Time Dependence of [^{3}H]-Vincristine Accumulation by L1210 Mouse Leukemic Cells. Effect of P-Glycoprotein Overexpression

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Abstract. Overexpression of P-glycoprotein (P-GP) accompanied by multidrug resistance (MDR) to diverse groups of cytostatics was developed by long-term adaptation of mouse leukemic cell line L1210 to vincristine. Two resistant sublines of cells characterized by ID_{50} values for vincristine 1.05 mg/l (L1210/VCR-1) and 2.3 mg/l (L1210/VCR-2), respectively, were used. The sensitive parental cell line L1210 had the ID_{50} value for vincristine around 0.01 mg/l. Overexpression of P-GP induced by the adaptation procedure was found to be accompanied by an increase in the mean cell diameter from 10.28 ± 1.60 μm (mean $\pm$ S_D, $n = 122$) for sensitive L1210 cells to 17.82 ± 2.59 μm ($n = 120$) and 37.26 ± 5.72 μm ($n = 121$) for L1210/VCR-1 and L1210/VCR-2 resistant cell sublines, respectively. Significant decrease in ability to accumulate [^{3}H]-vincristine from cultivation medium was observed for both resistant cell sublines in comparison to sensitive cells. Accumulation of [^{3}H]-vincristine by sensitive cells is secured only by passive diffusion of the drug across the plasma membrane. Contrary to that, active efflux of drug operating against its diffusion across the plasma membrane should be assumed as a factor influencing the [^{3}H]-vincristine accumulation by resistant cells. Indeed, the time dependence of [^{3}H]-vincristine accumulation by sensitive cells could be fitted using simple monoexponential kinetic dependence in contrast to biexponential kinetic dependences that are necessary for fitting [^{3}H]-vincristine accumulation by both resistant cell sublines. Kinetic analysis of the experimental data indicates that accumulation of [^{3}H]-vincristine by sensitive cells grows to a plateau reflecting probably the equilibrium of drug concentration in the intracellular and extracellular space. On the contrary, accumulation of [^{3}H]-vincristine by both resistant cell sublines was stabilized after an initial growth on a considerably lower level than it was observed for the sensitive cells in the equilibrium.

Key words: P-Glycoprotein — Multidrug resistance — Vincristine — Mouse leukemic cells — Drug accumulation

Introduction

The existence of neoplastic cells in the presence of several cytostatics such as vinca alkaloids (vincristine, vinblastine), anthracyclines (doxorubicine) and antibiotics (actinomycin-D) induces overexpression of a plasma membrane integral glycoprotein (Mr ~ 170 kD) termed P-glycoprotein (for review see Ban 1992; Vendrik et al. 1992). P-glycoprotein is a member of a transmembrane transport proteins and works as an ATP-hydrolyzing efflux pump, thereby reducing intracellular drug concentration (Hamada and Tsuro 1988; Deucharts and Ling 1988; Wienstein et al. 1990; Doige et al. 1992). Overexpression of P-GP results in the resistance of cells to a variety of unrelated chemotherapeutic agents, i.e. the multidrug resistance phenotype (Gottesman and Pastan 1988; Edicott and Ling 1989).

Periodical cultivation of neoplastic cell lines in a medium with progressively increasing concentrations of cytostatics induces overexpression of P-GP accompanied by MDR. Chinese hamster ovary cells resistant to doxorubicine (Hoban et al. 1992) and mouse leukemic cells L1210/VCR resistant to vincristine (Poleková et al. 1992) should be considered as examples of MDR sublines obtained by this procedure. Multidrug resistant L1210/VCR subline of mouse leukemic cells represents an advantageous model for the *in vitro* study of MDR (Barančík et al. 1994). Overexpression of P-GP accompanied by resistance to vincristine, vinblastine and actinomycin-D, morphological and cytochemical changes connected with P-GP overexpression and ability of several calcium entry blockers, neuroleptics, corticosteroids and local anesthetics to modulate resistance to vincristine were described for L1210/VCR cells previously (Poleková et al. 1992; Barančík et al. 1993; Barančík et al. 1994; Uhrík et al. 1994).

The aim of our report was to present kinetic description of [^{3}H]-vincristine accumulation by L1210 cells and to characterize the effect of P-GP overexpression on [^{3}H]-vincristine accumulation.

Abbreviations: P-GP, P-glycoprotein; MDR, multidrug resistance; VCR, vincristine; ID_{50}, median inhibition dose

Materials and Methods

Materials

Vincristine was supplied by Gedeon Richter Co. (Hungary). [^{3}H]-vincristine (1.85 MBq; 10.0 Ci.mmol^{-1}) was obtained from Amersham Corp. (UK).

Cultivation medium

RPMI 1640 medium, foetal bovine serum and gentamycin were obtained from Serva (Germany), University of Veterinary Medicine Brno (Czech Republic) and Pharmachim (Bulgaria).

All other chemicals were of p.a. grade and were obtained from Sigma (USA) and Lachema (Czech Republic).

Cells and culture conditions

Three sublines of L1210 mouse leukemia cells were used in the experiments: normal sensitive cell line L1210, and two multidrug resistant cell sublines L1210/VCR-1 and L1210/VCR-2, respectively. Both resistant cell sublines were obtained by long-term adaptation of cells to vincristine and exhibiting both P-GP overexpression and a lack of sensitivity to vincristine, vinblastine and actinomycin-D (Poleková et al. 1992). Cell cultivation was carried out in standard RPMI 1640 medium supplemented with 7% heat inactivated foetal bovine serum and gentamycin in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was exchanged every 3–4 days.

Effect of VCR on the viability of cells

Sensitive cell line L1210 and both vincristine resistant cell lines L1210/VCR-1 and L1210/VCR-2 were cultivated in the absence or presence of vincristine (0.005–6.000 mg/l). VCR was added directly to the growth medium with inoculum of cells (final concentration 10^4 cells/ml) in 96-well tissue culture plates. After a cultivation period of 3 days the cells were stained with methylene blue and counted in haemocytometer.

Accumulation of [^{3}H]-vincristine

Cells (2×10^5/100 μl) were incubated in full cultivation medium containing [^{3}H]-vincristine (0.2 mg/l, 0.25 mCi/l) for 5–180 min in humidified atmosphere of 5% CO_2 at 37 °C. After respective time intervals (30–180 min) the cells were centrifuged ($1200 \times g$), washed three times with PBS and resuspended in redistilled water. Aliquots were added to Bray scintillation solution and measured using a liquid scintillation counter 1214 Rackbeta (LKB, Sweden). Specific radioactivity was expressed in DPM (disintegration per minute)/10^6 cells.

Morphological characterization

Suspensions of cells after cultivation in the presence or absence of VCR (0.2 mg/l) were washed with PBS and fixed for 1 hour with 2% glutaraldehyde in 0.1 mol/l Na-cacodylate buffer, pH 7.2. After washings (3×10 min) with 0.1 mol/l cacodylate buffer, pH 7.2, the samples were postfixed in 0.1 mol/l cacodylate buffer containing 1% osmium tetraoxide (OsO_4). The samples were dehydrated in sequential changes of 35%, 70%, 96% and absolute ethanol and cleared in propylene oxide. After infiltration and embedding into Epon 812, ultrathin sections were cut with a Huxley LKB microtome, stained with basic lead citrate and uranyl acetate and finally examined under a TESLA 500 electron microscope at an accelerating voltage of 60 kV. Mean cell diameters were established micrometrically under a light microscope.

Data processing.

Experimental data of cell survival during cultivation in the presence of VCR were expressed as % from the control when VCR was omitted and were fitted using equation of exponential decay :

$$Y = Y_0 \exp[c \cdot \ln(0.5)/ID_{50}] \quad (1)$$

where Y_0 and Y are cell survival in the absence or presence of vincristine at concentration c in the cultivation medium (Y_0 was arbitrarily taken as 100%); ID_{50} is the median inhibition dose. Experimental data of the time dependence of [^{3}H]-vincristine accumulation by sensitive cells could be fitted by the monoexponential equation (2) reflecting the simple passive diffusion of drug across the plasma membrane as the only mechanism involved in the drug transplasmolemmal transport:

$$B = B_\infty [1 - \exp(-k_1 t)] \quad (2)$$

where B and B_∞ are the amount of accumulated [^{3}H]-vincristine at time t and $t \to \infty$, respectively; k_1 is the rate constant of passive diffusion of the drug across the plasma membrane.

The drug efflux activity of P-GP operating against the passive diffusion of the drug across the plasma membrane to the intracellular space in resistant cells is shown by biexponential equation (3) for the description of drug accumulation by both resistant cell lines:

$$B = \underline{B}_\infty [1 - \exp(-k_1 t)] [c + \exp(-k_2 t)] \quad (3)$$

where B and k_1 were described in equation (2); k_2 is the rate constant of drug efflux through the plasma membrane mediated by P-GP; $\underline{B}_\infty$ is the extrapolation of drug ac-

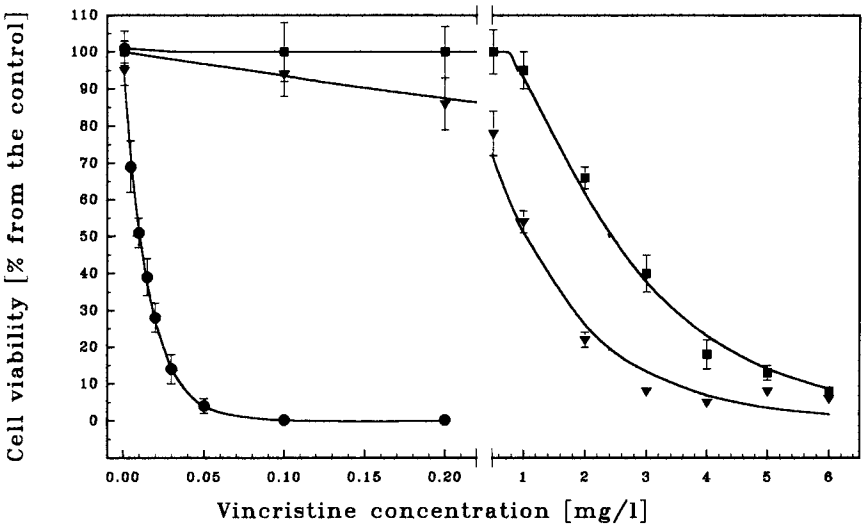

Figure 1. Cytotoxic effect of vincristine on sensitive L1210 (circles) and resistant L1210/VCR-1 (triangles) and L1210/VCR-2 (squares) cell lines. Viable cells were counted in a haemocytometer after cultivation of cells for 3 days in cultivation medium containing vincristine at respective concentration (for details see Materials and Methods). Data represent the mean ± S.E.M. from 6 independent values and are expressed as percentage from the control (i.e., when vincristine was absent). Nonlinear regressions of the data according to equation (1) yield ID_{50} values equal to 0.0104 ± 0.0009, 1.03 ± 0.08 and 2.39 ± 0.10 mg/l (value ± SD) for L1210, L1210/VCR-1 and L1210/VCR-2, respectively.

cumulation to $t \rightarrow \infty$ when the effect of drug efflux activity of P-GP was theoretically excluded; c is a constant including the effect of P-GP drug efflux activity on equilibrium accumulation of [^{3}H]-vincristine B_∞ given by equation (4):

$$B_\infty = \underline{B}_\infty \cdot c \tag{4}$$

The initial rate of [^{3}H]-vincristine accumulation is given by equation (5) derived from equation (2) for the sensitive cell line, and by equation (6) derived from equation (3) for both resistant cell lines:

$$v_0 = \lim_{t \to 0} \mathrm{d}B/\mathrm{d}t = B_\infty \, k_1 \tag{5}$$

$$v_0 = \lim_{t \to 0} \mathrm{d}B/\mathrm{d}t = \underline{B}_\infty \, k_1 \, (c + 1) \tag{6}$$

Experimental data were fitted according to the respective equation by nonlinear regression using Sigmaplot 5.0 (Jadel corporation) software.

Results

Both resistant cell lines (L1210/VCR-1 and L1210/VCR-2) showed lack of sensitivity to vincristine (Fig. 1) since ID_{50} values were shifted from 0.01 mg/l observed for L1210 cells to 1.03 and 2.30 mg/l observed for L1210/VCR-1 and L1210/VCR-2 cells, respectively. These changes in cell sensitivity to vincristine were accompanied by several changes in ultrastructure of the cells, namely in the plasma membrane region. Ultrastructural changes as a function of degree of cell adaptation to vincristine are shown in Fig. 2. The relatively smooth elliptic shape of L1210 cells (Fig. 2*A*) was found to be altered by numerous cytoplasmic protrusions of the cell surface in L1210/VCR-1 (Fig. 2*B*) and mainly in L1210/VCR-2 cells (Fig. 2*C*). On the other hand, adaptation of cells to vincristine and the growth of cells in the medium with vincristine induced observable increase in cell diameter (Fig. 2). This enhancement of the cell diameter was ascertained micrometrically under light microscope (Fig. 3). Mean cell diameter was found to be increased approximately two times for L1210/VCR-1 and approximately three times for L1210/VCR-2 cells in comparison to the sensitive L1210 cells.

The time course of [^{3}H]-vincristine accumulation by the sensitive L1210 cells represents a simple rectangular hyperbola (Fig. 4*A*) which may be fitted by a simple monoexponential kinetic dependence (equation 2). Contrary to that, biexponencial dependence (equation 3) was found to be necessary for the description of drug accumulation by both resistant cell lines.

Data in Fig. 4*A* were expressed as radioactivity accumulated after respective time intervals by 10^6 cells. Values of accumulated [^{3}H]-vincristine expressed in this way could not correlate with the intracellular concentrations of [^{3}H]-vincristine in the sensitive or both resistant cell lines due to marked differences in respective

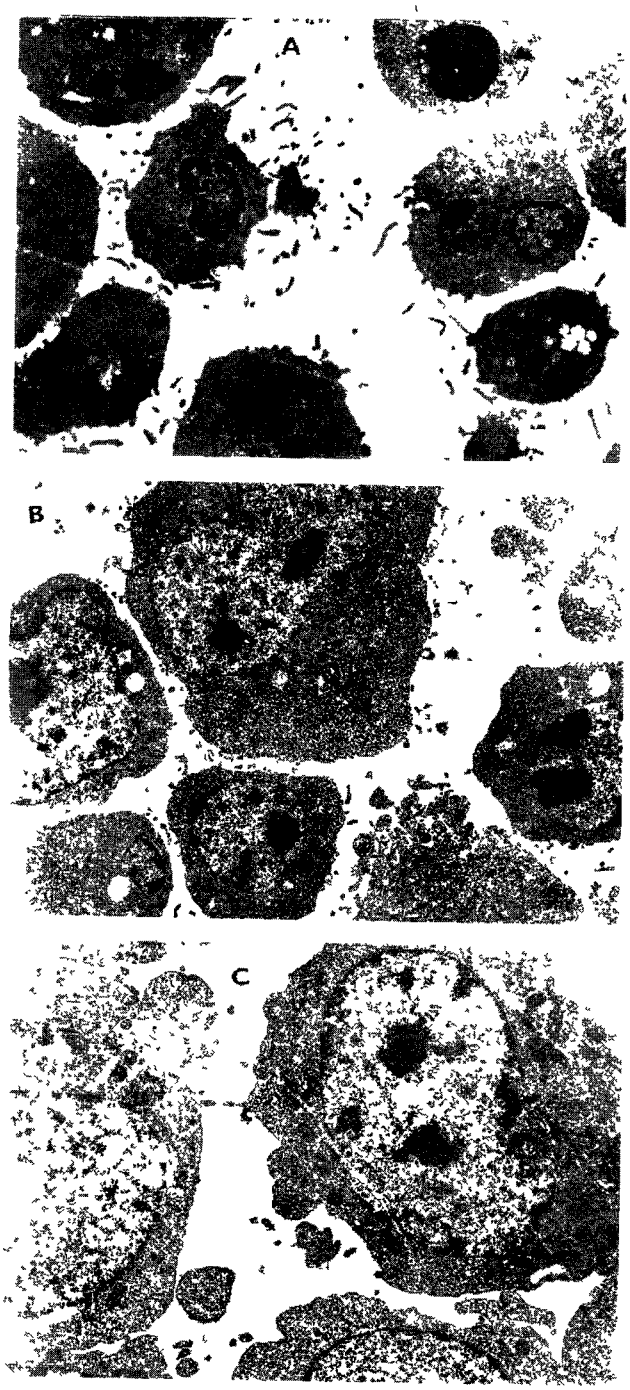

Figure 2. Ultrastructure of sensitive and both resistant cell lines *A* – sensitive L1210 cells *B* resistant L1210/VCR-1 cells *C* resistant L1210/VCR 2 cells Magnification × 3500

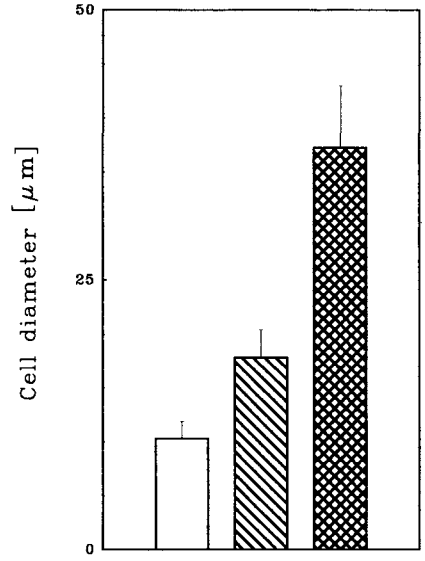

Figure 3. The mean cell diameters of sensitive L1210 (open bar), and both resistant L1210/VCR-1 (diagonally striated bar) or L1210/VCR-2 (crosshatch striated bar) cell lines. Data represent mean ± S.E.M. from 122, 120 and 121 independent values for L1210, L1210/ /VCR-1 and L1210/VCR-2 cells, respectively. The data differ significantly at $p < 0.02$.

mean cell diameter. From this point of view, it was reasonable to express the [^{3}H]-vincristine accumulation as accumulated radioactivity per quantity characterizing mean intracellular volume. Mean intracellular volume of cells should be proportional to the value (normalized cell diameter)^3 based on the idea that each alteration in the shape of a single cell is statistically eliminated for a large sum of cells. Normalized cell diameter represents the ratio of mean cell diameters of the resistant and sensitive cells. Therefore, [^{3}H]-vincristine accumulation by the sensitive and both resistant cell lines was expressed in Fig. 4B as DPM/{10^6 cells (normalized cell diameter)^3}. Parameters of equations (2) and (3) obtained for both expressions of the experimental data are shown in Table 1.

Discussion

The main difference between the resistant cell lines L1210/VCR-1 and L1210/VCR-2 and the sensitive cell line L1210 is in their ability to grow in a medium containing vincristine. The lack of sensitivity of resistant cell lines has been found to be connected with overexpression of P-glycoprotein, and may be considered as P-GP mediated MDR (Poleková et al. 1992; Barančík et al. 1993; Barančík et al. 1994; Uhrík et al. 1994). Cross-resistance of L1210/VCR-1 and L1210/VCR-2 cells to actinomycin-D and doxorubicine is in agreement with P-GP-mediated nature of this type of resistance.

Adaptation of L1210 cells to vincristine induced, besides the expression of P-GP, several changes in ultrastructure and marker enzyme contents as observed

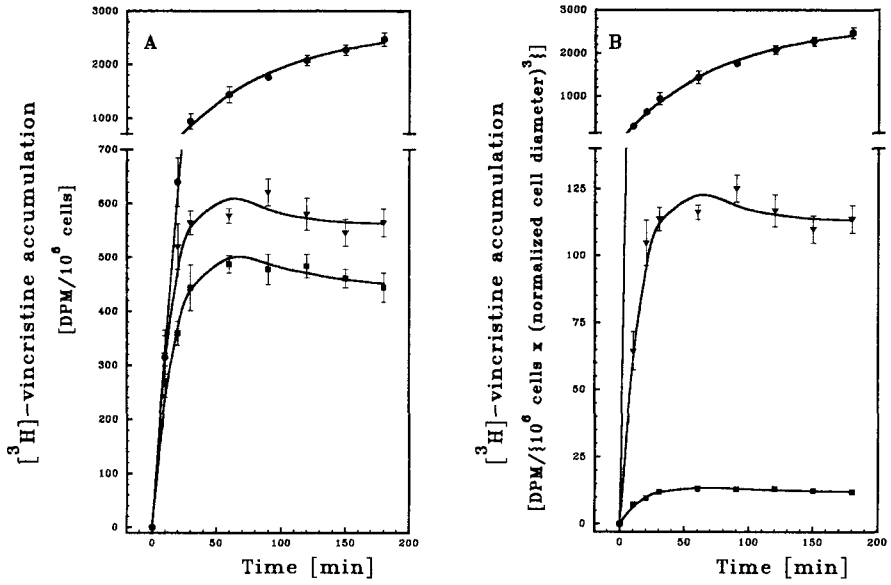

Figure 4. Accumulation of [^{3}H]-vincristine by sensitive L1210 (circles) and resistant L1210/VCR-1 (triangles) or L1210/VCR-2 (squares) cell lines. Data represent mean ± S.E.M. from 5 independent values and were fitted using equation (2) (L1210) or equation (3) (L1210/VCR-1, L1210/VCR-2) by nonlinear regression. Panel *A*: direct data expressed in DPM per 10^6 cells. Panel *B*: data normalized for mean cell volume expressed in DPM/[10^6 × cells (mean cell diameter)^3]. Kinetic parameters obtained by nonlinear regression are shown in Table 1.

histochemically, namely in the plasma membrane region (Uhrík et al. 1994). Similar ultrastructural changes as a function of degree of cell adaptation to vincristine are shown in Fig. 2. Moreover, both resistant cell lines showed an observable increase in mean cell diameter (Fig. 3) which may probably reflect the previously described stimulation of energetic metabolism (Poleková et al. 1992, Uhrík et al. 1994) of resistant cell lines under the influence of vincristine.

Drugs such as vincristine enter the cells by passive diffusion and their accumulation in the cells is limited by equilibrium between the intracellular and extracellular drug concentrations. Indeed, accumulation of [^{3}H]-vincristine by the sensitive cell line may be described by simple monoexponential kinetic dependence. Equation (2) reflects thus the equilibrium of passive diffusion as the only process (Fig. 4*A*). Drug efflux capability of P-GP in the resistant cells should operate against the cell drug accumulation. This efflux capability may be described by addition of $[c + \exp(-k_2 t)]$ to equation (2) resulting thus in equation (3) which adequately describes the process of [^{3}H]-vincristine accumulation by both resistant cell lines (Fig. 4*A*). Kinetic analysis of the experimental data indicates that

Table 1. Kinetic parameters [^{3}H]-vincristine accumulation by sensitive and both resistant cell lines.

Cells	$^*B_\infty$	$^*\underline{B}_\infty$	k_1 min^{-1}	k_2 min^{-1}	c
Direct data[a]					
L1210	2681	–	0.0128	–	–
	±192	–	±0.0009	–	–
L1210/VCR-1	561	1342	0.0213	0.0260	0.419
	±72	±98	±0.0019	±0.0021	±0.035
L1210/VCR-2	445	717	0.0269	0.0241	0.621
	±25	±56	±0.0031	±0.0022	±0.053
Normalized data[b]					
L1210	2681	–	0.0128	–	–
	±192	–	±0.0011	–	–
L1210/VCR-1	113	285	0.0203	0.0258	0.397
	±11	±27	±0.0013	±0.0015	±0.028
L1210/VCR-2	12	16	0.0294	0.0217	0.794
	±1	±2	±0.0025	±0.0018	±0.068

Experimental data were expressed as value ± S_D for degree of freedom 18–22.
*data were expressed in DPM per 10^6 cells (a) or DPM per [10^6 cells × (normalized cell diameter)^3] (b)

accumulation of [^{3}H]-vincristine by sensitive cells grows to the plateau reflecting probably the equilibrium of drug concentration in the intracellular and extracellular space. On the contrary, accumulation of [^{3}H]-vincristine by both resistant cell sublines was stabilized after an initial growth on a considerably lower level, reflecting probably the P-GP efflux capability that was observed for the sensitive cells in equilibrium. Thus, equilibrium concentration of vincristine in both resistant cell lines may be considered as a result of equilibrium between the P-GP drug efflux activity and passive diffusion of vincristine to the intracellular space. Expression of the data in Fig. 4*A* as radioactivity accumulated after respective time intervals by 10^6 of cells could not reflect the increase in intracellular volume induced by adaptation of cells to vincristine as it is documented on the increased mean cell diameter (Fig. 3). Therefore, data in Fig. 4*B* were expressed as DPM / {10^6 cells (normalized cell diameter)^3}. This type of transformation probably reflects better the differences between [^{3}H]-vincristine accumulation by these three cell lines than those applied in Fig. 4*A*. Fig. 4*B* shows real differences between [^{3}H]-vincristine accumulation by both resistant cell lines that could not be observed so expressively in Fig. 4*A*. The values of the parameters obtained by nonlinear fitting of data in Fig. 4 according to equations (2) and (3) are shown in Table 1. Strong decreases of

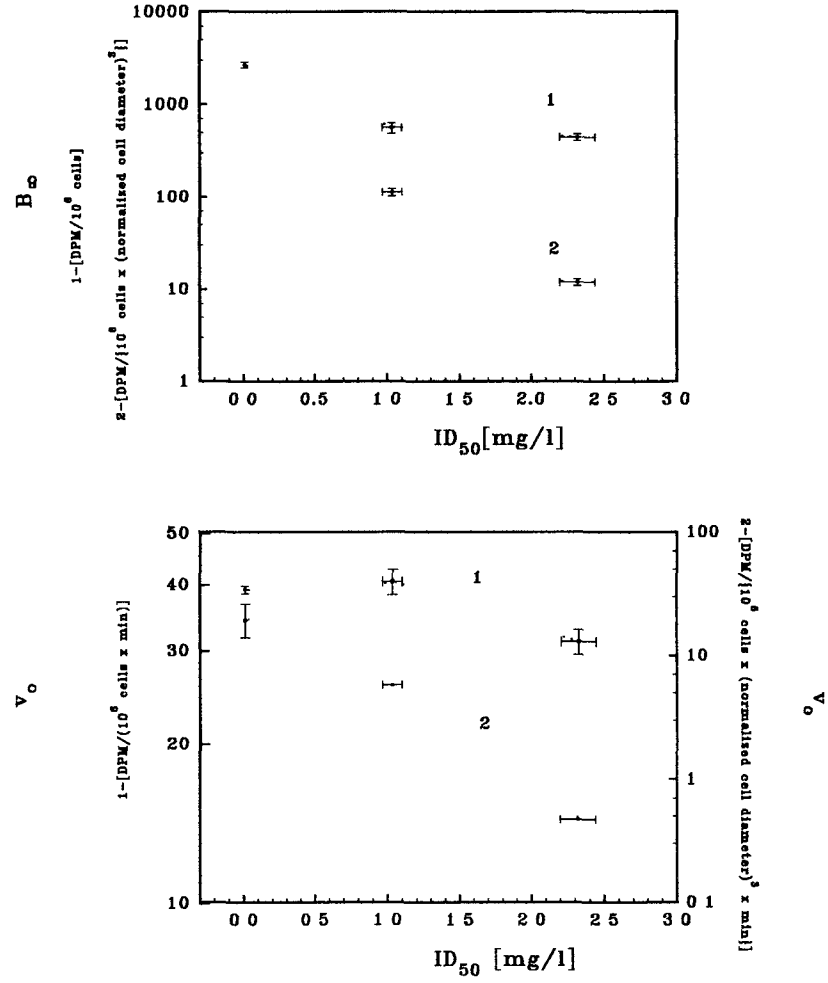

Figure 5. Trends of relation between ID_{50} values characterizing cytotoxic effect of vincristine on L1210 L1210/VCR 1 and L1210/VCR-2 cell lines (obtained from Fig 1) and kinetic parameters B_∞ (upper panel obtained from Table 1) or ι_0 (lower panel computed from the data in Table 1 according to equations (5) and (6)) 1 direct data 2 data normalized for mean cell volume (Fig 4)

B_∞ values were observed for both resistant cell lines in comparison to the sensitive cell line

The P-GP-mediated decrease in capability of accumulating [^{3}H]-vincristine observed in both resistant cell lines correlated with the lack of sensitivity of these cells to vincristine This correlation may be documented by almost linear tendency

of relation between B_∞ (Table 1) or v_0 (calculated from the data in Table 1 according to equations (4) and (5)) and ID_{50} value in semilogarithmic plot when data normalized for the mean cell volume were applied (Fig. 5). On the contrary, when direct data without normalization for cell volume were used (i.e., data fitted from Fig. 4*A*) no linearity was observed in relation of ID_{50} versus B_∞ or v_0. The latter fact indicates the requirement of data normalization of volume for a real characterization of differences in drug accumulation by the three cell sublines.

The linear tendencies shown in Fig. 5 indicate that the drug transporting activity of P-GP should be considered as a dominating process that secures the MDR of L1210/VCR-1 and L1210/VCR-2 cells. Multidrug resistance was often observed under P-GP overexpression (for review see Ban 1992; Vendrik et al. 1992). However, Batist et al. (1986) described an elevated expression of the anionic isoenzyme of glutathione-S-transferase in doxorubicine resistant MCF-7 cells. Additionally, two types of resistance acting on cellular topoisomerase enzymes have been recently described (Baguley et al. 1992). Tendency was found for coexpression of increased P-GP and glutathione-S-transferase and increased P-GP and decreased topoisomerase II expression in human kidney carcinoma and human breast carcinoma (Efferth et al. 1992). In contrast, haematological malignancies did not show such a coexpression of resistance markers (Efferth et al. 1992). Thus, in mouse leukemic cell line L1210 (as an example of haematological malignant cells), when MDR was secured by P-GP overexpression (Poleková et al. 1992; Barančík et al. 1994), only low level (if any) of glutathione-S-transferase or topoisomerase II overexpression should be expected. Since the verification of the latter assumption requires further study, it points out to the aptness of the resistant L1210/VCR-1 or L1210/VCR-2 cells as a model for studying of P-GP mediated MDR phenotype.

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