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Comparative uptake, retention and action of vincristine, vinblastine and vindesine on murine leukaemic lymphoblasts sensitive and resistant to vincristine

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1 The uptake and retention of vincristine (VCR), vinblastine (VBL) and vindesine (VDS) were evaluated comparatively with respect to their cytotoxic action on a murine lymphoblastic leukaemia (L5178Y).

2 The same parameters were measured on a derived subline of cells resistant to VCR (L5178Y/r) in order to determine whether the different degree of resistance to each alkaloid correlates with the amount of drug associated with the cells.

3 VCR was the most active on L5178Y cells ($IC_{50} = 5.8 \times 10^{-9}$ M) while the activity of VBL and that of VDS were similar (IC_{50} 4.4×10^{-8} M and 3.5×10^{-8} M, respectively). Nevertheless, a considerably larger amount of VBL was taken up by the cells compared to VDS, although there were no significant differences in their cytotoxic action.

4 The VCR resistant cell line also expressed resistance to VDS, whose IC_{50} was increased by a factor of 11.4, but not to VBL. However, the uptake and retention of the three alkaloids were similarly reduced in L5178Y/r cells regardless of the degree of resistance expressed.

5 Although a decreased drug uptake and/or retention by the cells provides an explanation for the resistance to vinca alkaloids, they do not seem to be the only factors accounting for the resistance shown by the cell line which we have isolated.

6 The results seem to indicate that part of the VBL taken up by the cells is not used to induce the cytotoxic effect, but is diverted to some cellular compartment(s) or rate controlling process(es) which are different from the target that mediates its cytotoxic action.

Introduction

The cellular pharmacology and mechanism of action of vinca alkaloids (vincristine, vinblastine and vindesine) used in cancer chemotherapy have not been clearly established. Their intracellular binding to tubulin with subsequent dissolution of microtubules and arrest of cells in mitosis are considered necessary to mediate their cytotoxic action (Creasey, 1979). However, although these alkaloids have only minor structural differences and behave in the same way at the level of drug-tubulin interaction (Himes *et al.*, 1976; Owellen *et al.*, 1977a), their toxicity and spectrum of clinical activity differ considerably.

There are various studies suggesting that divergences in the uptake and/or retention of these drugs by different tissues might be responsible for their selective toxicity (Noble *et al.*, 1977; Houghton *et al.*, 1984) and growth-inhibitory action on the cells. Also an impairment of drug uptake (Bruce *et al.*, 1969; Bulinski & Borisy, 1979; Bender *et al.*, 1982) and/or retention by the cells (Bleyer *et al.*, 1975; Beck *et al.*, 1983; Beck, 1984; Ganapathi *et al.*, 1986) has been suggested as a possible cause of the drug resistance shown by some tumour cells, but the mechanisms underlying these processes are poorly understood.

In the present study we evaluated comparatively the uptake and retention of vincristine (VCR), vinblastine (VBL) and vindesine (VDS) by L5178Y murine leukaemic lymphoblasts, with respect to their cytotoxic action on this cell line, with the aim of finding out whether or not the oncolytic response

induced by each one, parallels their affinity to the tumour cells. Furthermore, in order to determine whether the different degree of resistance shown to each drug correlates with their uptake and retention by the cells, the same parameters were measured on a cell line resistant to VCR (L5178Y/r) derived in our laboratory from L5178Y cells.

Methods

Cells

The L5178Y murine leukaemic lymphoblasts, originally obtained from Fischer & Sartorelli (1964), were maintained by weekly i.p. passage of 2×10^6 tumour cells into BDF1 male mice and through *in vitro* cultures in Fischer's medium supplemented with 10% horse serum.

The VCR-resistant line was derived from the L5178Y leukaemic lymphoblasts following the method described by Hill & Whelan (1982). Exponentially growing cells were exposed to 2.2×10^{-6} M VCR for 24 h, and then cloned in soft agar (Fischer & Sartorelli, 1964) in the presence of 2.2×10^{-7} M VCR. The cell line established from one individual colony was propagated in Fischer's medium supplemented with 10% horse serum in the continuous presence of 10^{-10} M VCR and stored frozen at −80°C. The VCR was removed from the culture medium 1 week before checking the sensitivity to different vinca alkaloids or any other experimental procedure.

The IC_{50}, defined as that concentration which reduces by 50% the population doubling number maximally obtained on control cultures, was measured for VCR, VBL and VDS, both on the parent line and the resistant cells isolated, through 72 h suspension cultures in the presence of increasing concentrations of each drug. The degree of resistance was calculated by comparing the IC_{50} values of each alkaloid on the parent and resistant cell lines.

The doubling time of both cell lines was measured in suspension cultures where the cell number was maintained below 5×10^5 cells ml^{-1} and the cell proliferation was exponential.

Determination of cell water and protein content

The intracellular water volume was measured on 9980 *g* pellets of 0.4 ml of cell suspensions (9×10^6 cells ml^{-1}) previously incubated (37°C, 4 min, 5% CO_2) in the presence of [^{14}C]-inulin (0.2 Ci ml^{-1}) and [^{3}H]-H_2O (1.72 Ci ml^{-1}). The intracellular water space was calculated as the difference between the total water and inulin distribution spaces in the pellet.

The protein content of the cells was measured by the procedure of Schacterle & Pollack (1973) using bovine serum albumin as a standard.

Uptake and retention of vinca alkaloids

Cells used for this study were removed from the ascitic fluid of mice which had received an inoculum of 2×10^6 parent cells or 10^7 resistant cells 8 or 10 days, respectively, before the uptake evaluation.

After the removal of erythrocytes by hypotonic shock with NaCl 0.2%, the cells were resuspended in Hank's balanced salt solution (HBSS), pH 7.4 at a density of 6–8 $\times 10^6$ cells ml^{-1} and preincubated for 25 min at 37°C in 5% CO_2 in a shaking water bath. The uptake experiments were initiated by adding the tritiated drug dissolved in HBSS to the cell suspension (1 : 3 v/v). The drug concentrations used were selected by referring to the serum peak concentrations found by different authors in pharmacokinetic studies (Dyke & Nelson, 1977; Owellen *et al.*, 1977c).

At graded time intervals, duplicate 0.4 ml samples of the incubation mixture were transferred to 1.5 ml microcentrifuge tubes containing 0.5 ml of a mixture of dibutyl phthalate and dioctyl phthalate (density = 1.0248) and centrifuged immediately at 9980 *g* for 30 s in an Eppendorf microcentrifuge (model 5414). Thereafter, the medium layer was aspirated, the tube washed with 1 ml water (×2) and finally the oil aspirated and each tube dried with a cotton plug to remove the oil and water residues. The pellet was digested with 0.5 M trichloroacetic acid and the uncapped tube and its contents transferred to a scintillation vial containing 10 ml of Unisolve 1 for determination of the radioactivity in a liquid scintillation spectrometer (LKB, 1217 Rackbeta). Parallel 10 s incubations at 4°C were carried out to evaluate the rapid non-specific adsorption of the drugs to the cell surface, and were considered as blank values to be substracted from the 37°C incubation values obtained.

The cellular drug retention was measured on cells preincubated for 30 min with the ^{3}H-labelled drug as described above. Then the cells were removed from the drug solution by centrifugation (10 min, 4°C, 1000 *g*) and immediately resuspended in drug-free HBSS (5–10 $\times 10^6$ cells ml^{-1}, 37°C). Thereafter, at graded time intervals between 1 and 40 min, 0.4 ml aliquots were removed and processed as above to quantitate the cell-associated radioactivity.

Throughout the uptake and retention studies other aliquots were removed from the incubation media to check the cell viability by the dye exclusion test, using trypan blue as the vital stain (Patterson, 1979). We confirmed that the unstained 'live' cells always exceeded 75% throughout the experiments.

Table 1 Duplication time and protein and water contents of L5178Y cells and the vincristine-resistant subline isolated (L5178Y/r cells)

	Cell line	
	L5178Y	*L5178Y/r*
Duplication time (h)	13.5	21
Protein content (μg per 10^6 cells)	153 ± 16	280 ± 35
Intracellular water content (μl per 10^6 cells)	0.33 ± 0.09	0.80 ± 0.17

All data are expressed as means ± s.e. mean and represent duplicate or quadruplicate determinations from two or three experiments. Where appropriate the data were subjected to statistical comparisons by use of two-tail Student's *t* test.

Materials

[G-^{3}H]-vincristine sulphate (4.8 Ci mmol^{-1}), [G-^{3}H]-vinblastine sulphate (11.5 Ci mmol^{-1}), desacetyl [G-^{3}H]-vinblastine amide sulphate (13 Ci mmol^{-1}), inulin [^{14}C]-carboxilic acid (7.92 Ci mmol^{-1}) and tritiated water (5 Ci ml^{-1}) were obtained from Amersham. Unlabelled desacetyl vinblastine amide (VDS) was obtained from Eli Lilly Co. and unlabelled vincristine (VCR) and vinblastine (VBL) were purchased from Sigma. Tissue culture media were purchased from Gibco and horse serum from Flow Laboratories. Scintillation cocktail (Unisolve 1) was from Koch-Light and all other chemicals were of reagent grade. HBSS was prepared with ultrapure water and had the following composition (mg l^{-1}): $CaCl_2 2H_2O$ 185.5, KCl 400, KH_2PO_4 60, $MgSO_4 7H_2O$ 200, NaCl 8000, $NaHCO_3$ 350, Na_2HPO_4 47.5, D-glucose 1000.

Results

Characteristics of tumour cells and evaluation of their sensitivity to vinca alkaloids

As has been mentioned previously, although the resistant cell line isolated was maintained in culture in the continuous presence of 10^{-10} M VCR, the drug was removed from the culture medium at least 1 week before any experimental evaluation, and the cells resuspended in fresh medium which was renewed twice a week in order to avoid the cytotoxic effect of the alkaloid released by the lysed cells (Lengsfeld *et al.*, 1982).

Some cellular characteristics are presented in Table 1, where it may be seen that the VCR-resistant cells isolated (L5178Y/r) have a doubling time which is longer than that of the parental cells from which they were derived. The cell volume is also increased, as evidenced by the larger protein and intracellular water content (Table 1) and by microscopic examination (data not shown).

The cytotoxicity of the three vinca alkaloids, VCR, VBL and VDS on the cell growth and the IC_{50} values presented in Figure 1 and Table 2 show that VCR is the most toxic alkaloid on L5178Y cells, while there was no significant difference between the cytotoxicity developed by VDS or VBL on these cells. Moreover, although VCR was the drug used to induce the resistance and was still the most active on L5178Y/r cells, the highest degree of resistance was developed to VDS, whose IC_{50} value was increased by a factor of 11.4. However, the IC_{50} for VCR only increased by a factor of 5, and there was no significant change in the sensitivity to VBL i.e., no cross-resistance to VBL was expressed.

Uptake and retention of vinca alkaloids by L5178Y cells

Figure 2 compares the time courses of uptake and retention of VCR, VDS and VBL by L5178Y murine

Table 2 IC_{50}* values for vinca alkaloids on L5178Y cells and on the vincristine-resistant subline isolated (L5178Y/r cells)

	Cell line		
Drugs	*L5178Y*	*L5178Y/r*	*Resistance degree*†
Vincristine,	5.8×10^{-9} M	2.9×10^{-8} M	5
Vindesine	3.5×10^{-8} M	4.0×10^{-7} M	11.4
Vinblastine	4.4×10^{-8} M	7.8×10^{-8} M	1.77

*IC_{50} represents the drug concentration which reduces by 50% the doubling number maximally obtained on control cultures.

†The degree of resistance was calculated by comparing the IC_{50} values of each alkaloid on the L5178Y/r cells versus L5178Y cells.

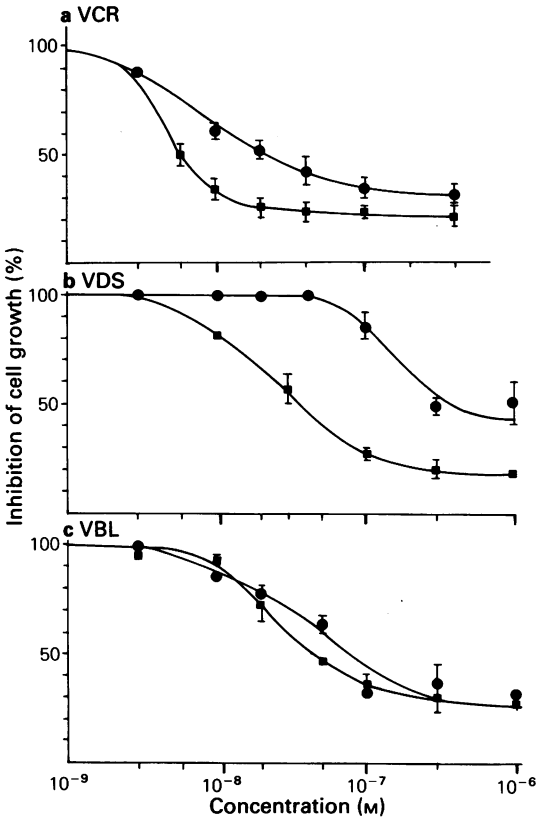

Figure 1 Cytotoxic activity of (a) vincristine (VCR), (b) vindesine (VDS) and (c) vinblastine (VBL). Exponentially growing L5178Y (■) and L5178Y/r (●) cells were incubated at 37°C with the alkaloids for 72 h as described in Methods. Each point is the mean of 4–8 determinations. Vertical lines represent s.e. mean.

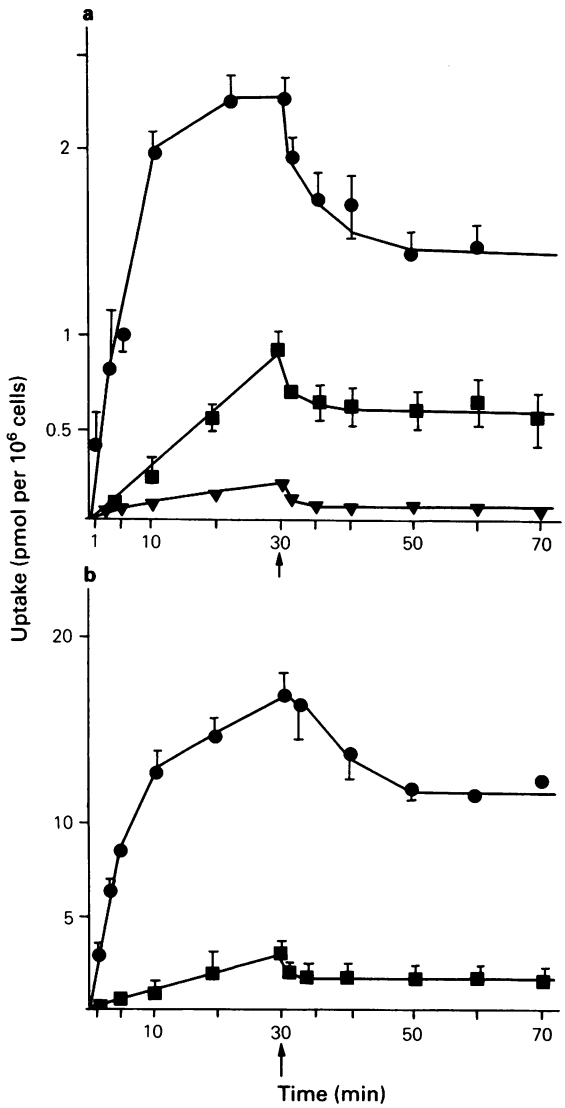

Figure 2 Uptake and retention of vinblastine (●), vincristine (■) and vindesine (▼) by L5178Y cells incubated for 30 min in the continuous presence of the drugs: (a) 7.6×10^{-8} M; (b) 2.4×10^{-7} M. Arrows indicate the cell wash and resuspension in drug-free medium to measure the cell-drug retention. Each point represents the mean value from two to four experiments with vertical lines showing s.e. mean.

leukaemic lymphoblasts at a drug concentration (7.6×10^{-8} M) which is close to the IC_{50} values of the alkaloids (Figure 2a). Although VBL was less toxic than VCR on the cell cultures, its uptake was consistently faster and greater than that of VCR throughout the time of its evaluation. Moreover, during the first 5–10 min of incubation the uptake of VBL was linear and thereafter it tended to equilibrate; this was not observed with VCR, probably because the time of its uptake evaluation was not sufficiently long. The time course of VDS incorporation shows that the rate of its accumulation at 37°C by L5178Y cells decreases with time, reaching a plateau within 40–80 min of incubation (data not shown). The uptake of VDS was considerably lower than that of VCR or VBL so that the amount of VDS taken up by the cells during the time of its evaluation did not exceed one tenth of the total amount of VBL found in the cells, although there was no significant difference between the IC_{50} of either drug.

As previously described, in order to evaluate the release and retention of the three vinca alkaloids by L5178Y cells, following 30 min of incubation in the

presence of drugs, the cells were removed from the incubation medium and resuspended in a drug-free medium. In these conditions, the behaviour of VCR, VBL and VDS on L5178Y cells was similar. The release of the three vinca alkaloids was most rapid during the first 10 min with the result that within this time the drug cell content decreased by 36% for VCR, 26% for VBL and 57% for VDS. Thereafter, the amount of drug retained by the cells did not change even though the incubation time was prolonged. Nevertheless, the total amount of VBL retained by the cells after equilibrium had been reached (1.47 pmol per 10^6 cells), was considerably greater than that of VCR (0.6 pmol per 10^6 cells) or VDS (0.078 pmol per 10^6 cells).

Both with the uptake and retention studies, similar observations were made when the incubations were carried out in the presence of higher concentration of VCR and VBL (Figure 2b).

Uptake and retention of vinca alkaloids by resistant cells (L5178Y/r)

The time courses of the uptake and retention of the three vinca alkaloids by the resistant cells are depicted in Figure 3. In order to compare the behaviour of both cell lines (L5178Y/r and L5178Y), the amount of each drug found in the cells was expressed with respect to the cell protein content to offset the different cell volumes measured in each cell line.

The time courses of the uptake of VCR, VBL and VDS were similar for both cell lines. The VCR incorporation was linear during the evaluation time, while the VBL and VDS rates of entry tended to decrease when the incubation was prolonged. Nevertheless, the total amount of the three drugs incorporated by the cells was always smaller in the resistant cell line with respect to the parental cells, even for VBL to which the cells did not express any resistance. In any case, the resistant cell line incorporated no more than 60% of either alkaloid when compared to that taken up by the parental cells in similar incubation conditions.

As in the parental cell line, the release of vinca alkaloids by the resistant cells was rapid during the first 10 min of their resuspension in drug-free HBSS, the content falling by 34%, 27% and 18% of the total amount of VCR, VDS and VBL incorporated in the cells, respectively. Thereafter, no more loss of any alkaloid was detected even though the incubation time was prolonged. Nevertheless, the total amount of VCR, VDS and VBL retained by the cells was always lower in the resistant cells with respect to the parent cell line from which they were derived, regardless of the degree of resistance expressed in the *in vitro* cell cultures.

Discussion

The similar IC_{50} values found for VDS and VBL on L5178Y cells cannot be accounted for by the amount of drug associated with the cells since, as has been

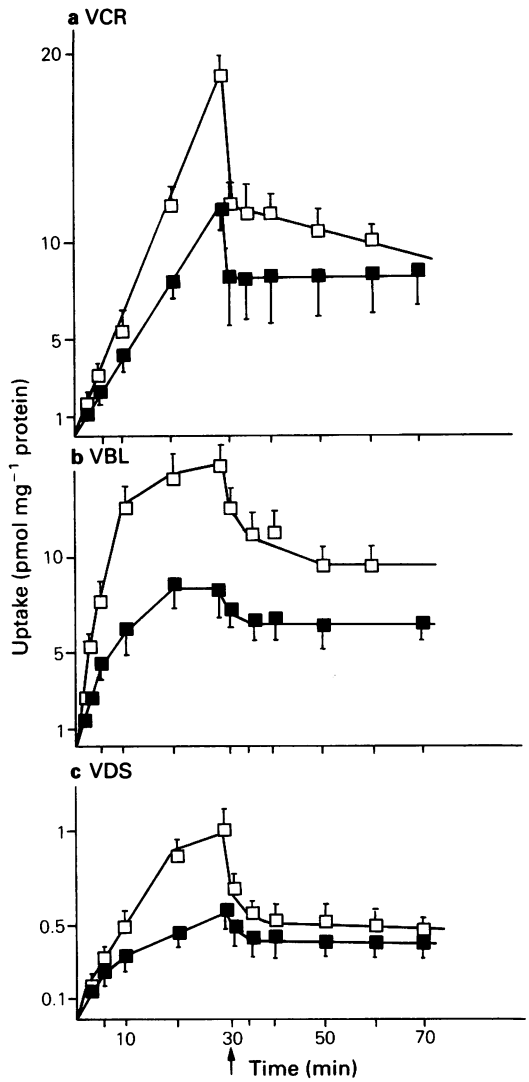

Figure 3 Comparison of the uptake and retention of vinca alkaloids by L5178Y cells (□) and the vincristine (VCR)-resistant subline isolated (L5178Y/r cells) (■). The drug uptake was evaluated for 30 min in the presence of (a) VCR (2.4×10^{-7} M), (b) vinblastine (VBL; 7.6×10^{-8} M) and (c) vindesine (VDS; 7.6×10^{-6} M). Arrow indicates the cell wash and resuspension in drug-free medium. Each point represents the mean value from two to four experiments with vertical lines showing the s.e. mean.

shown, VBL was incorporated in a higher concentration than VDS. This conclusion cannot be drawn for VCR, because the VCR uptake evaluation time was not long enough to reach a plateau as with VDS and VBL. Thus, the amount of VCR incorporated in the cells when the cytotoxic action was evaluated, 72 h in our case, could be similar or even higher than that of VBL (Ferguson *et al.*, 1984; Gout *et al.*, 1984) which would justify the different IC_{50} values obtained for each drug. It should be kept in mind that during the evaluation of the alkaloid cytotoxicity the cells were in the continuous presence of the drugs.

The differences observed in the amounts of VBL and VDS associated with the cells cannot be ascribed to a differential reactivity of these drugs with the microtubules, since it has been demonstrated *in vitro*, that these alkaloids are almost equieffective in blocking tubulin polymerization and the induction of microtubule distortions (Himes *et al.*, 1976; Owellen *et al.*, 1977a), effects to which their oncolytic action could be ascribed (Creasey, 1979). The difference cannot be attributed to the metabolic inactivation of VBL either, since the major metabolite derived from VBL, deacyl vinblastine (DVLD), is biologically even more active than the parent drug (Owellen *et al.*, 1977b).

All this seems to indicate that in L5178Y cells, a fraction of the VBL incorporated is diverted to some cellular compartment(s) or rate controlling process(es) which keeps the drug away from its target, hindering its cytotoxic action. The existence of this compartment(s) or rate controlling process(es) for 'inactive' drug is also suggested by the finding that VBL has the same IC_{50} values on the resistant and parental cells, though we have proved that the former cells take up a proportionally lower amount of alkaloid than the parental cells. This could mean: firstly, that the parental cells accumulated a fraction of alkaloid which was not used to induce the cytotoxic effect, and secondly, that in the resistant cell line those compartment(s) have a proportionally minor capacity to accumulate, retain or deviate the drug from its target.

Some of the cell-associated VBL which is not active on the tubulin could be partitioned into the lipid phase of cellular membranes, as suggested by Ferguson *et al.* (1984). This would explain the greater retention of VBL versus VCR and VDS, since it has been proved by Owellen *et al.* (1977a) that the octanol : water partition coefficient of VBL is much larger than those of VCR and VDS.

If the differences in the behaviour of VBL, VCR and VDS were due to the partitioning into the lipid phase of the cellular membranes, this could indicate a decrease in the lipid content or a change in the lipid pattern in the resistant cell line. This is a possibility that requires more detailed investigation, although Wright *et al.* (1985, 1986) have demonstrated some alterations in the content of ether-linked phospholipids in leukaemic lymphoblasts when they become resistant to VBL. Nevertheless, this does not seem to be the only reason for the differences in the level of alkaloid associated with the cells, because if this were the case, the drug content of the cells would re-equilibrate with the drug-free medium when the cells were washed. This did not occur under our experimental conditions, as 40 min after resuspension in drug-free medium the cells still retained 64% of the drug incorporated. This would imply that some other mechanism(s) besides the lipid partitioning might intervene in the accumulation of the drug in the cell, such as the interaction with some cellular protein, as has been proposed by Cornwell *et al.* (1986), or some energy-dependent processes which would modulate the cellular accumulation of the drug (Beck *et al.*, 1983; Bowman *et al.*, 1986; Jordan *et al.*, 1986; Wright *et al.*, 1986).

This kind of intracellular compartmentalization could also intervene in the association of VCR and VDS with the cell, but if this were so, it would work with less 'affinity' for VCR and VDS than for VBL. In fact, after the cell wash the amount of VBL retained by the L5178Y cells was still higher than that of VDS, although their cytotoxic action was the same.

Furthermore, although the resistance to vinca alkaloids has been atributed to a decreased membrane permeability and/or drug retention by the cells (Bruce *et al.*, 1969; Bleyer *et al.* 1975; Bender *et al.*, 1982; Beck *et al.*, 1983; Beck, 1984; Ganapathi *et al.*, 1986), some other factors seem to play a role in the resistance shown by the L5178Y/r which we have isolated. We have demonstrated that this cell line takes up and retains a smaller amount of VCR, VBL or VDS than the parental cells, regardless of the degree of resistance attained.

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