Biochemical Pharmacology, Vol. 24, pp. 633–639. Pergamon Press, 1975. Printed in Great Britain.

UPTAKE AND BINDING OF VINCRISTINE BY MURINE LEUKEMIA CELLS

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(*Received* 5 *May* 1974; *accepted* 23 *August* 1974)

Abstract—Uptake, metabolism and binding of tritium-labeled vincristine sulfate were studied in L1210, P388 and P388/VCR murine leukemia cells *in vitro*. Negligible metabolism of the drug by these cells was suggested by thin-layer chromatography experiments, in which more than 90 per cent of the label was recovered from cells incubated in tritiated vincristine, and greater than 95 per cent of the recovered tritium was found in the vincristine peak. Serial uptake measurements between 30 sec and 40 min disclosed a biphasic uptake pattern, the early component of which demonstrated Michaelis–Menten kinetics, temperature dependence, inhibition by metabolic poisons, and competitive inhibition by the structural analogue, vinblastine. Efflux experiments disclosed a fraction which was not free to leave the cell and progressively increased during uptake, but at a rate slower than that of entry of the alkaloid into the cells. Accumulation of total and of bound vincristine occurred most rapidly in P388 cells, the subline most sensitive among those studied to the cytotoxic effects of vincristine. The results implicate the presence of a carrier-mediated transport mechanism for translocation of the drug into the cells, and suggest that drug resistance in these cells is due at least in part to impaired accumulation and binding of vincristine within the cell.

Although vincristine (VCR) has been widely used as a cancer chemotherapeutic agent for more than a decade, the pharmacology and cellular pharmacokinetics of this agent have not yet been characterized. Recent acquisition of tritium-labeled VCR (^{3}H-VCR) has facilitated pharmacologic evaluation of this agent and prompted the studies summarized in this report. Experiments are described which evaluate the mechanism of transport, possible metabolism and binding of ^{3}H-VCR by murine leukemia cells *in vitro*. The results implicate the presence of an energy-dependent transport mechanism for translocation of the drug into the cells, and suggest that drug resistance in these cells is due at least in part to impaired accumulation and binding of VCR within the cell.

MATERIALS AND METHODS

^{3}H-VCR. Radioactive VCR was obtained from Dr. James P. Kutney at the University of British Columbia through NIH Contract N01-CM-23223 and was prepared labeled aromatically with tritium [1, 2]. The material was supplied in the lyophilized form as the free base with a specific activity of $2{\cdot}67 \times 10^7$ dis/min/mg. More than 95 per cent of the tritium label chromatographed with VCR, utilizing thin-layer Silica gel plates and an ascending ammonia–acetone solvent system [3]. The plate was spotted with 5 μl of the material to be chromatographed, exposed in one tank to concentrated ammonia vapor for 5 min, then developed in acetone in a second tank. After chromatography, the Silica gel was divided into 5-mm sections and scraped into scintillation vials for counting. Thin-layer chromatography disclosed decomposition of the parent compound at 4° in the dark within 7–14 days after reconstitution in aqueous solution. For each day of experimentation, 0·824 mg lyophilized drug was dissolved in 0·97 ml distilled water and 0·03 ml concentrated H_2SO_4. All experiments were conducted in a darkened room partially illuminated only by daylight.

Cells. L1210 murine leukemia cells were propagated in cell suspension in tissue culture and harvested during log-phase growth when the cell count was between 10^5 and 10^6/ml. P388 and P388/VCR cells were grown intraperitoneally in CDF_1 (BALB/cAnNxC57BL/KaLWN) mice and removed on day 7 after inoculation when red blood cell contamination of the ascitic fluid was negligible. The cells were washed twice in ice-cold phosphate-buffered 0·85% saline prior to resuspension in experimental media (see below).

Identification of accumulated material. To determine the extent of cell metabolism of ^{3}H-VCR during the longest experimental procedure, suspensions of L1210, P388 and P388/VCR cells were each incubated with 2 μM ^{3}H-VCR for 60 min. The cell fraction was then separated by centrifugation, washed twice with ice-cold saline, homogenized, and freeze-thawed three times. After centrifugation at 9000 *g* for 20 min, aliquots of the supernatant were chromatographed as previously described [3] and compared with chromatograms of the original ^{3}H-VCR. By this technique, more than 95 per cent of the tritium was found in the VCR peak in each of the cell sublines. Greater than 90 per cent of the total radioactivity added was recovered

from each of the supernatants. These observations suggested that metabolic conversion of ^{3}H-VCR by L1210, P388 or P388/VCR cells was negligible during any of the experimental conditions utilized in this study.

Incubation buffer and technique. The incubation buffer (referred to throughout as the "buffer solution") consisted of NaCl 125 mM, KCl 4·0 mM, K_2HPO_4 1·1 mM, $NaHCO_3$ 16·0 mM, $CaCl_2$ 1·9 mM and $MgCl_2$ 1 mM, and was aerated with 95% O_2–5% CO_2 for 15 min to adjust the pH to 7·4. All incubations were performed at 37°, unless otherwise mentioned, and a pH of 7·4 was maintained in the presence of 95% O_2–5% CO_2. Cytocrits were less than 1·0 as measured in a Shevky–Stafford centrifuge tube, and the cell counts were less than 2×10^7/cm^3. Extracellular VCR concentration ($[VCR]_e$) of 0·7 to 1·0 μM was selected from blood levels reported in rats 1 hr after intravenous injection of 1 mg/kg [3], and from calculation of plasma levels in man after a single intravenous dose of 2 mg/m^2 body surface area (2 mg/m^2 ÷ 1·5 l. plasma/m^2 ≃ 1·3 mg/l. or 1·4 μM if initial distribution is limited to plasma compartment). With L1210 and P388/VCR cells, $[VCR]_e$ declined less than 3 per cent during the first 4 min of incubation with ^{3}H-VCR, and less than 5 per cent during 40 min of incubation. Rapid uptake of ^{3}H-VCR by P388 cells resulted in decrements of $[VCR]_e$ in excess of 15 and 20 per cent at 4 and 40 min respectively.

Sampling procedure. Incubations were terminated by removing a 2 to 5-cm^3 aliquot of cell suspension, rapidly transferring the aliquot to a 13-ml conical tube containing 5 cm^3 of ice-cold 0·85% NaCl, and immediately centrifuging at 2000 *g* for 30 sec in an International model V unit. The centrifuge was stopped within 10 sec by the hand brake and the residual supernatant thoroughly aspirated. This was followed by two ice-cold 0·85% NaCl washes of 5-cc volume to remove extracellular VCR. Efflux experiments at 4° with and without washing the cells in ice-cold saline prior to resuspension indicated that less than 2 per cent of the ^{3}H-VCR was lost from the cells during the wash procedure (see also Fig. 4). Cell pellets were prepared according to the technique of Goldman *et al.* [4]. After removing the residual supernatant, the cell plug was drawn up into the tip of a Pasteur pipette and extruded onto a flexible polyethylene disc. The cell pellet was then dried overnight at 70° to constant weight, removed from the oven, and immediately separated from the polyethylene disc and weighed on a Cahn RG autoelectrobalance with a digital readout. Increase in weight during exposure to room temperature air was corrected by serial weighings and interpolation to time of removal from the oven. The weight of each pellet ranged from 0·7 to 2·0 mg. The pellet was then placed at the bottom of a scintillation vial and digested in 0·3 ml of 1 N KOH at 70° for 1 hr. After cooling to room temperature, 18 ml scintillation fluid containing toluene and methanol [4] was added and the vials were counted in a Beckman LS230 liquid scintillation unit. The average counting efficiency was 61 per cent and the quench variation between samples was negligible.

Measurement of initial uptake velocity. Cell suspensions were added to 13-ml conical tubes containing ^{3}H-VCR in buffer solution previously gassed with 95% O_2–5% CO_2 and warmed to 37°. The tubes were then stoppered and vigorously shaken in a 37° water bath. The incubation was terminated by centrifugation at 2000 *g* for 30 sec and the cells were processed as described above.

Measurement of VCR efflux. After incubation in ^{3}H-VCR, the cells were centrifuged and washed twice with ice-cold buffer. An aliquot of packed cells was then removed from the cold pellet for drying, weighing and counting (the pre-efflux or "0" time cell VCR value), and the remainder of the pellet was immediately and directly resuspended into 40 ml VCR-free buffer. Samples were taken and processed as described above.

Measurement of $[VCR]_e$. Initial and final extracellular VCR concentrations were measured in all experiments by centrifuging an aliquot of the cell suspension and counting 0·1-ml samples of the supernatant for ^{3}H-VCR.

RESULTS

Time course of VCR uptake in L1210 cells. The time course of ^{3}H-VCR uptake by L1210 cells *in vitro* was measured in 15 separate experiments, with $[VCR]_e$ of 0·7 to 1·0 μM. Serial measurements between 30 sec and 40 min after addition of ^{3}H-VCR to the cell suspension disclosed a biphasic uptake pattern (Fig. 1), with an early linear component between 1 and 4 min (Fig. 1, Insert) and a slower linear component after 10 min. In that the initial uptake component was thought to be related to entry of the vinca alkaloid into the cells, it was studied in detail to characterize the mechanism of

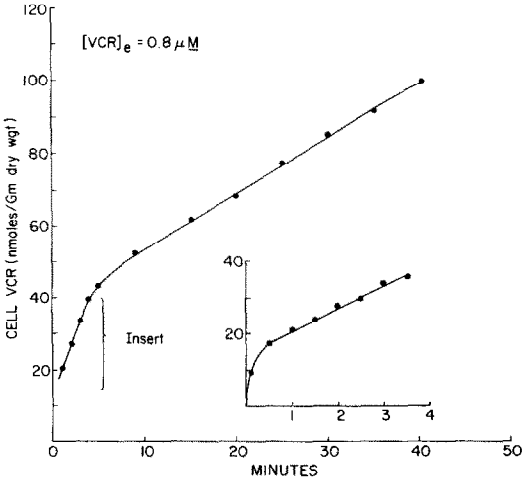

Fig. 1. Representative uptake of ^{3}H-VCR by L1210 murine leukemia cells *in vitro*. The initial linear component is amplified in the Insert.

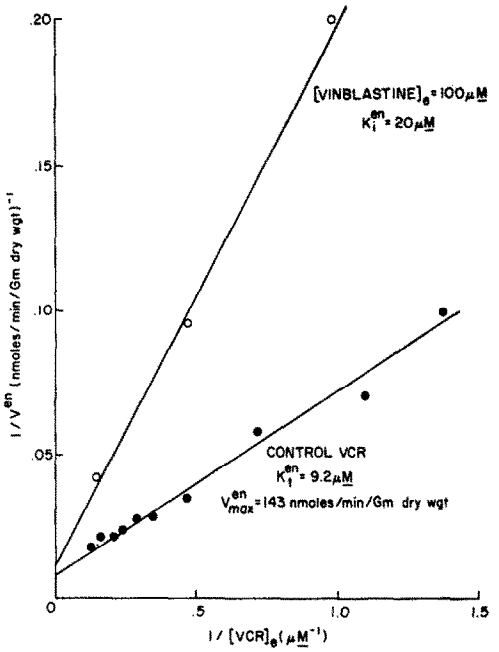

Fig. 2. Inverse of the velocity of the initial uptake component of ^{3}H-VCR uptake by L1210 cells vs the increase of extracellular ^{3}H-VCR concentration in the absence (closed circles) and presence (open circles) of vinblastine sulfate, 100 μM.

transport. The early uptake component will be referred to in this report as the "initial uptake component", although it is apparent that there is an earlier uptake phase within the first 15 sec (Fig. 1, Insert), a period during which limitations of the technique prohibit accurate measurements.

Kinetics of the initial uptake component in L1210 cells. The velocity of the initial uptake component in L1210 cells was measured as a function of $[VCR]_e$ between 0·8 and 8·0 μM. The initial uptake velocity appeared to be a hyperbolic function of $[VCR]_e$, with the velocity approaching an asymptote or maximum rate of entry (V_{max}^{en}). When the reciprocal of velocity was plotted as a function of the reciprocal of $[VCR]_e$, as in the lower line of Fig. 2, a straight line consistent with Michaelis–Menten kinetics was obtained. The V_{max}^{en} was measured at 143 nmoles/min/g dry weight and the concentration at which the velocity was one half the V_{max}^{en} was 9·2 μM (K_i^{en}).

Competition of VCR influx with structural analogue. The initial uptake component was measured in the presence and absence of vinblastine, which differs in molecular structure from VCR only in that in contains a methyl group in place of the formyl group attached to the nitrogen of the dihydroindole portion of the molecule (see Fig. 8). Vinblastine significantly reduced the initial velocity of VCR uptake by L1210 cells (Table 1). Moreover, the inverse plot of VCR in the presence of 100 μM vinblastine (Fig. 2, upper line) revealed that the slope was increased but the ordinate intercept unchanged, suggesting competitive inhibition.

Effects of temperature and of metabolic inhibitors on the initial uptake component. As seen in Fig. 3, the initial uptake component was highly temperature dependent, with a Q_{10} of 6·3 between 27 and 37°. Sodium fluoride 1 mM, *p*-chloromercuribenzoate 20 μM and ouabain 1 mM also reduced the initial velocity of VCR uptake by L1210 cells (Table 1).

Binding of VCR to L1210 cells. When L1210 cells were preloaded with ^{3}H-VCR, washed and resuspended in a large volume of VCR-free buffer solution at 37°, cell ^{3}H-VCR diminished rapidly to a plateau value which could not be reduced with additional washes and reincubation in VCR-free buffer solution

Table 1. Effect of metabolic inhibitors and VCR analogue on the initial velocity of VCR uptake by L1210 cells*

Inhibitor added	% Inhibition
Vinblastine sulfate, 0·1 mM	68 (±4·0)†
Sodium fluoride, 1 mM	50 (±1·0)
p-Chloromercuribenzoate, 0·02 mM	28 (±1·7)
Ouabain, 1 mM	18 (±1·4)

* Initial velocity was obtained from the slope of the linear regression curve derived from total cell tritium between 1 and 3 min after addition of ^{3}H-VCR. $[VCR]_e$ = 0·8 μM.

† Mean ± S.E.M.

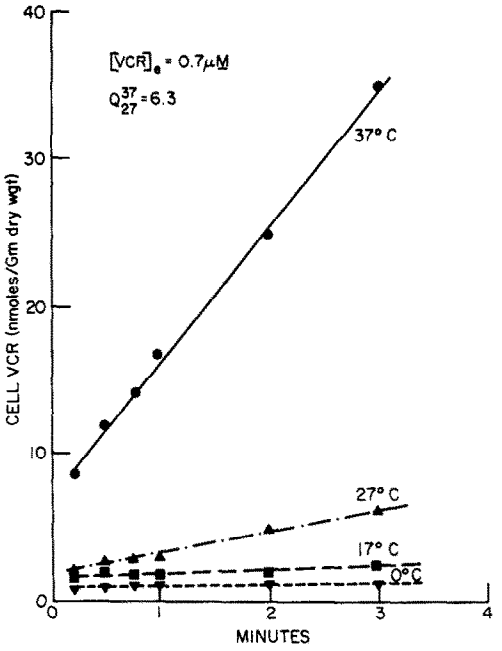

Fig. 3. Effect of temperature on the velocity of the initial uptake component in L1210 cells.

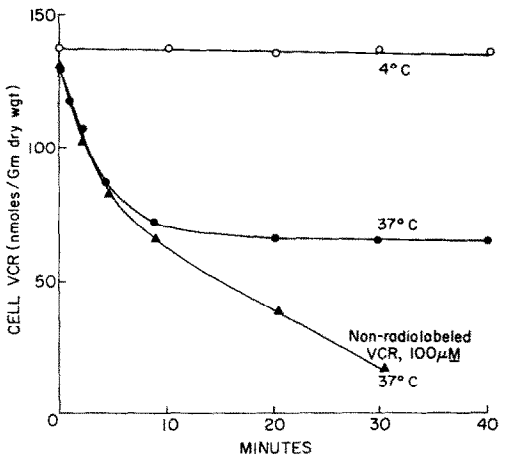

Fig. 4. Efflux of ^{3}H-VCR from L1210 cells. Cells were incubated with 0·9 μM ^{3}H-VCR for 40 min, washed in ice-cold saline and divided into three portions. One portion was resuspended in a large volume 37° VCR-free buffer (solid circles), one portion was resuspended in VCR-free buffer at 4° (open circles), and the third portion was resuspended in 37° buffer containing non-radiolabeled VCR at 100 μM (closed triangles). The points at time zero were obtained from cells taken from the washed pellet immediately prior to resuspension.

(Fig. 4). This plateau level was considered to be alkaloid which was not free to leave the cells and is referred to as "bound" VCR. Efflux was totally inhibited at 4° (Fig. 4). ^{3}H-VCR could be released from binding sites by resuspension in non-radiolabeled VCR at 100 μM (lowest curve in Fig. 4).

Efflux of ^{3}H-VCR from L1210 cells in 37° buffer solution was performed after intervals of uptake ranging from 30 sec to 60 min. In each instance, the plateau level was reached within 15 min and no additional VCR could be removed from the cells thereafter. Serial effluxes during the same uptake disclosed bound VCR by 30 sec and a progressive increase in the bound component thereafter (Fig. 5). During the initial uptake component, bound VCR accumulated more slowly than did total VCR (Fig. 5), averaging 36, 37, 37 and 41 per cent of the total VCR at 1, 2, 3 and 4 min, respectively (means of four experiments). During the second uptake component, total and bound VCR increased in a parallel fashion (Fig. 5). Saturation of the cellular binding capacity for VCR was not observed with L1210 cells during 60 min of incubation in 1 μM ^{3}H-VCR.

Binding vs transport during the initial uptake component. Since significant levels of bound VCR were detected during the initial uptake component, studies were undertaken to determine whether changes in the initial linear component could have been due to changes in binding, rather than to alterations in transport. All experimental conditions producing a diminished velocity of the initial uptake component (27°, 17°, 0°, vinblastine, ouabain, sodium fluoride, *p*-chloromercuribenzoate) were repeated in triplicate for quantitation of both total and bound VCR during exposure to these experimental conditions. Except for vinblastine and *p*-chloromercuribenzoate, there were no significant changes in bound ^{3}H-VCR during the first 3 min of uptake. Vinblastine and *p*-chloromercuribenzoate reduced bound VCR during the initial uptake component, but the decrement in bound VCR could not account for more than half of the reduction in total VCR. Results of these studies suggest that changes in the initial uptake component observed with temperature, metabolic inhibitors and a structural analogue were due primarily to alterations in the transport mechanism.

Comparison of uptake and efflux in P388, P388/VCR and L1210 cells. L1210, P388 and P388/VCR cells were compared for VCR transport and binding characteristics. L1210 and P388/VCR cells are totally resistant to the cytotoxic effects of VCR, whereas P388 cells are readily killed *in vivo* and *in vitro* by VCR. P388/VCR is a drug-induced VCR-resistant cell subline of P388.

The initial uptake component in P388 and P388/VCR cells (measured between 1 and 3 min in P388 cells) was found to be temperature-dependent, saturable, and attenuated by metabolic inhibitors, i.e. analogous to the effects observed in L1210 cells. Quantita-

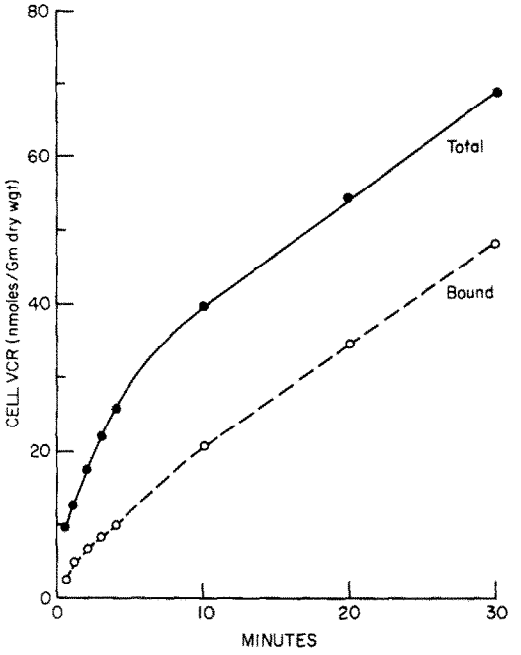

Fig. 5. Total and bound ^{3}H-VCR as a function of uptake interval. "Bound" values were derived from efflux experiments in which aliquots of cells were removed from the incubation flask, washed and resuspended for 30 min in a large volume of VCR-free buffer. Both uptake and effluxes were performed at 37°. Uptake was performed with 0·7 μM ^{3}H-VCR.

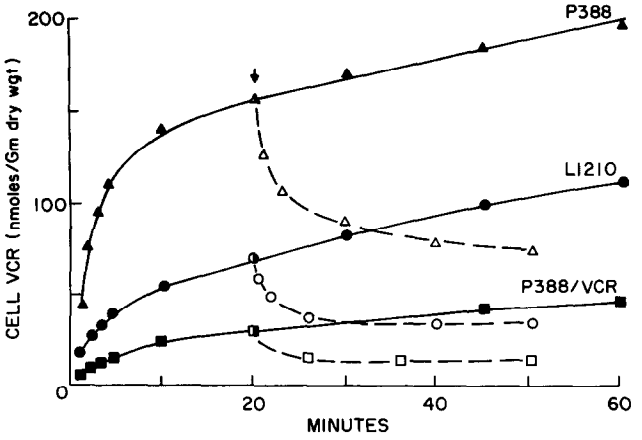

Fig. 6. Comparison of uptake and efflux of ^{3}H-VCR in three different sublines of murine leukemia cells. [VCR]$_e$ was 0·8 μM during uptake. Both uptake and efflux were conducted at 37°.

tive evaluation of the uptake kinetics in P388 cells was prohibited, however, by the rapidity of uptake and the reduction in [VCR]$_e$ noted with the experimental conditions utilized in these studies.

As seen in Fig. 6, the drug-sensitive P388 cells demonstrated significantly greater uptake and binding of VCR than did the drug-resistant P388/VCR and L1210 cell lines. Also, both free and bound VCR accumulated at a significantly faster rate in P388 than in P388/VCR or L1210 cells (Fig. 7). Moreover, the binding capacity of P388 cells for VCR was exceeded in some experiments during the experimental period, whereas there was no evidence for saturation of binding capacity in any experiment with the drug-resistant cells (Fig. 7).

DISCUSSION

VCR is a dimeric periwinkle alkaloid (Fig. 8) with a molecular weight of 824, making it one of the largest molecules among the clinically useful antitumor agents requiring entry into the cell for cytotoxic effect. Once within the cell, the periwinkle alkaloids bind with high affinity to microtubules [5], forming protein–drug precipitates [6–8] which are thought to be the filamentous structures and crystals observed in electron micrographs [9–11]. The microtubule subunit to which the alkaloid binds appears to be a soluble, cytoplasmic protein of approximately 120,000 molecular weight [12].

Vinca alkaloids apparently exert their cytotoxic effect by interacting with microtubular elements of the mitotic apparatus, thereby impairing formation of a functional mitotic spindle and arresting cell division in metaphase [13–17]. Interaction with microtubules of cilia and flagella may interfere with cell movement, and binding to filamentous protein structures immediately beneath the cell membrane may change cell shape [18–20] or alter membrane function [21–23].

Studies described in this report suggest that in murine leukemia cells, VCR is: (1) rapidly accumulated by a carrier-mediated transport system, (2) negligibly metabolized, (3) tightly bound to or within the cell at a rate which appears slow in relation to transport and (4) transported and bound more slowly in cells resistant to the cytotoxic effects of VCR than in cells which are readily killed by the drug.

Demonstration of a carrier-mediated transport mechanism for translocation of VCR into the cell would help explain how this relatively large molecule enters the cell. In this study, such a mechanism was evidenced by the effects of temperature, a structural analogue, metabolic inhibitors and extracellular VCR concentration on the initial velocity of VCR uptake. Another consideration compatible with an energy-dependent mechanism is calculation of the intracellular concentration of free VCR achieved by the cell. The latter can be estimated from the amount of ^{3}H-VCR

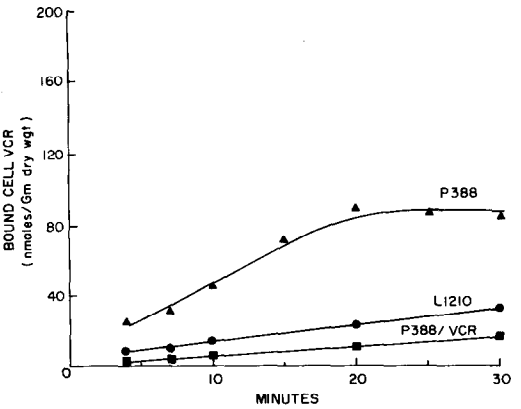

Fig. 7. Comparison of the accumulation of bound ^{3}H-VCR in three different sublines of murine leukemia cells at 37°.

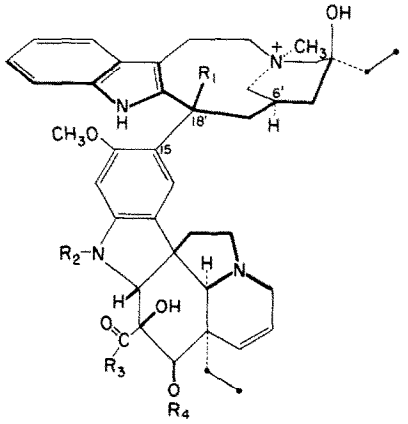

Fig. 8. Conformational structure of vincristine methiodide with $R_1 = COOCH_3$, $R_2 = CHO$, $R_3 = OCH_3$ and $R_4 = COCH_3$ (according to J. W. Moncrief and W. N. Lipscomb, *Acta crystallogr.* **21**, 322, 1966). In vinblastine, $R_2 = CH_3$.

which was free to leave the cell in efflux experiments, assuming that the exchangeable VCR is unbound (low-affinity binding cannot be excluded) and homogenously distributed within the cell water. Twenty to 50 nmoles ^{3}H-VCR/g dry wt of cells exited rapidly from L1210 cells which had been preloaded for 10–40 min in 0·7 to 1·0 μM $[VCR]_e$ (Figs. 4, 5 and 6). Since the cells contain 3·73 to 3·90 ml water/g dry wt [4], the intracellular VCR concentration can be estimated as between 5·2 and 13·1 μM, or from 5·2- to 18·7-fold higher than the extracellular concentration.

Murine leukemia cells are not unique in their capacity for rapid accumulation of a vinca alkaloid. Rat blood platelets [18, 24], Ehrlich ascites cells [25] and S180 sarcoma cells [25] have been shown to accumulate tritiated vinblastine rapidly, and to be unable to metabolize the vinca compound. All of these studies were performed with vinblastine, however, and none have distinguished between transport and binding of the alkaloid by the individual cell types.

The efflux experiments described in this study indicate that a fraction of the total VCR within the murine leukemia cells binds tightly to a site or sites within the cell. This observation is compatible with a high affinity binding site such as the microtubular protein receptor, but identification of the receptor will have to await subcellular localization of the bound ^{3}H-VCR, studies of which are currently in progress. It will also be important to determine whether binding varies with phases of the cell cycle, a possibility suggested by George *et al.* [14], and by the fact that mitotic spindles are present in the cell for a very short portion of the cell cycle.

Drug resistance has been related to alterations in transport mechanisms, receptor sites, intracellular activation and inactivation, and other factors. Creasey [26] noted that vinblastine-resistant Ehrlich ascites cells demonstrated both reduced uptake of tritiated vinblastine and slower conversion of the drug to bound, alkali-labile material. The study described here extends this observation to VCR in VCR-resistant murine leukemia cells. Additional experiments will be necessary to determine whether the smaller amount of bound VCR in resistant cells is secondary to a slower rate of VCR influx, fewer receptor sites available for binding, or a lower affinity of the receptor site for the alkaloid.

Although it is not clear why murine leukemia cells should have a carrier-mediated transport mechanism for the translocation of VCR into the cell, demonstration of its presence may ultimately permit explanations as to why certain tumors are or become resistant to this drug. Characterization of the transport and binding processes may also provide new approaches toward the clinical use of the vinca alkaloids.

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