral form of the molecule and therefore its kinetics of uptake is as low as its pK_a is low and its lipophilicity high (Frezard and Garnier-Suillerot, 1991a,c; Harrigan et al., 1993). We have previously determined the kinetics of anthracycline uptake by cells and therefore the influx coefficient k_+ which is equal to 2×10^{-10}, 20×10^{-10}, 35×10^{-10}, 40×10^{-10} and higher than 70×10^{-10} s^{-1} $(cells/ml)^{-1}$ for daunorubicin, 8-(*S*)-fluoro-idarubicin, pirarubicin, idarubicin and 4′-epi-2′-bromo-daunorubicin, respectively (Garnier-Suillerot, 1995; Mankhetkorn et al., 1996a). On the contrary, the kinetics of P-glycoprotein-mediated efflux of anthracyclines does not depend on their lipophilicity (Mankhetkorn et al., 1996a). Our data show that when the kinetics of uptake is fast, as it is for idarubicin, 8-(*S*)-fluoro-idarubicin, 4′-epi-2′-bromo-daunorubicin and pirarubicin, P-glycoprotein which, in the presence of verapamil, still has 10% of its normal efficiency, only very slightly changes the intracellular drug concentration. The 10% of P-glycoprotein functionality remaining is not enough to pump the drug out from the cells to any extent. However, in the case of daunorubicin which enters the cells slowly, the 10% functionality of the pump plays an important role, being sufficient to remove efficiently the drug from the cells.

All these data clearly demonstrate the role of the kinetics of drug uptake, and therefore of the hydrophobicity and pK_a, not only in the cytotoxicity of the drug towards resistant cells (Coley et al., 1989, 1992; Facchetti et al., 1991; Garnier-Suillerot, 1995; Mankhetkorn et al., 1996a) but also in the ability of verapamil to restore intracellular incorporation to a level comparable with that observed in parent cells. This held for verapamil, which is unable to completely block the P-glycoprotein-mediated efflux of anthracycline, but did not hold for cyclosporin A which is apparently able to completely block P-glycoprotein.

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