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Rapid kinetics of the interaction between daunomycin and drug-sensitive or drug-resistant P388 leukemia cells

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The initial stages of the interaction of daunomycin (DNM) with drug-sensitive (P388/S) and drug-resistant (P388/100) cells have been defined by a rapid kinetics stopped-flow procedure. The process can be described by two kinetic components. The faster component accounts for rapid occupation of cell surface sites by DNM, as supported by experiments with liposomes with different surface charge. On the other hand, the effect of verapamil in the assays, suggests that the slower component is involved in the transport of the drug into the cells. Our observations are consistent with a loss in the control of the passive permeability to the drugs in the drug-resistant tumor cells.

Rapid kinetics; Anthracycline resistance; P388 cell

1. INTRODUCTION

Reduced intracellular drug accumulation in drug-resistant tumor cells with respect to their parental drug-sensitive counterparts constitutes a hallmark of multi-drug resistance (MDR) [1–2]. Thus, alterations in drug transport through the cell membrane are expected to be the basis of the mechanisms of resistance to the anti-neoplastics [3]. In this regard, the overexpression of P-glycoproteins in drug-resistant cells acting as drug efflux pumps represents a well-supported mechanism to reduce intracellular drug accumulation in MDR cells [4]. However, recent reports demonstrate the existence of drug-resistant cell lines which do not overexpress P-glycoproteins [5,6]. Moreover, in some instances the level of expression of P-glycoproteins does not correlate with alterations in drug accumulation [7,8]. In murine leukemia P388 cells, differences in drug transport and efflux between drug-sensitive and drug-resistant cells can not be explained exclusively on the basis of the pump activity of P-glycoproteins [9]. These premises support the notion that, in addition to drug efflux mediated by P-glycoproteins, other membrane transport alterations should be invoked to explain the differences in drug accumulation between drug-sensitive and drug-resistant cells.

Transport of the anti-cancer anthracyclines in tumor cells has been difficult to evaluate because of the rapidity of the process and the enormous extent of intracellular binding of the drugs [10]. Hence, resistance due to decreased intracellular binding might be difficult to distinguish from resistance due to decreased drug transport. In the present study, a rapid kinetics procedure has been applied to define the initial steps in the interaction of the anthracycline DNM with murine leukemia P388 cells, as well as to provide a quantitative estimation of the possible kinetic differences of such interactions between daunomycin (DNM)-sensitive and DNM-resistant P388 cells.

2. MATERIALS AND METHODS

DNM-sensitive P388 murine leukemia cells (P388/S) and a 90–100-fold resistant, stable sub-line (P388/100) were grown and maintained as described in [11].

Fast kinetics measurements for the initial interaction of DNM with the P388/S and P388/100 cells have been made using a HiTech stopped-flow spectrophotometer (model SF-51) set up in the fluorescence mode and operating with a 150-W xenon lamp. The process was initiated by rapidly mixing 0.1 ml of a suspension of 2.10^6 cells/ml in 10 mM HEPES buffer, pH 7.2, containing 130 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBS solution), with an equal volume of 10 μM DNM in the same solution. Experiments in the presence of verapamil (VRP) were done by incubating the cell suspensions with 5 μM of the drug for 2 h prior to the acquisition of the kinetic traces. The samples were excited at 478 nm and fluorescence emission was monitored by using a glass OG-530 cutoff filter (50% transmission at 525 nm). Visual monitoring and storage of the data were obtained by using an interfaced Apple IIe computer. A total of 200 points were collected for each trace and fitted by a nonlinear regression method using the algorithm of Marquardt to equations containing a single exponential term:

Abbreviations: DNM, daunomycin; MDR, cellular multi-drug resistance; P388/S, daunomycin-sensitive P388 cells; P388/100, 100-fold daunomycin-resistant P388 cells; VRP, verapamil; PCL, positively charged liposomes; NCL, negatively charged liposomes.

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$$F(t) = A_1\,[\exp(-k_1 \cdot t)] + C \quad (1)$$

or the sum of two exponentials terms:

$$F(t) = A_1\,[\exp(-k_1 \cdot t)] + A_2\,[\exp(-k_2 \cdot t)] + C \quad (2)$$

where $F(t)$ is the fluorescence at time t, C is the equilibrium fluorescence level, A_1, A_2 and k_1, k_2, represent the amplitudes and the apparent rate constants, respectively, associated with each of the terms expressed in equations **1** or **2**. Cell viability at the end of the experiments was similar to that of the starting samples (>95%).

Long-term uptake of DNM by the P388 cells was carried out as described in [12]. Briefly, cell suspensions (10^6 cells/ml) in HBS were incubated with 3 μM solutions of DNM, in the absence or in the presence of 5 μM VRP at 25°C. In the latter, the cells were in the presence of VRP for 2 h before the addition of DNM. Upon 90 min of incubation, aliquots were removed and analyzed by flow cytometry in an Epics Profile I instrument equipped with a 0.75 W argon laser, set at 488 nm. The concentration of drug associated to each cell is proportional to the mean fluorescence intensity (mean fluorescence channel) determined in the histograms. A total of 10,000 cells were measured during each sample analysis. Cell viability remains unaffected during the assays (>95%).

Positively (PCL)-, neutral- and negatively (NCL) charged liposomes were from Avanti Polar Lipids. Neutral liposomes contain egg phosphatidylcholine (63 μmol) and cholesterol (9 μmol). The composition of PCL and NCL liposomes was the same as that of neutral liposomes with, in addition, dicetyl phosphate (18 μmol) or stearylamine (18 μmol), respectively. The preparation of these liposomes has been previously described [13].

3. RESULTS

The kinetics of the initial stages for the rapid interaction between the lipophilic anthracycline, DNM, and murine leukemia P388 cells has been determined by stopped-flow techniques under conditions which preserve cell viability (>95%). The assay is based upon the change in the fluorescence quantum yield when the drug incorporates into hydrophobic environments [14], such as those provided by the lipid bilayer of the cell membranes. This allows for a direct, continuous and rapid monitoring of the early steps of drug association to the cells, restricted only by the time required to obtain an adequate mixing between cells and DNM (~0.1 s).

Fig. 1A shows the continuous recording of the fluorescence decays corresponding to the initial interaction of 5 μM DNM with the P388/S and P388/100 cells. The traces reveal that following a rapid decay during the first few seconds upon mixing, there is a slower decrease in the fluorescence signal with time. Computer fitting of the experimental traces to exponential equations containing one or more exponential terms, indicates that the interaction of DNM with the P388 cells can be satisfactorily described by the sum of two exponential terms (solid lines in Fig. 1A represent fits of the traces to equation **2** in Materials and Methods). In fact, addition of a third exponential term in equation **2**, results in neither improvement of the fit nor better statistical determinations, giving nonsensical values of the amplitudes, and was therefore unwarranted (not shown). The kinetic parameters obtained upon fitting the traces to equation **2** are summarized in Table I. It is observed that the two kinetic components contribute significantly to the overall process (comparable amplitude values, A_1 and A_2, in Table I) and that the apparent rate constant k_1 associated to the faster step is ~40–45% more rapid in the P388/100 than in the P388/S cells. On the other hand, similar k_2 values corresponding to the apparent rate constant of the slower component, were obtained for both cell lines (Table I). The two components in the double exponential process can be visualized separately by computer simulation (based on the kinetic parameters obtained upon fitting the experimental recordings to equation **2**), allowing for an easier comparison of the individual steps involved in the interaction of DNM with the P388/S and P388/100 cells (Fig. 1B and C, respectively).

The observed total amplitude of the fluorescence

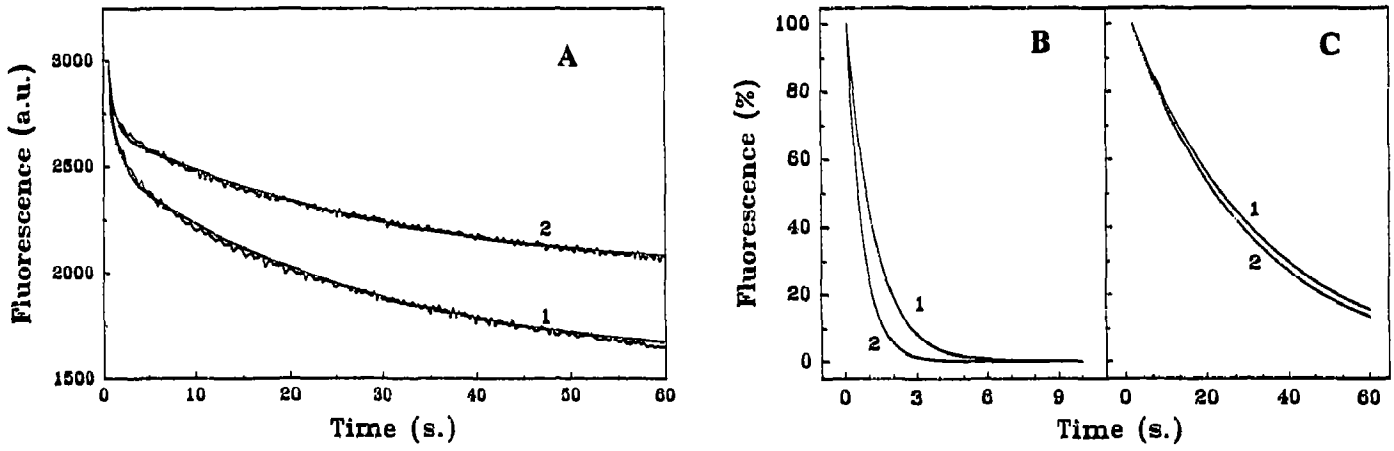

Fig. 1. Stopped-flow recordings corresponding to the fluorescence decay of DNM upon rapid mixing with the P388 cells. Final concentration of DNM was 5 μM; final density of cells was 1.10^6 cells/ml. (A) Comparison of the kinetics of the initial interaction between DNM and P388/S (1) and P388/100 (2) cells. Fits to equation **2** in Materials and Methods are represented by solid lines. (B and C) Individualized computer simulation analysis of the two terms of equation **2**, fast (B) and slow (C), corresponding to the initial interaction of DNM with the P388/S (1) and P388/100 (2) cells.

Table I

Kinetic parameters corresponding to the initial steps in the interaction of DNM with the P388 cells obtained upon fitting the experimental traces to equation 2 given in Materials and Methods

Parameter*	P388/S	P388/100
No VRP		
A_1	868 ± 52	741 ± 64
k_1	0.856 ± 0.235	1.510 ± 0.297
A_2	969 ± 6	695 ± 5
k_2	0.031 ± 0.004	0.034 ± 0.001
C	1181 ± 8	1962 ± 7
VRP		
A_1	856 ± 101	1121 ± 71
k_1	1.283 ± 0.016	1.299 ± 0.024
A_2	820 ± 8	483 ± 6
k_2	0.037 ± 0.001	0.055 ± 0.001
C	927 ± 11	1362 ± 4

*Determination of the kinetic parameters in the absence (No VRP) and in the presence (VRP) of 5 μM VRP, respectively.
The values corresponding to the amplitudes (A_1 and A_2) and to the apparent rate constants (k_1 and k_2), are given as fluorescence arbitrary units and s^{-1} ± S.D., respectively. (n=6).

change seems to be greater in the P388/S cells than in the P388/100 cells. This is reflected in term C of equation 2, which represents the extrapolated equilibrium fluorescence intensity of the reacting system (cells + DNM), and which predicts that the association of DNM to the P388/S should be greater than that to the P388/100 cells. Such an expectation is in qualitative agreement with the results obtained in long-term uptake experiments of DNM by the P388 cells using laser flow cytometry under steady-state conditions (Fig. 2A).

It seems reasonable to assume that the faster component in the interaction of DNM with the cells should involve cell surface interactions. To explore this possibility, we have analyzed the influence of different surface environments on the kinetics of the association of DNM to liposomes having either positive (PCL), neutral, or negative surface charge (NCL). These experiments were done at pH 7.2, at which most of the drug is present as a cationic form (pK_a 7.6–8.2 [10]). Fig. 3 illustrates the kinetic differences in the interaction between DNM and the three types of liposomes. It can be observed that the apparent rate constant for the faster component follows the sequence: PCL>neutral>NCL. This observation seems consistent with the fact that P388/100 cells have a more cationic surface environment than the P388/S cells [11], thus suggesting that the surface charge may play an important role in determining the rate at which interaction between DNM and cells occurs.

Solubilization of DNM into the lipid bilayer of the cell membranes following drug adsorption onto the cell surface is considered the mechanism by which anthracyclines enter tumor cells, i.e. by passive diffusion [10,15]. On this basis, we have tentatively ascribed the slower step of the interaction of DNM with the cells to a component involved in the passive permeability to the drug. The simplest experimental design to measure the rate of the diffusion of DNM through the P388 cells implies initial conditions in which the drug is present only in the extracellular medium. In this regard, an added advantage of using stopped-flow techniques is that, at the time scale used in the experiments, the extracellular concentration of DNM should be sufficiently high with respect to that inside the cells as to maintain a steep gradient that would favor unidirectional diffusion of the drug into the cells, thus making processes such as dissociation of DNM from cellular targets or drug efflux by passive diffusion negligible. Nevertheless, even at low concentrations of intracellular drug, the occurrence of active drug efflux mediated by resistance-related P-glycoproteins, which are overexpressed in the P388/100 cells [16], could mask the correct determination of the rate of diffusion of DNM into these cells. To avoid this

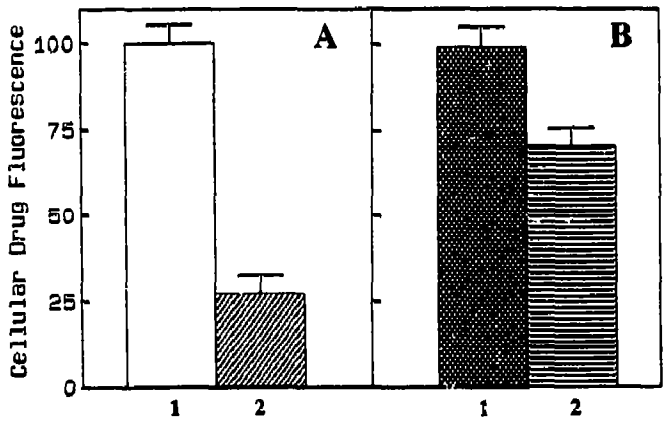

Fig. 2. Long-term uptake of DNM by the P388/S cells (1) and P388/100 cells (2), in the absence (A) or in the presence (B) of 5 μM VRP. The Y-axis indicates the normalized fluorescence of DNM (mean fluorescence channel in the histograms) associated with each cell population. 100% refers to the fluorescence of DNM in the P388/S cells in the absence of VRP. Bars ± S.D.

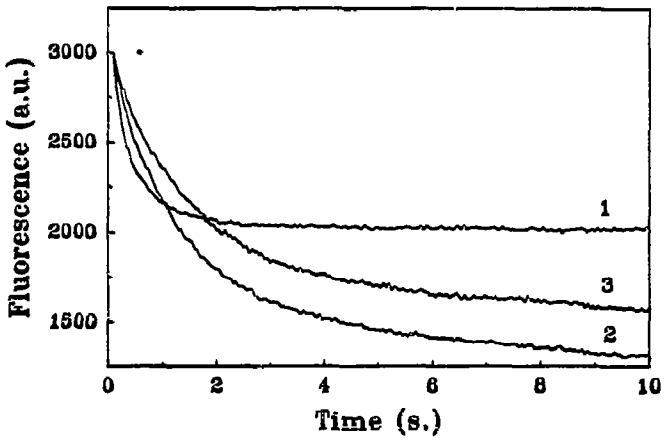

Fig. 3. Kinetics of the interaction between DNM and artificial lipid vesicles with different surface charge: (1) PCL; (2) neutral; (3) NCL liposomes; at pH 7.2 and 25°C.

possibility, the above stopped-flow experiments were also done in the presence of VRP, a calcium channel blocker able to prevent the active efflux of drugs by P-glycoproteins [17]. Control experiments indicated that VRP at the concentration of 5 μM used in the assays does not perturb the fluorescence properties of DNM (not shown). Fig. 4A shows the interaction of DNM with the P388/S and the P388/100 cells in the presence of VRP, a process that, similarly to what it has been previously observed in the absence of VRP, can be also described by the sum of two exponential terms such as those in equation 2. Under these conditions, the estimated values for the apparent rate constant of the fast component, k_1, are similar in the P388/S and P388/100 cells (Table I). Although we have no explanation for a direct effect of VRP on the adsorption of DNM to the cells, it has been described that VRP may alter some of the properties associated to the cell surface, such as the membrane potential [18]. Furthermore, the presence of VRP consistently increases the apparent rate constant of the slow step, k_2, in the two cell lines. This effect, however, is much more pronounced in the P388/100 than in the P388/S cells (Table I), suggesting that active drug efflux mediated by P-glycoproteins is indeed causing an underestimation in the determination of the rate at which the slower component, defined by k_2, occurs. As above, the differences in the interaction between DNM and P388/S or P388/100 cells in the presence of VRP are better visualized when the two phases are individually analyzed by computer (Fig. 4B and C), respectively.

The effect of VRP on the equilibrium fluorescence intensity (C values) is also indicated in Table I. It is observed that while VRP slightly modifies the predicted amount of DNM associated to the P388/S obtained upon extrapolation, it substantially increases the extent of drug association to the P388/100 cells, a finding that should be expected from a resistance-reverting agent [17]. Moreover, long-term uptake assays of DNM by the P388 cells in the presence of VRP by laser flow cytometry confirm, as stated by others [19], that accumulation of DNM increases in the P388/100 but remains practically unaltered in the P388/S cells (Fig. 2B).

4. DISCUSSION

In the present report we have analyzed the initial stages of the interaction of DNM with leukemia P388 cells in an attempt to illustrate whether acquisition of resistance to anthracyclines by these cells is accompanied by alterations in the permeability to the drugs. This has been done by rapid kinetics which, while minimizing the extent of intracellular binding of DNM in the timescale used in the experiments, allow the determination of unidirectional passive diffusion of the drug into the cells.

In spite of using a very simple experimental design, our results reveal that the interaction between DNM and the P388 cells is complex and needs to be described by at least two kinetic components referred in the text as fast and slow, respectively. The fast component seems to involve rapid adsorption of DNM to the cell surface, a suggested common event shared by several anticancer drugs, including *Vinca* alkaloids and colchicine [2,20] anthracyclines [21], etc., and presumably due to complex formation between phospholipids and the sugar residues of the drugs [22]. The differences observed in the faster component between P388/S and P388/100 cells are consistent with the different surface environment previously reported in isolated plasma membrane fragments from both types of cells [11], as well as with reports describing alterations in the membrane potential, which is usually decreased in MDR cells [23]. In fact, VRP, which is known to restore the

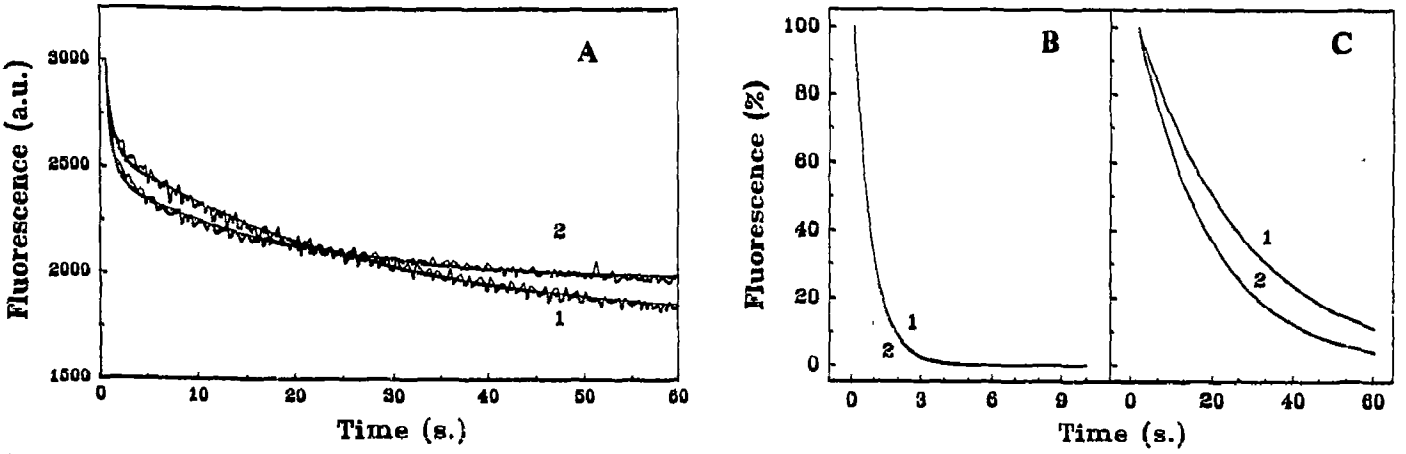

Fig. 4. Stopped-flow recordings describing the initial interaction of DNM with the P388 cells in the presence of 5 μM VRP. Final conditions for DNM and cell suspensions were as in Fig. 1. (A) DNM fluorescence decay upon rapid interaction with the P388/S (1) and P388/100 (2) cells. Solid lines represent the fits of the experimental traces to equation 2. (B and C) Individualized computer analysis of the two terms, fast (B) and slow (C), corresponding to the interaction of DNM with the P388/S (1) and P388/100 (2) cells.

membrane potential of anthracycline-resistant P388 cells [18], abolishes the differences estimated in the rapid component between the P388/S and P388/100 cells.

Nevertheless, the most intringuing finding arises from the substantial increase observed in the apparent rate constant of the slow component (k_2) in the P388/100 cells, upon inhibition of the active drug efflux of P-glycoproteins by VRP. The observation suggests that the slow step in the interaction of DNM with the cells represents a kinetic component involved in the membrane permeability to the drug. In our interpretation and according to our experimental design, while the slower kinetic component observed in the P388/S cells should reflect, exclusively, entrance of the drug by passive diffusion, in the P388/100 cells, and in the presence of VRP it should represent the net balance from two opposite processes: inward passive diffusion and active efflux mediated by P-glycoproteins. Under these conditions, i.e. in the absence of VRP, the rates for the movement of DNM accross the plasma membranes are similar in the P388/S and in the P388/100 cells. On the contrary, when the activity of P-glycoproteins is prevented by VRP, k_2 would represent the 'true' apparent rate constant for passive diffusion of DNM into the P388/100 cells. These observations are in apparent discrepancy with previous studies reported by others [21,24]. Nevertheless, the considerable differences in the time-scale and time- resolution used previously with respect to those employed here makes it very difficult to compare the kinetic data. A comparison of the k_2 values between the P388/S and P388/100 cells in the presence of VRP indicates that acquisition of resistance to anthracyclines by the P388 cells is accompanied by a considerable increase in their passive permeability to the drug.

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REFERENCES

[1] Fine, R.L. and Chabner, B.A. (1986) in: Multidrug Resistance (Pinedo, H.M. and Chabner, B.A., eds.) Cancer Chemotherapy Annual 8, pp. 117–128, Elsevier, Amsterdam.
[2] Gerlach, J.H., Kartner, N., Bell, D.R. and Ling, V. (1986) Cancer Surv. 5, 25–46.
[3] Fry, D.W. and Jackson, R.C. (1986) Cancer Surv. 5, 47–79.
[4] Endicott, J.A. and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171.
[5] Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. and Pastan, I. (1987) Proc. Natl. Acad. Sci. USA 84, 265–269.
[6] McGrath, T. and Center, M. (1987) Biochem. Biophys. Res. Commun. 145, 1171–1176.
[7] Bates, S.E., Mickley, L.A., Chen, Y., Richert, N.D., Rudick, J., Biedler, J.L. and Fojo, A.T. (1989) Mol. Cell Biol. 9, 4337–4344.
[8] Mickley, L.A., Bates, S.E., Richert, N.D., Currier, S., Tanaka, S., Foss, F., Rossen, N. and Fojo, A.T. (1989) J. Biol. Chem. 264, 18031–18040.
[9] Alabaster, O., Woods, T., Ortiz-Sanchez, V. and Jahangeer, S. (1989) Cancer Res. 49, 5638–5643.
[10] Siegfried, J.M., Burke, T.G. and Tritton, T.R. (1985) Biochem. Pharmacol. 34, 593–598.
[11] Escriba, P.V., Ferrer-Montiel, A.V., Ferragut, J.A. and Gonzalez-Ros, J.M. (1990) Biochemistry 29, 7275–7282.
[12] Krishan, A., Sauerteig, A., Gordon, K. and Swinkin, C. (1986) Cancer Res. 46, 1768–1773.
[13] Ferrer-Montiel, A.V., Gonzalez-Ros, J.M. and Ferragut, J.A. (1992) Biochim. Biophys. Acta (in press).
[14] Burke, T.G. and Tritton, T.R. (1985) Biochemistry 24, 1768–1776.
[15] Willingham, M.C., Cornwell, M.M., Cardarelli, C.O., Gottesman, M.M. and Pastan, I. (1986) Cancer Res. 46, 5941–5946.
[16] Garcia-Segura, L.M., Ferragut, J.A., Ferrer-Montiel, A.V., Escriba, P.V. and Gonzalez-Ros, J.M. (1990) Biochim. Biophys. Acta 1029, 191–195.
[17] Ford, J.M and Hait, W.N. (1990) Pharmacol. Rev. 42, 156–199.
[18] Vayuvegula, B., Slater, L., Meador, J. and Gupta, S. (1988) Cancer Chemother. Pharmacol. 22, 163–168.
[19] Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, I. (1981) Cancer Res. 41, 1967–1972.
[20] Skovsgaard, T. (1978) Cancer Res. 38, 4722–4727.
[21] Skovsgaard, T. (1978) Biochem. Pharmacol. 27, 1221–1227.
[22] Duarte-Karim, M., Ruysschaert, J.M. and Hildebrand, J. (1976) Biochem. Biophys. Res. Commun. 71, 658–663.
[23] Hasmann, M., Valet, G.K., Tapiero, H., Trevorrow, K. and Lampidis, T. (1989) Biochem. Pharmacol. 38, 305–312.