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CELL CYCLE DEPENDENT UPTAKE AND RELEASE OF ANTHRACYCLINE BY DRUG-RESISTANT AND DRUG-SENSITIVE HUMAN LEUKAEMIC K562 CELLS

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Abstract—The appearance of cellular resistance to antitumor drugs is a major problem in cancer chemotherapy. This results from the overexpression of the *mdr* 1 gene which encodes the 170 kDa P-glycoprotein or multidrug transporter. The uptake and release of 4′-*O*-tetrahydropyranyladriamycin by drug-sensitive and drug-resistant K562 cells in the different phase of the cycle have been determined. Synchronized cells were obtained by centrifugal elutriation. The kinetics, as well as the amount of drug intercalated inside the nucleus and free in the cytoplasm, have been determined using a spectrofluorometric method that we have developed and that does not compromise cell viability. The kinetics of active efflux of the drug under the effect of P-glycoprotein has been determined. We have calculated that the number of 4′-*O*-tetrahydropyranyladriamycin molecules, which are actively effluxed per cell and per second, is constant whatever the cell cycle phase.

The appearance of cellular resistance to antitumor drugs is a major problem in cancer chemotherapy. The problem is that the tumor cells become resistant not only to the drug which has been used during the treatment but also to other drugs which are structurally and functionally unrelated [1, 2]. This results from expression of the *mdr* 1 gene which encodes the 170 kDa P-glycoprotein or multidrug transporter [3, 4]. It has been shown that the purified protein exhibits ATPase activity [5], suggesting that P-glycoprotein may function as an energy-dependent drug efflux pump [6, 7]. Cells exhibiting the multidrug resistance phenotype can be made more sensitive to the cytotoxic effects of drugs by treatment with calcium channel blockers such as verapamil [8, 9]. The search for agents which can potentiate drug sensitivity at clinically achievable concentrations is a very important point. This requires the determination of the precise role of P-glycoprotein and of cellular factors regulating its activity. In this context we wondered whether P-glycoprotein overexpression and functionality could be associated with any specific phase of the cell cycle.

The present study is devoted to P-glycoprotein functionality i.e. to the determination of the kinetics of active efflux of drug by cells in the different cycle phase. For this purpose we have determined the uptake and release of 4′-*O*-tetrahydropyranyladriamycin (THP-ADR) by drug-sensitive and drug-resistant K562 cells in the different phase of the cell cycle. Viable synchronized cells were obtained using the method of elutriation. This method, which does not use exogenous compounds for synchronization, avoids effects. The kinetics, as well as the amount of drug intercalated inside the nucleus and free in the cytoplasm, have been determined using a spectrofluorometric method that we have developed and that does not compromise cell viability [10–14]. We have thus determined the active efflux of the drug under the effect of P-glycoprotein in the different phase of the cell cycle and shown that the number of THP-ADR molecules, which are actively effluxed per cell and per second, is constant whatever the cell cycle phase.

MATERIALS AND METHODS

Drugs and chemicals. Purified THP-ADR was kindly provided by Laboratoire Roger Bellon (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11{,}500\ M^{-1}\,cm^{-1}$. As anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just prior to use. All other reagents were of the highest quality available, and deionized double-distilled water was used throughout the experiments. Unless otherwise stated buffer solutions were 9.5 mM Hepes buffer (plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, with 5 mM glucose) at pH 7.15. In order to provide better conditions for the cell survival, a pH_e (extracellular pH) lower than 7.4, which is more currently used for this type of experiment, was chosen.

Absorption spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer and fluorescence spectra on a Jobin Yvon, JY3CS spectrofluorometer.

Cell lines and cultures. K562 cells (see the American Type Culture Collection catalogue of cell

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lines and hybridomas, Rockeville, MD, U.S.A.) were a gift of Dr Tapiero (Departement de Pharmacologie Cellulaire, ICIG, 94800 Villejuif, France). They were grown in RPMI medium (Sigma), containing 10% heat-inactivated fetal calf serum (Sigma), 2 mM L-glutamine (Sigma), streptomycin (0.1 mg/mL) and penicillin (100 U/mL) at 37° in a humidifier atmosphere with 5% CO_2. Cultures initiated at a density of 10^5 cells/mL grew exponentially to about 10^6 cells/mL in 3 days. For the assays and in order to have cells in the exponential growth phase, cultures were initated at 5×10^5 cells/mL and the cells were used 24 hr later; they were then at about 8×10^5 cells/mL. Cell viability was assessed by Trypan blue exclusion, and cell counting and cell diameter were determined using a Coulter channelyzer 256.

Synchronization of cell cultures. Cells were separated by centrifugal elutriation carried out with a Beckman JE-6 elutriation rotor using a J2-21 centrifuge. The separation medium was pumped through the system using a Cole Parmer Masterflex pump with a No. 7016-20 pump head. Fractionation of the cell suspension was performed, with some modifications, by the method described by Zhu and Paul [15].

Briefly, 10 mL of Ca^{2+}/Mg^{2+} depleted Hepes buffer containing exponentially growing cells (1.3–1.8×10^7 cells/mL), were gently injected through the afferent flow tube while the rotor was running at 2000 rpm. The cells concentrated in the rotor chamber were submitted to a counterflow of increasing flow rates from 14 to 28, 36, 40.5, 44, 46, 50 and 60.5 mL/min. During elutriation, the centrifuge chamber was maintained at 15°. Elution of the cells was monitored at 280 nm using a continuous flow spectrophotometer UA 5 (ISCO). The cells collected in centrifuge tubes (50 mL) were concentrated by centrifugation at 150 *g* for 10 min at 4°. All the fractions eluting at the same flow rate were pooled.

Determination of cell synchrony. The DNA content of cells from each fraction was determined by FACS (Becton Dickinson) analysis after staining the nuclei with propidium iodide. One mililiter of a staining mixture prepared extemporaneously (0.5 mg of propidium iodide, 10 mg of sodium citrate, 15 μL of 20% Triton X-100, 5 mg of RNAse, diluted in 20 mL of distilled water) was added to the pellet obtained after centrifugation of one aliquot of each fraction containing about 5×10^5 cells. Preparations were kept in the dark at 0° until FACS analysis procedure.

Determination of the initial rate of uptake of THP-ADR by the cells. Anthracycline uptake by the cells was followed using a fluorometric method that we have developed [10–14]. Using this method it is possible (i) to accurately quantify the amount of anthracycline intercalated inside the nucleus at the steady-state and at the equilibrium state and (ii) to follow the kinetics of drug uptake as incubation of cells with the drugs proceeds without compromising cell viability. This method, which takes 5–10 hr less than procedures currently in use [16, 17], is based on the observation that fluorescence of anthracycline is only quenched when intercalated between the base pairs of DNA and that transport across the cell

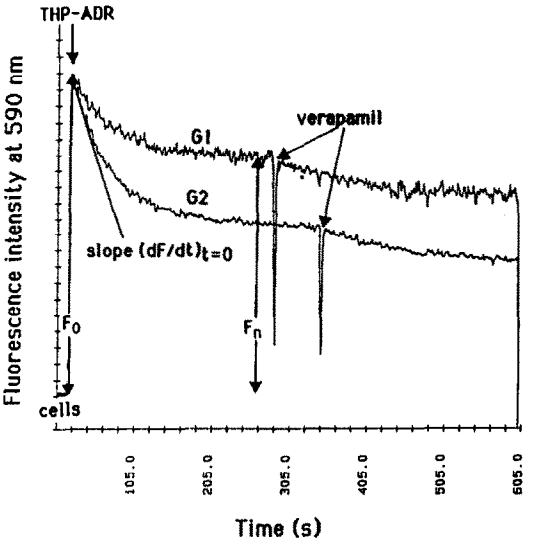


Fig. 1. Uptake of THP-ADR by drug-resistant K562 cells in phase G1 and G2, respectively. *F*, fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. Cells (2×10^6) were suspended in a cuvette filled with 2 mL buffer at pH_e = 7.2 under vigorous stirring. At $t = 0$, 20 μL of a 100-μM stock THP-ADR solution was added to the cells yielding a C_T = 1 μM THP-ADR solution; the fluorescence intensity was then F_0. The slope of the tangent to the curve $F = f(t)$ at $t = 0$ was $(dF/dt)_{t=0}$ and the initial rate of uptake $V_+ = (dF/dt)_{t=0} \cdot (C_T/F_0)$. Once the steady state was reached, the fluorescence was F_n 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 the steady state was reached 10 μM verapamil were added.

membrane is the rate-limiting step [10]. The measurements were done using an excitation wavelength at 480 nm and slit widths of 10, 10, 2 and 2 nm. The fluorescence emission was measured at 590 nm.

All experiments were conducted in 1-cm quartz cuvettes containing 2 mL of buffer at pH_e 7.15. The temperature was maintained at 37° using a circulating thermostated water bath. In a typical experiment, 2×10^6 cells were suspended in 2 mL of glucose containing Hepes buffer under continuous stirring. Stock anthracycline solution (20 μL) were quickly added to this suspension yielding an anthracycline concentration equal to C_T μM. The decrease of fluorescence intensity (*F*) at 590 nm, was followed as a function of time. The initial rate of uptake (V_+) was determined using the following equation:

$$(V_+)_{t=0} = (dF/dt)_{t=0} \cdot C_T/F_0,$$

where $(dF/dt)_{t=0}$ is the slope of the tangent to the curve $F = f(t)$ as shown in Fig. 1, F_0 is the fluorescence intensity at $t = 0$ of a C_T μM anthracycline solution.

It has been previously shown that anthracycline derivatives, which are weakly basic, are transported across bilayers as the deprotonated species

[10, 12, 13, 17, 18]. The initial rate of uptake $(V_+)_{t=0}$ can be written as:

$$(V_+)_{t=0} = P^0_+ . S.n.([D^0]_e)_{t=0}, \quad (1)$$

where P^0_+ is the permeability constant for the neutral form of the drug, S is the membrane exchange area per cell, n is the number of cells in 1 cm^3 and $([D^0]_e)_{t=0}$ the neutral drug concentration at $t = 0$. $[D^0]_e$ is easily calculated using the Henderson–Hasselbach equation and taking into account that the initial drug concentration is 1 μM, the extracellular $pH_e = 7.15$ and the pK_a of deprotonation equal to 7.7 at 37°.

Determination of the amount of THP-ADR intercalated between the base pairs in the nucleus at the steady state and at the equilibrium state. After about 10 min, the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to F_n (Fig. 1). The drug cell system was thus in a steady state and the drug concentration C_n intercalated between the base pairs in the nucleus was $C_n = C_T.(F_0 - F_n)/F_0$. Once the steady state was reached, cell membranes were permeabilized with 0.05% Triton X-100 yielding the equilibrium state which was characterized by a new value of the fluorescence intensity, F_N which depends on the value of pH_e, [11–13]. The concentration C_N of drug intercalated between the base pairs in the nucleus at the equilibrium was $C_N = C_T.(F_0 - F_N)/F_0$.

Under our conditions Triton-X100 was not able to release anthracycline from its intercalative binding site to DNA and the quenching of anthracycline fluorescence, by the interaction of the drug with cells was not due to the formation of non-fluorescent drug metabolites [10].

Determination of the free THP-ADR concentration in the cytosol at the steady state. For this determination the following points have been taken into account: (i) the free drug in the cytosol is in thermodynamic equilibrium with the drug bound to the nucleus, (ii) the binding constant for the drug–nucleus interaction is the same for drug-sensitive and drug-resistant cells [6, 12], (iii) pH_i (intracellular pH) is equal to 7.25 for both drug-sensitive and drug-resistant cells [12].

In the case of drug-sensitive cells, the free drug concentration in the cytosol is easily calculated. At the steady state, the free drug concentration in the buffer outside the cells is:

$$C_e = C_T - C_n \text{ [10, 12]}$$

$$C_e = [DH^+_e]_s + [D^0_e]_s$$

where $[DH^+_e]_s$ and $[D^0_e]_s$ are the concentration of drug in the protonated and neutral form, respectively. Using the Henderson–Hasselbach relation, $[D^0_e]_s$ can be expressed as a function of C_e, the pH_e and the pK_a of deprotonation of the drug:

$$[D^0_e]_s = C_e 10^{pH_e - pK_a}/(1 + 10^{pH_e - pK_a}).$$

At the steady state there is a transmembrane equilibrium of the neutral form of the drug: $[D^0_e]_s = [D^0_i]_s$ where $[D^0_i]_s$ is the free neutral drug concentration in the cytosol,

$$[D^0_i]_s = C_i 10^{pH_i - pK_a}/(1 + 10^{pH_i - pK_a})$$

and then the free drug concentration in the cytosol of drug-sensitive cells can be expressed as

$$C_i = C_e 10^{pH_e - pK_a}(1 + 10^{pH_i - pK_a})/10^{pH_i - pK_a}(1 + 10^{pH_e - pK_a}). \quad (2)$$

The drug–nucleus interaction can be represented by the simple equilibrium:

$$\text{Drug}_{\text{(free in the cytosol)}} + \text{DNA}_{\text{(nucleus)}} \rightarrow \text{Drug}_{\text{(bound to the nucleus)}}$$

with a "stability constant"

$$\text{"}K\text{"} = \mathscr{C}_n/[\text{DNA}].C_i \quad (3)$$

where $[\text{DNA}] = Q/NV$ is the DNA (b.p.) concentration in the nucleus (Q is the total amount of DNA present, V the volume nucleus and N the number of cells in 1 L), $\mathscr{C}_n$ is the local molar concentration of drug bound to the nucleus, $\mathscr{C}_n = C_n/N.V$ (C_n is the overall molar concentration of drug bound to the nucleus).

If Q_1 is the amount of DNA present in phase cells G1, the amount of DNA present in cells in phase G2 will be $2 \times Q_1$ and in phase S a mean value of $1.5 \times Q_1$ can be used. For each cell fraction, the amount of DNA present can be written as $Q = \alpha Q_1$ where $1 < \alpha < 2$ and $\text{"}K\text{"} = C_n/\alpha.N.Q_1.C_i$. Within our experimental conditions, $\mathscr{K} = \text{"}K\text{"}.N.Q_1 = C_n/\alpha$. C_i must be a constant and the same for drug resistant- and drug-sensitive cells. It follows that $\mathscr{K} = (C_n/\alpha C_i)_R = (C_n/\alpha C_i)_S$. We used this relation to determine the free drug concentration in the cytosol of resistant cells.

Determination of the kinetics, V_a, of P-glycoprotein-mediated efflux of THP-ADR at the steady state. At the steady state, whatever the type of cells i.e. either drug-sensitive or drug-resistant, the kinetics of drug influx is equal to that of drug efflux. We have already proposed that for the drug-resistant cells, the efflux should be composed of two terms: a passive efflux of the neutral form of the drug and an active efflux of the drug due to P-glycoprotein [13]. The kinetics are $(V_-)_s$ and $(V_a)_s$, respectively. It follows that:

$$(V_+)_s = (V_-)_s + (V_a)_s$$

where $(V_+)_s$ and $(V_-)_s$ stand for the passive influx and efflux of the neutral form of the drug at the steady state, respectively, and $(V_a)_s$ for the active efflux at the steady state. In the following we will make the reasonable assumptions: (i) that the permeability coefficients are the same for the passive influx and efflux: $P^0_+ = P^0_-$, (ii) that these values are the same at $t = 0$ and at the steady state. It follows that,

$$(V_a)_s = P^0_+ . S.n.([D^0]_e - [D^0]_i)_s. \quad (4)$$

Using Eqn (1), $(V_a)_s$ can be written as:

$$(V_a)_s = (V_+)_{t=0}([D^0]_e - [D^0]_i)_s/([D^0]_e)_{t=0}. \quad (5)$$

Direct determination of the P-glycoprotein-mediated efflux of THP-ADR. Cells (10^6 per mL) were incubated for 30 min in the presence of 10 mM N_3^- and in the absence of glucose. In these conditions, there was no ATP synthesis and the P-glycoprotein mediated active efflux of THP-ADR was blocked

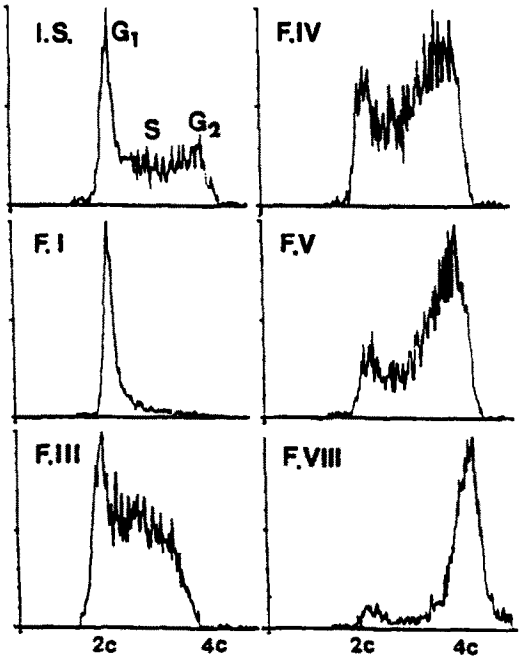
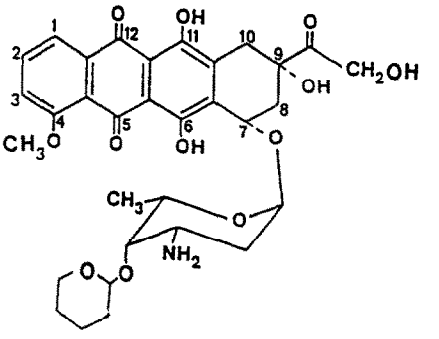


Fig. 2. Flow cytometric analysis of selected fractions after centrifugal elutriation of K562 cells. Abscissa; DNA content; ordinate: relative cell number.



THP-Adriamycin

Scheme 1.

[19]. The incorporation of THP-ADR in these energy-depleted resistant cells thus compared to that observed in sensitive cells. At the steady state the fluorescence signal was F'_n and the concentration of drug bound to the nucleus $C'_n = C_T(F_0 - F'_n)/F_0$. The subsequent addition of 5 mM glucose gave rise to the ATP synthesis via the glycolysis pathway [20] and, after about 30 sec, one observed an increase of the fluorescence signal due to the release of drug from the cells. At the new steady state, the fluorescence intensity was F_n. At the equilibrium state, after the addition of Triton X-100, the fluorescence intensity was F_N. The rate of active efflux V_a was determined as $V_a = (dF/dt)_{glu} \cdot C_T/F_0$, where $(dF/dt)_{glu}$ was the slope of the tangent to the curve $F = f(t)$ at the time corresponding to the glucose addition.

RESULTS

Fractionation of K562 cells

During the loading of the separation chamber, performed at the flow rate of 14 mL/min, a preliminary fraction containing most of the cellular debris and damaged cells was collected. Eight subpopulations (Fractions I–VIII) were isolated after increasing the flow rate from 28 to 60.5 mL/min. For each fraction, cell volume and DNA content was estimated using a Coulter counter and a flow cytometer, respectively.

The flow cytometric analysis of the successive elutriated subpopulations, obtained after fractionation of the resistant K562 cell population, is illustrated in Fig. 2. The first fraction elutriated at 28 mL/min, is composed of a fairly pure G1 population presenting a mean diameter value of 13.4 ± 0.3 μm. Fractions II–IV show a preferential enrichment of cells in S phase. From fraction V, a progressive increase of percentage of cells in G2 is observed until a value of about 80% obtained in fraction VIII. This last fraction corresponds to the pool of the first four tubes collected with the flow rate 60.5 mL/min. The mean value of the G2 cell diameter is estimated to be 16.8 ± 0.3 μm.

Comparison of the uptake of THP-ADR by drug-resistant cells in the G1 and G2 phases, respectively

The structure of THP-ADR used in the present study is shown in Scheme 1. The visible absorption spectrum exhibits a maximum absorbance at 480 nm. The fluorescence spectra are obtained by excitation at 480 nm.

The kinetics of uptake of THP-ADR by G1 and G2 phase cells, respectively, was recorded in the following conditions: a constant initial concentration of drug equal to 1 μM was added to 10^6 cells/mL suspended in a glucose containing Hepes buffer at $pH_e = 7.15$ and 37°. Figure 1 shows the records of two typical experiments. Once the steady state was reached, 10 μM verapamil were added. After some minutes a new steady state was reached. As can be seen, the initial rate of uptake (V_+) of THP-ADR by G2 phase cells, is about twice that of cells in phase G1. The overall concentration of drug (C_n) intercalated between the base pairs of DNA at the steady state is also twice as important in G2 compared to G1. The subsequent addition of 0.05% Triton X-100 yielded the equilibrium state [10].

Comparison of the uptake of THP-ADR by drug-resistant and drug-sensitive G1 phase cells

Figure 3 shows the uptake of THP-ADR by drug-sensitive and drug-resistant cells in phase G1. As can be seen, the initial rates of uptake are, within the limit of experimental errors, the same for both types of cells. However, whereas in the case of drug-sensitive cells the fluorescence intensity at 590 nm decreased smoothly as a function of time to reach a steady state, in the case of drug-resistant cells, the

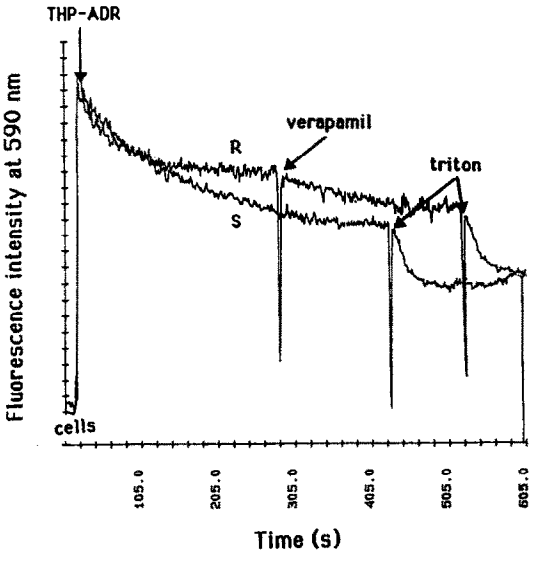


Fig. 3. Uptake of THP-ADR by drug-resistant (R) and drug-sensitive (S) K562 cells in phase G1. F, fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. The experimental conditions are the same as in Fig. 1. Once the steady state was reached, 10 μM verapamil were added to drug-resistant cells and 5 μL 20% Triton X-100 to drug-sensitive and drug-resistant cells.

decrease of fluorescence intensity appeared to be biphasic with a sudden stop of the uptake of the drug. As it is well known, at the steady state, the amount of drug in the nucleus is lower for drug-resistant cells than for drug-sensitive cells. The subsequent addition of 10 μM verapamil to drug-resistant cells gave rise immediately to an increase of the uptake, and a new steady state was reached in which the amount of drug incorporated into the nucleus was close to that obtained for sensitive cells. After the addition of Triton, the same amount of drug was incorporated inside the nucleus of both sensitive and resistant cells.

Uptake of THP-ADR by drug-sensitive and drug-resistant cells as a function of phase

The uptake of the THP-ADR was measured for the different elutriated fractions. For each fraction, the following parameters were determined: (i) the percentage of cells in phase G1, S and G2, respectively, (ii) the mean radius of cells, (iii) the initial rate of uptake $(V_+)_{t=0}$, (iv) the overall molar concentration C_n of drug bound to the nucleus at the steady state, (v) the overall molar concentration C_n^v of drug bound to the nucleus after addition of 10 μM verapamil, (vi) the overall molar concentration C_N of drug bound to the nucleus at the equilibrium state i.e. after permeabilization of the plasmic membrane by addition of Triton X-100.

The data of two typical experiments performed with drug-sensitive and drug-resistant cells are shown in Tables 1 and 2 and Fig. 4. We can already notice that, in drug-resistant cells, the C_n value in G2 phase is twice that in G1 phase. This is not the case for drug-sensitive cells where the C_n value is multiplied by a factor of 1.4 only.

Free drug concentration C_i in the cytosol of sensitive and resistant cells as a function of phase

The free drug concentration in the cytosol of drug-sensitive cells was determined as indicated in Materials and Methods. The values obtained for the different fractions are reported in Table 1. In order to determine C_i in the cytosol of drug resistant cells, we have first determined the amount Q of DNA present in each fraction and then $\alpha = Q/Q_1$ where Q_1 is the amount of DNA present in phase cells G1. The α value calculated for each fraction of drug-sensitive cells are reported in Table 1 together with the value of $\mathcal{K} = C_n/\alpha.C_i$. As was expected, the $\mathcal{K}$ value did not depend on the phase and a mean value of 1.0 ± 0.1 was obtained (Table 1). C_n and α were also measured for the drug-resistant cells and C_i was calculated using the relation $C_i = \mathcal{K}.\alpha/C_n$ (Table 2).

Passive efflux of THP-ADR by drug-sensitive cells at the steady state

At the steady state, the kinetics of passive efflux of THP-ADR are $(V_-)_s = (V_+)_{t=0}$ $([D^0]_i)_s/$

Table 1. Uptake of THP-ADR by drug-sensitive K562 cells in different phases of cell cycle

Fraction	% Cells in phase G_1	S	G_2	α	$(V_+)_{t=0}$ (nM/sec)	C_n (μM)	C_i (μM)	$\mathcal{K}$
I	86	14	0	1.07	3.9	0.44	0.47	0.88
II	49	51	0	1.25	4.8	0.51	0.41	0.99
III	32	68	0	1.34	6.4	0.50	0.42	0.89
IV	15	47	38	1.61	7.9	0.59	0.34	1.06
V	10	36	54	1.72	8.6	0.57	0.36	0.92
VI	13	35	52	1.69				
VII	7	21	72	1.82	10.6	0.63	0.31	1.11
VIII	25	24	51					

α: coefficient characteristic of the amount of DNA present in cell nucleus.
$(V_+)_{t=0}$: initial rate of uptake at $t = 0$ (±10%).
C_n: overall molar concentration of drug bound to the nucleus at the steady state.
C_i: molar concentration of free drug in the cytoplasm.
$\mathcal{K}$: $C_n/\alpha.C_i$.

Table 2. Uptake of THP-ADR by drug-resistant K562 cells in different phases of cell-cycle

Fraction	% Cells in phase G_1	S	G_2	α	ϕ μM	$(V_+)_{t=0}$ nM/sec	C_n (μM)	C_n^v (μM)	C_i (μM)	$(V_a)_s$ (nM/sec)	$a \times 10^{-6}$
I	79	21	0	1.10	13.4	3.5	0.23	0.38	0.21	1.81	1.1
II	47	53	0	1.26	14	3.4	0.25	0.44	0.20	1.74	1.0
III	34	63	3	1.36	14.6	4.2	0.27	0.44	0.20	2.02	1.2
IV	20	50	30	1.55	15.2	4.7	0.30	0.46	0.19	2.20	1.3
V	11	40	49	1.69	15.8	5.7	0.42	0.55	0.25	2.17	1.3
VI	15	29	56	1.70	16.4						
VII	9	30	63	1.80	16.6	7.5	0.48	0.59	0.27	1.45	0.9
VIII	8	15	77	1.84	16.8	6.7	0.45	0.59	0.24	1.75	1.0

α: coefficient characteristic of the amount of DNA present in cell nucleus.
ϕ: mean cell radius ($\pm 0.3\ \mu$m).
$(V_+)_{t=0}$: initial rate of uptake at $t = 0$ ($\pm 10\%$).
C_n and C_n^v: overall molar concentration of drug in the nucleus at the steady state in the absence and presence of 10 μM verapamil, respectively ($\pm 5\%$).
C_i: molar concentration of free drug in the cytoplasm of the steady state.
$(V_a)_s$: kinetics of active efflux.
a: number of molecules actively efflux per cell and per second.

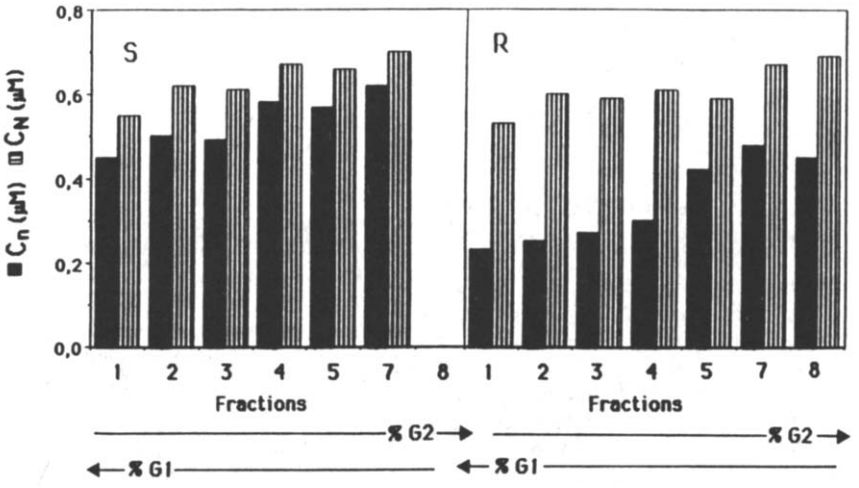


Fig. 4. Overall molar concentration of drug inside the nucleus in the different cell fractions obtained after elutriation. C_n (■) and C_N (▨) are the overall molar concentration of drug at the steady state and equilibrium state (after the addition of 0.05% Triton X-100), respectively, when 10^6 cells/mL of either drug-sensitive (left) or drug-resistant (right) are incubated wtih 1 μM THP-ADR, at pH_e 7.15 and 37°. The proportion of cells in phase G1 decreases from Fraction I to VIII whereas, the proportion of cells in phase G2 increases from Fraction I to VIII.

$([D^0]_e)_{t=0}$. Mean values of 2.2 ± 0.1 and 3.7 ± 0.2 nM/sec were determined for cells in phase G1 and G2, respectively. This means that the number of molecules which are passively effluxed per cell and per second is 1.8 higher in G2 phase than in G1. As the membrane exchange area per cell is only 1.4 higher in G2 than in G1, this data suggests that the permeability constant for the neutral form of the drug is slightly higher in G2 than in G1.

Kinetics of P-glycoprotein-mediated efflux of THP-ADR in the different phase

The kinetics of P-glycoprotein-mediated efflux were determined by two different methods: (i) at the steady state, (ii) directly after the addition of glucose to cells which were previously incubated with 1 μM THP-ADR in the presence of N_3^- and absence of glucose. We have first checked that there was a good correlation between the data obtained by both methods. However, the method dealing with the steady state was preferred because it cannot give rise to metabolic perturbation. The data of a typical experiment in which V_a has been determined at the steady state are shown in Table 2.

DISCUSSION

Multidrug-resistant cells are characterized by

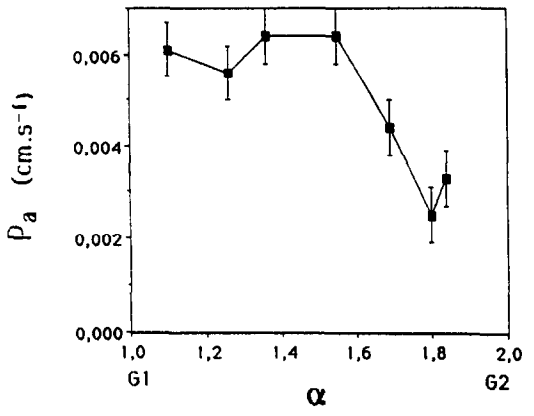


Fig. 5. Variation of the permeability coefficient for the active efflux of THP-ADR as a function of cell phase. $P_a = (V_a)_s/n.C_i.S$, $(V_a)_s$: kinetics of active efflux at the steady state; n: cell number/mL; C_i: free drug concentration in the cytoplasm at the steady state; S: membrane exchange area per cell; α: coefficient characteristic of the amount of DNA present in cell nucleus. Results are the mean of two experiments ± SD.

impaired drug accumulation associated with an increase expression of a 170-kDa glycoprotein termed P-glycoprotein, which removes various lipophilic drugs from the cells in an ATP-dependent manner [2, 20–24]. Despite the amount of work which has appeared on that subject, the mechanism of outward transport of drug by P-glycoprotein is still poorly understood. In this study, we have compared the active outward transport of an anthracycline derivative, THP-ADR, under the effect of P-glycoprotein, in the different phases of the drug-resistant cell cycle. For this purpose, centrifugal elutriation, that allows the isolation of cell subpopulation according to specific cell cycle phases in a short time and with minimal metabolic perturbation, was used [25]. Different studies dealing with K562 cells have already appeared that used this fractionation method [15, 26, 27]. THP-ADR (Scheme 1) has a relatively low pK_a (7.7) of deprotonation [11] and consequently, enters the cell very rapidly [13]. Its uptake and release by cells can be easily followed using the spectrofluorometric method that we have developed [10–14]. We have assessed efflux indirectly by measuring the kinetics of uptake of drug at the initial time and at the steady state and by calculating the intracellular concentration of free drug, taking into account that the major binding site for anthracycline inside the cell is the nucleus. The active efflux was also determined directly after the preliminary incubation of THP-ADR with energy-depleted cells and the subsequent addition of glucose to yield ATP synthesis and reactivation of P-glycoprotein. Both methods gave similar results.

As can be seen in Table 2, the value of $(V_a)_s$ calculated for the different fractions is nearly constant whatever the cycle phase. This data can also be expressed as the number of molecules of THP-ADR which are actively effluxed per cell and per second. A constant value equal to $(1.1 \pm 0.2) \times 10^6$ molecules/cell/sec is found.

By analogy with the expression of the kinetics of passive diffusion equation [1], the kinetics of active efflux at the steady state can be expressed as:

$$(V_a)_s = P_a.S.n.C_i \quad (6)$$

where P_a is the permeability coefficient for the active efflux of THP-ADR, S the membrane exchange area is taken equal to the total plasmic area. As can be seen in Fig. 5, where P_a has been plotted as a function of α, the value of P_a for G2 phase cells is only 50% of the value of P_a for cells in phase G1.

These data show that the amount of P-glycoprotein, which is functionally active at the beginning of cell cycle and pumped out THP-ADR, is not modified during cell cycle. To account for this observation the following mechanisms can be evoked: (i) like the vast majority of the many different proteins and RNA molecules present in a cell, P-glycoprotein is synthesized continuously throughout interphase but the newly synthesized P-glycoprotein is not functional before mitose, (ii) P-glycoprotein is synthesized late in G2. Experiments have been undertaken to quantify the amount of P-glycoprotein present in the different phases.

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REFERENCES

1. Fojo AT, Whang-Peng J, Gottesman MM and Pastan I, Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells. *Proc Natl Acad Sci USA* **82**: 7661–7665, 1985.
2. Bradley G, Juranka PF, and Ling V, Mechanisms of multidrug resistance. *Biochim Biophys Acta* **948**: 87–128, 1988.
3. Riordan JR, Deuchars K, Katner N, Alon N, Trent J and Ling V, Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* **316**: 817–819, 1985.
4. Roninson IB, Chin JE, Choi K, Gros P, Housman DE, Fojo A, Shen D-W, Gottesman MM and Pastan I, Isolation of human *mdr* DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc Natl Acad Sci* **83**: 4538–4542, 1986.
5. Hamada H and Tsuruo T, Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance. 170- to 180-kilodalton glycoprotein is an ATPase. *J Biol Chem* **263**: 1454–1458, 1988.
6. Skovsgaard T, Mechanism of resistance to daunorubicin in Ehrlich ascites tumor cells. *Cancer Res* **3**: 1785–1791, 1978.
7. Inaba H and Johnson RK, Uptake and retention of adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia *Biochem Pharmacol* **27**: 2123–2130, 1978.
8. Tsuruo T, Iida H, Tsukagoshi S and Sakurai Y, Potentiation of vincristine and adriamycin effects in human hemopoietic tumor cell lines by calcium antagonists and calmodulin inhibitors. *Cancer Res* **43**: 2267–2272, 1983.
9. Kessel D and Wilberding C, Mode of action of calcium

antagonists which alter anthracycline resistance. *Biochem Pharmacol* **33**: 1157–1160, 1984.
10. Tarasiuk J, Frezard F, Garnier-Suillerot A and Gattegno L, Anthracycline incorporation in human lymphocytes. Kinetics of uptake and nuclear concentration. *Biochim Biophys Acta* **1013**: 109–117, 1989.
11. Frezard F and Garnier-Suillerot A, Comparison of the binding of anthracycline derivatives to purified DNA and to cell nuclei. *Biochim Biophys Acta* **1036**: 121–127, 1990.
12. Frezard F and Garnier-Suillerot A, Determination of the osmotic active drug concentration in the cytoplasm of anthracycline-resistant and -sensitive K562 cells. *Biochim Biophys Acta* **1091**: 29–35, 1991.
13. Frezard F and Garnier-Suillerot A, Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **196**: 483–491, 1991.
14. Tarasiuk J and Garnier-Suillerot A, Kinetic parameters for the uptake of anthracycline by drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **204**: 693–698, 1992.
15. Zhu JD and Paul J, Chomatin structure of genes during the cell cycle. *Biosci Rep* **5**: 401–406, 1985.
16. Burke TG, Morin MJ, Sartorelli AC, Lane PE and Tritton TR, Function of the anthracycline amino group in cellular transport and cytotoxicity. *Mol Pharmacol* **31**: 552–556, 1987.
17. Skovgaard T and Nissen NI, Membrane transport of anthracyclines. *Pharmacol Ther* **18**: 293–311, 1982.
18. Dalmark M, Characteristics of doxorubicin transport in human red blood cells. *Scand J Clin Lab Invest* **41**: 633–639, 1981.
19. Beck WT, Cellular pharmacology of vinca alkaloid resistance and its circumvention *Adv Enzyme Regul* **22**: 207–227, 1984.
20. Beck WT, Cirtain MC and Lefko JL, Energy-dependent reduced drug binding as a mechanism of vinca alkaloid resistance in human leukemic lymphoblasts. *Mol Pharmacol* **24**: 485–492, 1983.
21. Roninson IB, *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*. Plenum, New York, 1991.
22. Beck WT, The cell biology of multidrug resistance. *Biochem Pharmacol* **36**: 2879–2887, 1987.
23. Pastan I and Gottesman MM, Multiple-drug resistance in human cancer. *N Engl J Med* **316**: 1388–1393, 1987.
24. Moscow JA and Cowan KH, Multidrug resistance. *J Natl Cancer Inst* **80**: 14–20, 1988.
25. Kauffman MG, Noga SJ, Kelly TJ and Donnenberg AD, Isolation of cell cycle fractions by counterflow centrifugal elutriation. *Anal Biochem* **191**: 41–46, 1990.
26. Hann SR, Thompson CB and Eisenman RN, *c-myc* oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature* **314**: 366–369, 1985.
27. Iqbal MA, Plumb M, Stein J, Stein G and Schildkraut CL, Coordinate replication of members of the multigene family of core and H1 human histone genes. *Proc Natl Acad Sci USA* **81**: 7723–7727, 1984.