Pergamon

Biochemical Pharmacology, Vol. 47, No. 10, pp. 1851–1857, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0037-L

CORRELATION BETWEEN THE SHORT-TERM MEASUREMENTS OF DRUG ACCUMULATION IN LIVING CELLS AND THE LONG-TERM GROWTH INHIBITION

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(*Received* 5 *July* 1993; *accepted* 10 *January* 1994)

Abstract—The basic distinguishing feature of all cells expressing functional P-glycoprotein-multidrug resistance (P-gp-MDR) is a decrease of steady state drug levels as compared to drug-sensitive controls. Recently it has been pointed out that there appears to be a discrepancy between the amount of drug accumulated at steady state by drug-sensitive and highly resistant cells and their degree of resistance. These observations could suggest two things: (1) that factors other than drug accumulation may be important in MDR, (2) that they reflect a discrepancy between the short-term measurements of drug accumulation at 60 min versus long-term (72 hr) growth inhibition. Due to the different experimental conditions and the different type of cells used it is very difficult to compare the literature data. For this reason we have investigated the effect of 12 compounds in overcoming resistance in relation to drug accumulation. We have used a spectrofluorometric method which allows the determination of the nuclear drug accumulation directly on living cells. Our data clearly establish that, at least for the compounds used in that study, there is a very good correlation between their ability to increase drug accumulation, measured at short-term, and their ability to reverse MDR, but no correlation with their ability to inhibit protein kinase C activity. In addition, their efficiency to reverse MDR correlates with their pK_a values, the efficiency being the highest when the pK_a is the lowest.

Key words: multidrug resistance; drug accumulation; Adriamycin; protein kinase C

The acquired resistance of tumor cells to anthracyclines, vinca alkaloids, actinomycin D, taxol, and a variety of other natural cytotoxic drugs is an important clinical problem in cancer therapy. Since these drugs are structurally and functionally different from each other, the term MDR† is frequently used to describe this type of resistance. In most cell lines, MDR is associated with the presence of a 170-kDa glycoprotein in the plasmic membrane, the P-glycoprotein (P-gp) [1].

The basic distinguishing feature of all cells expressing functional P-gp-MDR is a decrease of steady state drug levels as compared to drug-sensitive controls [2]. Biochemical and pharmacological data strongly indicate that P-gp, which is overexpressed in resistant cells, renders cells resistant to lipophilic cytotoxic drugs by serving as an efflux pump [1, 3–5]. This mechanism of P-gp-mediated drug efflux appears to be associated with drug resistance both in cell culture and in clinical cancer.

Recently it has been pointed out that there appears a discrepancy between the amount of drug accumulated at steady state by drug-sensitive and highly resistant cells and their degree of resistance [3]. These observations could suggest two things: (1) that factors other than drug accumulation may be important in MDR, or (2) that they reflect a discrepancy between the short-term measurements of drug accumulation at 60 min versus long-term (48 hr) growth inhibition [4]. Due to the different experimental conditions and the different type of cells used it is very difficult to compare the literature data. This was one of the reasons why we have undertaken the measurements of the short-term accumulation of one type of drug, THP-ADR, within one type of drug-resistant cells, K562, in the presence of various concentrations of different inhibitors concomitantly with the long-term growth inhibition.

Our data clearly establish that, at least for this type of drug and this cell line, there is a good correlation between the short-term drug accumulation and the long-term growth inhibition. We have also observed that there is a good correlation between the pK_a values, the efficiency of the modulator to reverse MDR being the highest when the pK_a is the lowest.

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 $\varepsilon_{480} = 11{,}500\ M^{-1}\ cm^{-1}$. Stock solutions were prepared just before use. Verapamil, chlorpromazine, promethazine, fluphenazine, prochlorperazine, promazine, trifluoropromazine, were from Sigma (St Louis, MO, U.S.A.), phenothiazine, phenoxazine, quinine, quinidine, ethopropazine, trifluoroperazine, thioridazine, were from Aldrich.

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† Abbreviations: THP-ADR, 4′-*O*-tetrahydropyranyl-adraimycin; MDR, multidrug resistance; PKC, protein kinase C; P-gp, P-glycoprotein.

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 $MgCl_2$, 5 mM glucose) at pH 7.25.

Absorption spectra were recorded on a Cary 219 spectrophotometer and fluorescence spectra on a Jobin Yvon JY 3CS spectrofluorometer. Experiments were conducted in 1-cm quartz cuvette containing 2 mL of buffer under continuous stirring. The temperature was controlled at 37° using a circulating thermostated water bath.

Cell lines and cultures. Anthracycline-sensitive and -resistant erythroleukemia K562 cells were a gift from Dr Tapiero (Departement de Pharmacologie Cellulaire, ICIG, 94800 Villejuif, France). They were grown in RPMI (flow) medium supplemented with L-glutamine and 10% FCS at 37° in a humidified atmosphere of 95% air and 5% CO_2. Cultures initiated at a density of 10^5 cells/mL grew exponentially to about 10^6 cells/mL grew exponentially to about 10^6 cells/mL in 3 days. For the short-term measurements of drug accumulation, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/mL and cells were used 24 hr later; they were then at a density of about 8×10^5 cells/mL. Cell viability was assessed by trypan blue exclusion. The number of cells was determined by Coulter counter analysis.

For the long-term growth inhibition, cells were grown in culture as described above. The ability of a molecule to reverse MDR was assessed as follows: 1×10^5 cells/mL were cultured in the presence of various THP-ADR concentrations and in the simultaneous presence or absence of chemotherapy modulator. We first checked that the modulators alone, at the concentrations used, have no effect on the cell proliferation. The IC_{50} values were determined by plotting the percentage of cell growth inhibition versus the logarithm of the antitumor drug concentration: IC_{50} is the drug concentration that inhibits cell division by 50% after 72 hr; the percentage of cell growth inhibition is defined as $[(N_0-N_x)/(N_0-1)] \times 100$, where N_0 and N_x are the number of cells in the absence and presence of THP-ADR at concentration x, respectively.

A "resistance factor" was obtained by dividing the IC_{50} of resistant cells by the IC_{50} of the corresponding sensitive cells. The resistance factors obtained were 28 and 8 for Adriamycin and THP-ADR, respectively.

Drug accumulation. Determination of the amount of drug bound to the nucleus at the steady state. Short-term measurement of anthracycline accumulation was followed using a spectrofluorometric method previously described [6–10]. Using this method it is possible to accurately quantify the amount of anthracycline intercalated inside the nucleus at the steady state as incubation of the cells with the drugs proceeds without compromising cell viability. Briefly, this method is based on the observation that anthracycline fluorescence is only quenched (95%) when intercalated between the base pairs of DNA, and that transport across the cell membrane is the rate-limiting step [6]. The measurements were done using an excitation wavelength of 480 nm and slit widths of 10, 10, 2, 2 nm, and the fluorescence emission was measured at 590 nm.

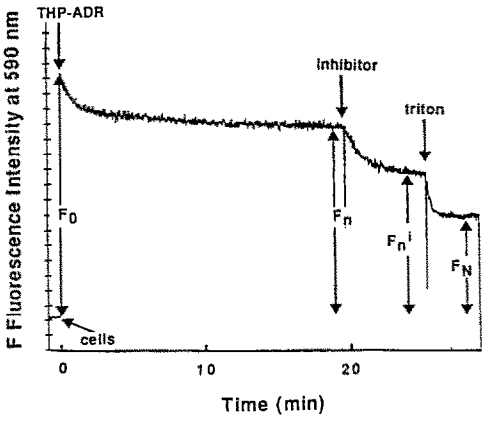

Fig. 1. Uptake of THP-ADR by drug-resistant K562 cells. F, fluorescence intensity at 590 nm ($\lambda = 480$ nm), was recorded as a function of time. 2×10^6 cells were suspended in a cuvette filled with 2 mL buffer at $pH_e = 7.25$ under vigorous stirring. At $t = 0$, 20 μL of a 100-μM stock (THP-ADR) solution was added to the cells yielding a $C_T = 1$ μM THP-ADR solution. The fluorescence intensity was then F_0. 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, i μM inhibitor was added. At the new steady state, the fluorescence intensity was F_n^i. The addition of 0.05% Triton X-100 yielded the equilibrium state. The fluorescence intensity was then F_N.

All experiments were conducted in 1-cm quartz cuvette containing 2 mL of buffer. 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 at pH 7.25, under continuous stirring. 20 μL of the stock anthracycline solution was quickly added to this suspension yielding an anthracycline concentration C_T equal to 1 μM. The decrease of fluorescence intensity F at 590 nm was followed as a function of time. After about 20 min, the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to F_n (Fig. 1). The drug–cells system was thus in a steady state and the overall concentration C_n of drug intercalated between the base pairs in the nucleus was $C_n = C_T \cdot (F_0 - F_n)F_0$. Once the steady state was reached, the inhibitor at concentration $[i]$, was added and a new steady state was reached, the fluorescence intensity being F_n^i. The overall concentration C_n^i of drug intercalated between the base pairs in the nucleus was then $C_n^i = C_T \cdot (F_0 - F_n^i)/F_0$. An aliquot of the solution was then taken away and cell viability was assessed by trypan blue exclusion. It was always higher than 98%. Cell membranes were then permeabilized by the addition of 0.05% Triton X-100 yielding the equilibrium state which was characterized by a new value F_N of the fluorescence intensity. The overall concentration C_N of drug intercalated between the base pairs in the nucleus was then $C_N = C_T \cdot (F_0 - F_N)/F_0$.

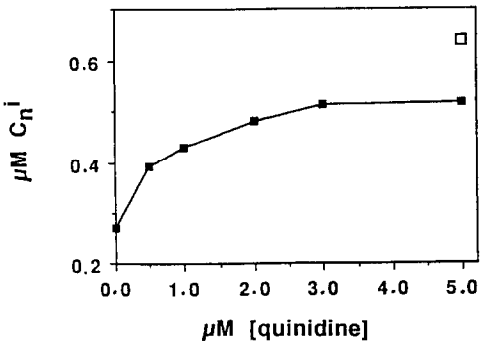

Fig. 2. Effect of quinidine on the THP-ADR accumulation in living cell nuclei. C_n^i, the overall concentration of THP-ADR bound to the nucleus at the steady state has been plotted as a function of the quinidine concentration (filled square), the open square corresponds to the overall concentration of THP-ADR bound to the nucleus at the equilibrium state after the addition of Triton. It does not depend on the quinidine concentration. The experimental conditions are described under Materials and Methods. Each point represents the average of three determinations.

RESULTS

Determination of the overall concentration of THP-ADR bound to the nucleus at the steady state in the presence of various concentrations of inhibitor

The overall concentration C_n of THP-ADR bound to the nucleus of drug-resistant cells was determined at the steady state in the presence of inhibitors at different concentrations, as indicated under Materials and Methods. The inhibitors used were quinine, quinidine, verapamil and phenothiazine derivatives bearing either a piperazinic chain (trifluoroperazine, fluphenazine, prochlorperazine), an aliphatic chain (chlorpromazine, trifluoropromazine, promethazine, promazine, ethopropazine) or a piperidinic chain (thioridazine).

Figure 2 shows the typical plot of C_n^i, the overall concentration of drug bound to the nucleus at the steady state, as a function of the inhibitor concentration in the case where the inhibitor was quinidine. As can be seen, the amount of drug bound to the nucleus increased as the concentration of quinidine increased. In the following, $(C_n)_{R0}$ and $(C_n)_{Ri}$ will stand for the overall concentration of THP-ADR bound to the nucleus of resistant cells in the absence and in the presence of inhibitor, respectively. $(C_n)_{Ri}$ can be expressed as a function of $(C_n)_{R0}$ and $(C_n)_S$ as

$$(C_n)_{Ri} = (C_n)_{R0} + [(C_n)_S - (C_n)_{R0}]\cdot\alpha \qquad (1)$$

where $(C_n)_S$ is the overall concentration of drug bound to the nucleus of sensitive cells and α is the fold increase in THP-ADR incorporation in the presence of inhibitor. α varies between 0 (in the absence of inhibitor) and 1 (when the amount of drug in resistant cells is the same as in sensitive cells).

The inhibitor concentrations required to obtain $\alpha = 0.5$ are reported in Table 1. As most of the cytotoxic assays have been performed in the presence of 5 μM inhibitor, the α values obtained in the presence of 5 μM inhibitor are also reported in Table 1.

Determination of IC_{50} in the presence of 5 μM inhibitor

We first checked that, at 5 μM concentration, none of the inhibitors used in the study was cytotoxic. IC_{50}, the THP-ADR concentration required to inhibit 50% of cell proliferation, was then determined in the presence of 5 μM of each inhibitor as indicated under Materials and Methods. The values thus obtained are reported in Table 1. $(IC_{50})_{RI}$, the IC_{50} value of resistant cells obtained in the presence of a concentration [i] of inhibitor can be expressed as

$$(IC_{50})_{RI} = (IC_{50})_{R0} + [(IC_{50})_S - (IC_{50})_{R0}]\cdot\beta \qquad (2)$$

where $(IC_{50})_{R0}$ and $(IC_{50})_S$ are the THP-ADR concentrations required to inhibit 50% of resistant cell and sensitive cell proliferation in the absence of inhibitor. Under our experimental conditions, $(IC_{50})_{R0} = 32$ nM and $(IC_{50})_S = 4$ nM. The values obtained in the presence of 5 μM inhibitor are shown in Table 1 as well as the β value calculated using Eqn(2).

Relation between α and β

In order to quantify the relation between the intranuclear THP-ADR concentration in short-term measurement and IC_{50}, we have calculated the values of the α and β parameters (Table 1) defined by Eqns (1) and (2) when the experiments were performed in the presence of 5 μM inhibitors. As can be seen in Fig. 3, there is a linear relation between α and β proving that, at least for the modulators used in this study, their ability to enhance drug cytotoxicity is directly correlated with their ability to increase drug incorporation. In addition, this correlation is present despite the fact that the two sets of experiments were performed using different experimental conditions: the THP-ADR concentrations were either 1 μM or ranged from 1 to 50 nM and the incubation time was either 1 hr or 72 hr.

Relation between the inhibitor pK_a values and their ability to modulate THP-ADR incorporation

Almost all of the modulators are lipid-soluble compounds at physiological pH. In addition, based on the data shown in Table 1, it appears that there is a relationship between the inhibitor pK_a values and their ability to modulate THP-ADR incorporation. This proves to be the case, as can be seen in Fig. 4 which is a plot of α, obtained in the presence of a constant inhibitor concentration (5 μM), versus the pK_a of the modulators. A good correlation is obtained for nine of the compounds tested ($P > 0.95$) (prochlorperazine, fluphenazine and thioridazine do not fit the correlation well). As can be noticed α is higher when the pK_a is lower.

Relation between the ability of modulators to reverse MDR and to inhibit PKC

As the structure relationships of phenothiazines and related drugs for inhibition of PKC has been recently studied [11], we find it interesting to compare the inhibitor concentration which is required

Table 1. Effect of various inhibitors on the short-term measurements of THP-ADR accumulation in drug-resistant K562 cells and on their long-term growth inhibition

Inhibitor	$[i]$ μM* ($\alpha = 0.5$)	α† ($[i] = 5$ μM)	IC_{50}nM‡ ($[i] = 5$ μM)	β§ ($[i] = 5$ μM)	pK_a‖	$[I]_{PKC}$‖ (μM)
Sensitive cells						
None	($\alpha = 1$)		4			
Resistant cells						
None	($\alpha = 0$)		32			
Promazine (1)	21	0.26	17	0.53	9.4	46
Chlorpromazine (2)	7.0	0.35	26	0.21	9.3	50
Trifluoropromazine (3)	12	0.31	20	0.43	9.2	170
Prochlorperazine (4)	6.2	0.42	16	0.57	8.1	44
Trifluoroperazine (5)	2	0.72	8.7	0.83	8.1	100
Fluphenazine (6)	4.5	0.50	12.6	0.69	8.1	93
Promethazine (7)	13.2	0.28	19	0.46	9.1	290
Ethopropazine (8)	18	0.18	25	0.25		
Thioridazine (9)	2	0.73	15	0.61		
Verapamil (10)	2	0.75	6.6	0.91	8.2	
Quinine (11)	6.8	0.45	18	0.5	8.6	
Quinidine (12)	3	0.76	11	0.75	8.3	

* and † are related to the short-term measurements of drug accumulation: *$[i]$ ($\alpha = 0.5$) are the inhibitor concentrations which cause a half-maximal increase in cellular THP-ADR accumulation; † α values obtained in the presence of 5 μM inhibitor (according to Eqn (1)). ‡ and § are related to the long term-growth inhibition: ‡ THP-ADR concentration required to inhibit 50% of cell proliferation in the presence of 5 μM inhibitor: § and β values obtained in the presence of 5 μM inhibitor (according to Eqn (2)). ‖ pK_a value of the inhibitor (taken from Refs. [31–33]). ¶ IC_{50} for PKC inhibition (taken from Ref. [11]).

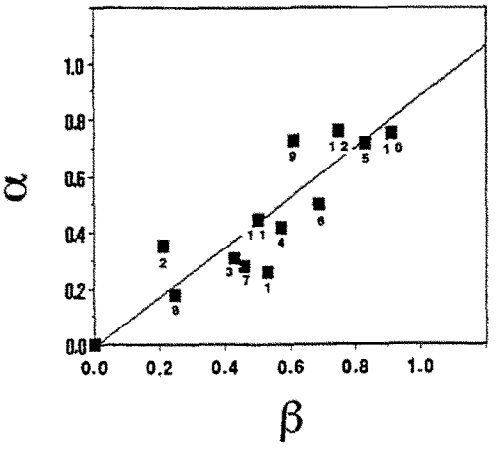

Fig. 3. Correlation between the short-term measurements of THP-ADR accumulation and the long-term growth inhibition. α, the parameter related to the short-term measurements, is defined by Eqn (1) and β related to long-term growth inhibition by Eqn (2). The numbers indicate the nature of the inhibitor used according to Table 1. The line drawn has been least-squares fitted to the data ($P > 0.90$.)

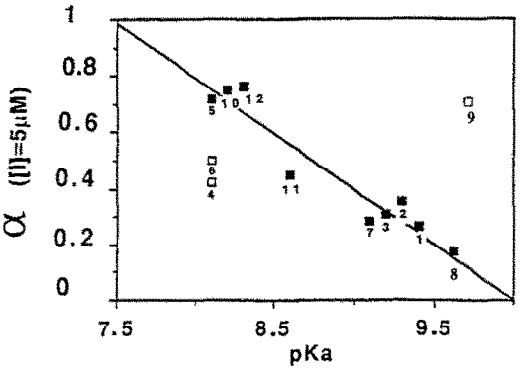

Fig. 4. Relation between the inhibitors' pK_a values and their ability to increase THP-ADR incorporation in resistant cells. The values of the parameter α, related to the short-term measurements (Eqn (1)) performed in the presence of 5 μM inhibitors have been plotted as a function of pK_a. The numbers indicate the nature of the inhibitor used according to Table 1. The line drawn (opened squares not included) has been least-squares fitted to the data ($P > 0.95$).

to inhibit 50% PKC activity [11] to the inhibitor concentration required to obtain $\alpha = 0.5$ in our short-term drug accumulation measurements (Table 1). As can be seen in Fig. 5, there is absolutely no correlation between the ability of these molecules to increase drug accumulation (or drug cytotoxicity) and their ability to inhibit PKC activity.

DISCUSSION

It is well-documented that a variety of compounds have the ability to enhance the cytotoxic activity of antitumor compounds in the multidrug-resistant cell line. Among them we can cite verapamil [12], phenothiazine calmodulin inhibitors like trifluoroperazine [13], dihydropyridines [14–16], chloroquine derivatives [17, 18], reserpine [19] and cyclosporin A [20]. The mechanism of action of

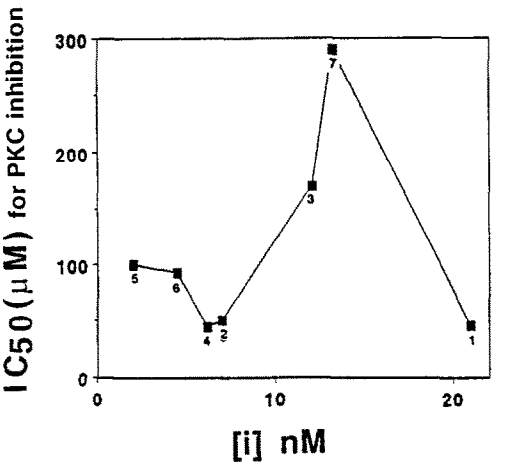

Fig. 5. Lack of correlation between the ability of some phenothiazine derivatives to inhibit PKC activity and their ability to increase THP-ADR incorporation in resistant cells. IC_{50} for PKC inhibition (taken from Ref. [30]) has been plotted as a function of the inhibitor concentrations which cause a half-maximal increase in cellular THP-ADR incorporation. The numbers indicate the nature of the inhibitor used according to Table 1.

these agents has not been fully established [14, 21, 22] but in most cases they inhibit the function of P-gp by preventing the efflux of anticancer drug substrates. These compounds represent a wide range of chemical structures and drug classes. The structures of many of these agents and descriptions of their P-gp inhibitory effects have been presented in a recent review [23]. It has been shown by Beck *et al.* that most of these compounds have in common certain chemical and physical features and they have established physical–chemical "rules" for a compound to be a good modulator of P-gp-MDR [24]: such compounds should have two planar aromatic rings, a tertiary nitrogen and be hydrophobic. However, it has been recently observed that compounds such as cyclosporin that do not possess these structural features can enhance the cytotoxicity of antitumor compounds.

The aim of our work was not to discuss the structural characteristics of compounds that modulate P-glycoprotein-associated multidrug resistance, as this has largely been done previously [25], but to determine if there was a correlation between the amount of inhibitor able to enhance the cytotoxic effect of antitumor drug in long-term growth and the inhibitor concentration able to increase the intranuclear antitumor drug concentration at short-term measurements.

The relation between intracellular Adriamycin levels and cytotoxicity in Adriamycin-sensitive and -resistant cells has been the object of several studies. Thus, it has been demonstrated that the primary mechanism of Adriamycin resistance, in some ovarian cancer cells from clinically refractory patients, is not a transport defect leading to a lowest accumulation of drug such as has been described for cells with *in vitro* induced resistance to Adriamycin [26]. Also, a higher incidence of DNA single- and double-strand breaks in sensitive P388 cells than in resistant cells which cannot be explained by the differences in drug uptake has been observed [27]. On the other hand, Ganapathi *et al.* [28] have shown that in resistant P388 cells that were a 100-fold more resistant to the cytotoxic effects of ADR than were sensitive cells, the cellular accumulation of ADR was 2-fold to 4-fold lower in resistant cells than in sensitive cells. Schuurhuis *et al.* [29] have demonstrated, in two multidrug resistant cell lines, that Adriamycin resistance was mainly due to an impaired ability of Adriamycin to affect cellular targets critical for cytotoxicity rather than to an impaired drug accumulation and that resistance modifiers like verapamil act by increasing the effectiveness of intracellular Adriamycin by inducing redistribution of the drug from the cytoplasm to the nucleus of a multidrug-resistant cell.

In our studies we have used THP-ADR for the reason that this molecule enters the cells very rapidly: for instance, when 10^6 cells/mL are incubated with 1 μM drug, the steady state is reached within less than 30 min at 37°. When similar experiments are performed with daunorubicin or Adriamycin, the steady state is reached within about 1 and 4 hr, respectively. K562 cells were used because the resistant K562 cells are well characterized multidrug-resistant cells that overexpress a 170-kDa P-glycoprotein [30]. As can be seen in Table 1, the inhibitors which are the most efficient at increasing the intranuclear THP-ADR incorporation are trifluoroperazine, thioridazine, verapamil and quinidine (for these four compounds the concentration required to obtain $\alpha = 0.5$ does not exceed 3 μM). Trifluoroperazine, verapamil and quinidine are also the inhibitors which at the same concentration (5 μM) yield the minimum IC_{50} values. From our data we have obtained a good correlation between α and β (Fig. 1). This means that the toxic effect of the drug is correlated to its nuclear accumulation and that the short-term measurements of nuclear drug accumulation can be used to predict long-term growth inhibition.

Our data also show that the best modulator would be that exhibiting the lowest pK_a value [31–33]. This may suggest that this is the neutral form of the molecule which inhibits the P-gp-mediated efflux of anthracycline derivatives.

From our data we can also make a comment about the involvement of PKC in the MDR phenotype. PKC involvement has been implied by previous studies that demonstrated that many MDR cell lines contain higher PKC activity than do wild-type cells and by the use of protein kinase inhibitors to decrease drug resistance [34, 35]. Recently it has been shown that staurosporine, which is a potent protein-kinase inhibitor, enhances accumulation of vincristine in multidrug-resistant cells [36]. However, Miyamoto *et al.*, using different staurosporine derivatives, did not find a clear correlation between their activities on PKC and the vinblastine resistance of P388/ADR cells and suggested that the promotion of drug accumulation may be provided by particular characteristics of these compounds rather than by their inhibitory activity on protein kinase [37]. Our data show that there is absolutely no correlation

between the ability of phenothiazines to increase drug accumulation (or drug cytotoxicity) and their ability to inhibit PKC activity.

In conclusion, we have shown that, at least for the modulators used in this study, there is a good correlation between their ability to reverse MDR and to increase drug accumulation measured at short-term but no correlation with their ability to inhibit PKC activity.

Acknowledgements—This investigation was supported by l'ARC (Association pour la Recherche contre le Cancer), l'Université Paris Nord and le Centre National de la Recherche Scientifique. E.P. Is grateful to CNPq (Bresil) for a fellowship.

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