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CELLULAR UPTAKE AND TUBULIN BINDING PROPERTIES OF FOUR VINCA ALKALOIDS

WILLIAM D. SINGER and RICHARD H. HIMES*
Department of Biochemistry, University of Kansas, Lawrence, KS 66045-2106, U.S.A.

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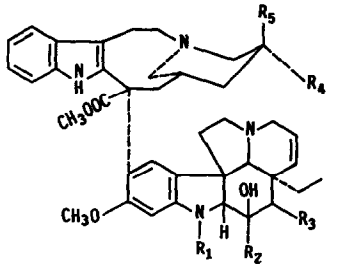
Abstract—The *in vitro* effects of four Vinca alkaloids, vinblastine (VLB), vincristine (VCR), vindesine (VDS) and vinepidine (VPD), on B16 melanoma proliferation, binding to bovine brain tubulin and B16 melanoma cell extracts, and uptake by the B16 cells were compared. The relative binding affinities to bovine brain tubulin were VPD > VCR ≈ VDS > VLB with the K_a for VPD being about 4-fold higher than that for VLB. On the other hand, the relative effects on B16 cell proliferation were exactly the opposite. Differences were found in the degree of concentration of the four alkaloids by the cells: 100-fold for VLB, 50-fold for VCR and VDS, and 20-fold for VPD. At the extracellular concentrations of drugs which inhibit proliferation by 50%, the intracellular concentration would still be far less than the tubulin concentration. Thus, it is likely that all of the Vinca alkaloids would be bound to tubulin and difference in uptake rather than K_a values is the major factor in determining the relative effectiveness of the drugs. L cells showed 50% the sensitivity of B16 melanoma cells toward VLB and 30% the sensitivity toward VPD. The L cells also concentrated these drugs to a lesser extent than did the B16 cells.

Vinblastine (VLB†) and vincristine (VCR) are members of the group of *Catharanthus roseus* antimitotic alkaloids referred to as Vinca alkaloids. These two compounds are commonly used in cancer chemotherapy against a variety of tumors. Inhibition of mitosis is a result of the binding of alkaloids to tubulin and microtubules. Numerous studies have shown the intracellular disruption of microtubules by VLB and VCR and, at high enough concentrations, the formation of paracrystals containing tubulin [see Refs. 1–4 for reviews]. At very low concentrations, in one cell line, the Vinca drugs can stop cell proliferation in M-phase without disrupting the spindle apparatus, presumably by inhibiting addition of tubulin to microtubule ends [5]. *In vitro*, the Vinca alkaloids bind to tubulin producing aggregation of the protein [6], inhibit microtubule assembly [7], inhibit the addition of tubulin dimers to the ends of microtubules at steady state [8], bind directly to the walls of microtubules [9], and cause the disruption of the wall structure of microtubules with the resulting formation of microtubule protofilaments coiled into spiral-like structures [10].

Various semisynthetic derivatives of VLB and VCR have been made including vindesine (VDS; deacetylvinblastine amide) and vinepidine (VPD; 4′-deoxyepivincristine) (see Fig. 1), and their activities against tumors, cells in culture, and the *in vitro* polymerization of tubulin have been examined. The relative effectiveness of the four Vinca alkaloids VLB, VCR, VDS and VPD in inhibiting tumor growth and cell proliferation varies and depends on the system being studied. In inhibiting microtubule dynamics at steady state, VPD is the most effective of the four followed by VCR, VDS and VLB, but in inhibiting B16 melanoma cell proliferation in culture, VLB is the most effective and VPD the least [11].



	R_1	R_2	R_3	R_4	R_5
VLB	CH_3	$COOCH_3$	$OCOCH_3$	CH_2CH_3	OH
VDS	CH_3	$CONH_2$	OH	CH_2CH_3	OH
VCR	CHO	$COOCH_3$	$OCOCH_3$	CH_2CH_3	OH
VPD	CHO	$COOCH_3$	$OCOCH_3$	H	CH_2CH_3

Fig. 1. Structures of four Vinca alkaloids. Abbreviations: VLB, vinblastine; VDS, vindesine; VCR, vincristine; and VPD, vinepidine.

The purpose of this study was to determine whether differences in drug uptake by one cell line,

* Corresponding author. Tel. (913) 864-3813; FAX (913) 864-5321.

† Abbreviations: VLB, vinblastine; VDS, vindesine; VCR, vincristine; VPD, vinepidine; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; PEM buffer, 0.1 M Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9; PBSA, phosphate-buffered saline without divalent cations; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); and ELISA, enzyme-linked immunosorbent assay.

B16 melanoma, could explain the differential effects of the four Vinca alkaloids on cell proliferation, or whether the differences could be due to differences in affinity for tubulin.

MATERIALS AND METHODS

Materials. VLB and VCR were purchased from Sigma (St. Louis, MO); VDS and VPD were gifts of Eli Lilly & Co. (Indianapolis, IN). Dulbecco's modified Eagle's Minimal Essential Medium and donor calf serum were obtained from Hazleton (Lenexa, KS). Inulin [^{14}C]carboxyl was purchased from American Radiolabeled Chemicals (St. Louis, MO) and [^{3}H]H_2O from Amersham (Arlington Heights, IL). Anti-β tubulin monoclonal antibody was derived from ascites tumors grown in Balb-C mice injected with hybridoma cells provided by Bonnie Neighbors (University of Colorado, Boulder, CO). Horseradish peroxidase-conjugated goat anti-mouse IgG + IgA + IgM and ABTS were purchased from Zymed (South San Francisco, CA).

Preparation of tubulin. Tubulin was obtained from bovine brain by a modification of the microtubule assembly–disassembly procedure of Shelanski *et al.* [12], followed by phosphocellulose chromatography [13]. Purified tubulin in PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9) was drop-frozen in liquid nitrogen and stored at −80°. Before use, thawed tubulin was cleared of particulate debris by centrifugation at 27,000 *g* for 10 min at 0°, and its concentration was determined by the method of Bradford [14].

Binding of Vinca alkaloids to tubulin. Binding was measured by a previously described gel column centrifugation technique [15, 16], except that radioactive compounds were not used. Samples containing 0.5 mL of tubulin (2 μM) with various concentrations of the Vinca alkaloids in PEM were incubated for 20 min on ice. Unbound drug was then removed by centrifuging duplicate 0.2-mL aliquots through Sephadex G-25 columns (0.6 mL) packed in 1-mL syringes. A 50-μL aliquot of the eluate was used for protein determination and 2.7 mL of ethanol was added to 0.3 mL of the remainder to release the bound alkaloid. After a 30-min incubation at −20° the precipitated protein and Pipes were removed by centrifugation and the ethanol was vacuum-evaporated. The residue was dissolved in 50% methanol in 10 mM KH_2PO_4 (pH 4.5) and the amount of Vinca compound present was determined by reversed-phase HPLC [17]. Chromatography conditions were as follows: Beckman Ultrasphere C_{18} column; isocratic solvent system, 50% methanol in 10 mM KH_2PO_4 (pH 4.5); flow rate, 1.2 mL/min; and detector, 220 nm. The area of the peak due to the alkaloid was determined using either a Shimadzu Chromatopak CR 601 integrator or a Houston Hipad digitizing tablet and Sigmascan (Jandell Scientific) software. The quantity of bound Vinca drug in the eluate was determined from standard curves and used to calculate free concentrations in the original solution. The association constants for tubulin–Vinca binding were calculated according to the method of Scatchard [18]. Association constants were calculated by linear regression analysis using Enzfitter (Elsevier-BIOSOFT) software. Data points up to a value of 1.0 for Vinca bound/tubulin were used.

Tubulin aggregation. Tubulin aggregation in the presence of the Vinca drugs was examined by gel exclusion HPLC [16]. Purified tubulin was polymerized for 30 min at 37° at a concentration of 6.9 mg/mL in PEM buffer containing 0.5 mM GTP. The resultant microtubules were pelleted at 200,000 *g* for 4 min at 37° in Beckman TL-100 ultracentrifuge, resuspended and cold-depolymerized in PEM buffer, and centrifuged again at 4° to remove any cold-stable assembly products. Samples containing 2 μM tubulin and 5 μM Vinca alkaloid were prepared and incubated for 20 min at room temperature. The relative degree of tubulin aggregation was then monitored by passing a 100-μL aliquot through a TSK G-3000 SW column equilibrated with the reaction buffer containing the alkaloid (flow rate = 1 mL/min, detection at 280 nm). The elution times for molecular weight standards (BioRad) in PEM were: aggregate (void volume), 6.4 min; thyroglobulin (670 kDa), 6.4 min; γ-globulin (158 kDa), 9.8 min; ovalbumin (44 kDa), 11.3 min; myoglobin (17 kDa), 13.3 min; and vitamin B_{12} (1.35 kDa), 15.9 min.

Cell proliferation experiments. Murine B16 melanoma and NCTC mouse clone 939 (Strain L, Earle) cells were grown in monolayer cultures in 5% CO_2 at 37° in Dulbecco's modified Eagle's MEM containing 7% donor calf serum. Frozen cells were thawed and grown for at least 15 passages before an experiment. In the proliferation experiments 1-mL aliquots of medium containing 4.2×10^4 cells were added to wells in a 24-well plate. After 24 hr the medium was replaced by medium lacking or containing different concentrations of the drugs and the cells were incubated for another 43 hr. Cells were loosened with 0.05% trypsin–0.02% EDTA in phosphate-buffered saline without divalent cations (PBSA), suspended in medium, and counted in a Coulter counter.

Binding of Vinca alkaloids to cell-free extract. Cells were seeded in 40 mL of medium (1×10^5 cells/mL) in milk dilution bottles and grown for 4 days. They were subsequently harvested in 0.2 mM EDTA in PBSA by scraping with a rubber policeman. The cells were washed by three cycles of centrifugation and resuspension in PBSA. After a fourth centrifugation, the cells were resuspended in an equal volume of 2 × PEM buffer (0.2 M Pipes, 2 mM EGTA, 2 mM $MgSO_4$, pH 6.9). The suspension was sonicated and debris was removed by two centrifugation steps at 4°, the first at 27,000 *g* for 10 min and the second at 100,000 *g* for 45 min. Samples containing 500 μL of the supernatant (4.4 mg protein/mL) with either a 0.3 or 0.75 μM concentration of the Vinca drugs were incubated on ice for 20 min. The amount of bound drug was then determined by the HPLC assay described above except that standards were prepared by adding known alkaloid concentrations to cell extracts, followed by precipitation with ethanol.

Determination of intracellular water content. Water content was determined as described by Rivera-Fillat *et al.* [19]. Cells grown for 48 hr in twelve 2-day-old 100 mm culture dishes were loosened with

0.05% trypsin–0.02% EDTA in PBSA and suspended in 40 mL of medium. The cells were collected by centrifugation, resuspended in 6.5 mL of medium, and counted in a Coulter counter. The medium was distributed to three centrifuge tubes such that they contained 2.1, 4.2, and 6.3×10^7 cells, respectively. Following another centrifugation step, the pellets were taken up in 1 mL of medium containing 1×10^6 cpm each of $[^3H]H_2O$ and inulin ($[^{14}C]$carboxyl). After a 30-min incubation at room temperature the samples were centrifuged, the supernatant was removed carefully, and the pellets were resuspended in 1 mL of PBSA. Aliquots were removed for scintillation counting, and the cell volume was calculated by taking the ^3H content as the sum of the intra- and extracellular H_2O contents and the ^{14}C content as the extracellular volume.

Accumulation of Vinca alkaloids in cells. Cells were grown in 100 mm culture dishes to a density of approximately 1×10^7 cells/dish. At this time the medium was replaced with 4 mL of fresh medium lacking or containing 0.1 or 1 μM alkaloid. After a 90-min incubation at 37° the medium was removed and the plates were washed quickly three times with PBSA. The cells were detached with trypsin–EDTA, suspended in 1 mL PBSA, counted in a Coulter counter, and sonicated.

To remove substances interfering with HPLC analysis, Vincas were extracted by an ion-pairing procedure [20]. Sonicated samples (1 mL) were cleared of protein by the addition of 2 mL of acetonitrile. After centrifugation to remove precipitate, 2.7 mL of the supernatant was evaporated at 60° under a stream of N_2. To each sample was added 6 mL of 0.4 M phosphate buffer, pH 3, containing 0.05 M sodium octylsulfate and 3 mL of chloroform. The mixtures were agitated for 30 min on an orbit shaker; then 2.5 mL of the organic phase was evaporated at 30° under a stream of N_2. The residue was dissolved in 50% methanol in 10 mM KH_2PO_4 (pH 4.5) and the amount of Vinca present was determined by HPLC. The recovery of the drugs added to sonicates by this procedure was greater than 90%.

Determination of tubulin content of B16 melanoma cells. A direct enzyme-linked immunosorbent assay (ELISA) was used to determine cellular tubulin content. The primary antibody, an anti-β tubulin antibody, was obtained from the ascites cells as described above. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG + IgA + IgM and ABTS was used as the chromogen.

RESULTS

Inhibition of cell proliferation by the four Vinca alkaloids. In a previous study of the relative effects of Vinca alkaloids on B16 melanoma cell proliferation the drugs were compared at a single concentration [11]. To obtain a better comparison of the effectiveness we determined proliferation after 43 hr in the presence of different concentrations of the alkaloids (Fig. 2). The data clearly show that the cells were most sensitive to VLB and least sensitive to VPD. Little difference was found between VCR and VDS. The concentrations required for 50% inhibition of proliferation under the conditions used were 4.3 nM (VLB), 9.9 nM (VDS), 11 nM (VCR), and 60 nM (VPD). Previous results had shown a slightly greater inhibition by VDS when compared to VCR [21].

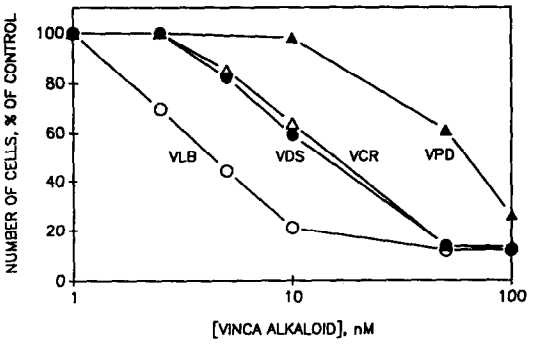


Fig. 2. Inhibition of B16 melanoma cell proliferatioin by the four Vinca alkaloids. Cells were grown for 24 hr before being placed in medium containing the drugs. After 43 hr of incubation the cells were counted. At this time the control contained 6.3×10^5 cells/well. Details are given under Materials and Methods. The data are from one experiment, but similar experiments were done at least four times with similar results.

Binding of the four Vinca alkaloids to bovine tubulin. Although binding of VLB and VCR to tubulin has been studied previously, no information on VPD binding is available and VDS binding has been studied using an impure tubulin preparation and a competitive binding assay. Moreover, because of the influence of solution components on the experimentally determined apparent affinity constant for the tubulin–Vinca alkaloid complex [16, 22, 23], it was important to compare binding using the same tubulin preparation under identical solution conditions. Binding to bovine brain tubulin was measured using the centrifugation–filtration technique and HPLC analysis for the bound alkaloid. Scatchard plots of the data together with apparent K_a values are presented in Fig. 3. Although there were not large differences in the constants, the order of binding affinity was always VPD > VCR ≈ VDS > VLB.

It has been shown that binding of Vinca drugs to tubulin is linked to a protein aggregation process [22]. A qualitative measure of binding is the degree of aggregation produced by the Vinca alkaloid, which can be monitored by size exclusion HPLC [16]. Figure 4 shows the result of such an experiment in which the ratio of Vinca drug to tubulin was maintained at a 2.5:1 molar ratio during chromatography. Tubulin samples usually contain a small amount of aggregated material together with the dimer. In the presence of VLB it is apparent that the sample consists of multiple species with molecular weights greater than that for the dimer. The elution profile in the presence of VLB is similar to that published previously [16]. The degree of aggregation, as indicated by the amount and the

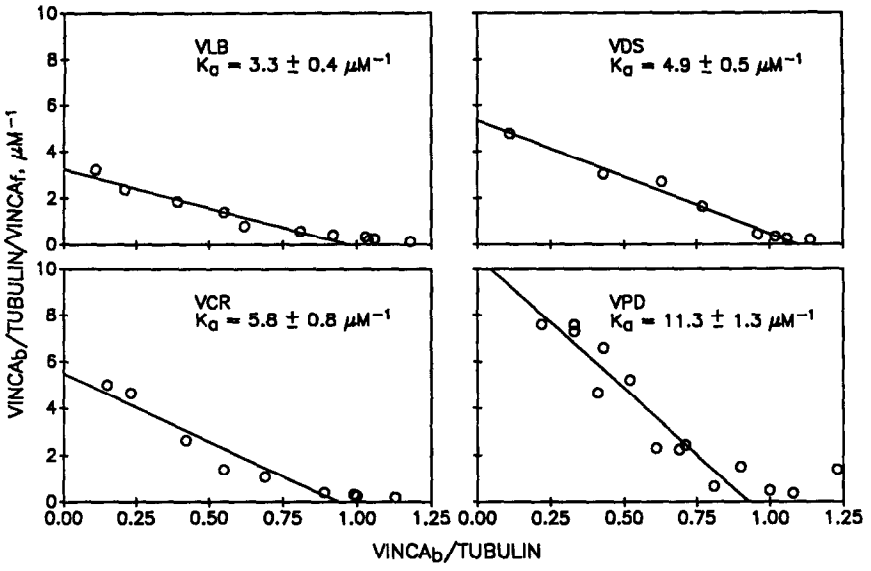


Fig. 3. Binding of four Vincas to bovine brain tubulin. Various concentrations of the Vinca alkaloids were incubated with 2 μM tubulin for 20 min at 0° after which tubulin-bound Vinca (Vinca$_b$) was separated from free Vinca (Vinca$_f$) by column centrifugation. Procedures used are presented under Materials and Methods. Results from one experiment are presented as Scatchard plots. Data points from Vinca$_b$/tubulin ≤ 1 were used to determine the best fit to the line by linear regression. K_a values for the individual Vinca alkaloids were obtained two to four times in other experiments and always showed the order of K_a values as shown in this figure.

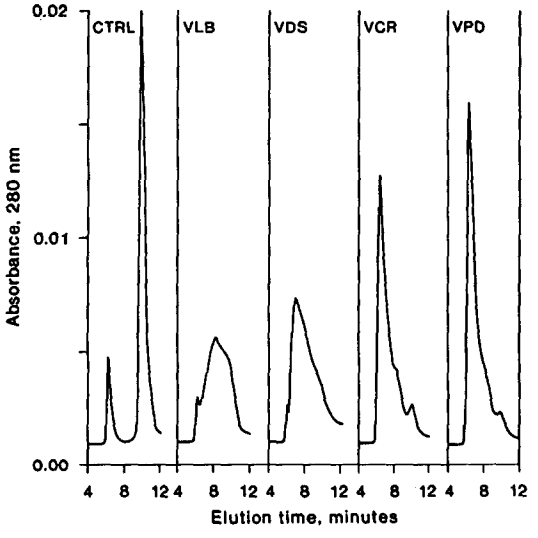


Fig. 4. Vinca-induced aggregation of bovine brain tubulin. Tubulin (2 μM) was incubated in the absence or presence of 5 μM Vinca alkaloids and then placed on a size exclusion HPLC column. The elution buffer (PEM) contained 5 μM Vinca alkaloid. Details are given under Materials and Methods. These data are the result of a single experiment.

time of elution of the fastest-eluting species, increased as the drug was changed to VDS, VCR and finally VPD. The result is consistent with the relative K_a values shown in Fig. 3.

Table 1. Binding of Vinca alkaloids to B16 cell-free extracts

	Vinca bound (μM)	
Alkaloid	0.3 μM	0.75 μM
VLB	0.20 (0.23)	0.50 (0.53)
VDS	0.22 (0.25)	ND*
VCR	0.28 (0.26)	0.65 (0.60)
VPD	0.29 (0.27)	0.65 (0.64)

Binding was measured at two alkaloid concentrations, 0.3 and 0.75 μM and a protein concentration of 4.4 mg/mL. Details are given under Materials and Methods. The numbers in parentheses are calculated values using a tubulin content of 4% of the total protein and the K_a values reported in Fig. 3. The results are from a single experiment. Abbreviations: VLB, vinblastine; VDS, vindesine; VCR, vincristine; and VPD, vinepidine.

* Not determined.

Binding of the Vinca alkaliods to B16 melanoma cell-free extract. It is possible that the relative binding affinity of the four alkaloids to B16 tubulin is not the same as to bovine brain tubulin. To test this we examined binding to cell-free extracts at two alkaloid concentrations. With the use of an ELISA assay we determined that tubulin constituted about 3–4% of the protein of a 100,000 *g* supernatant from a cell extract. Table 1 presents the results of the binding experiments using 0.3 and 0.75 μM alkaloid. At the protein concentration used, the tubulin concentration

Table 2. Uptake of Vinca alkaloids by B16 cells

Drug	Uptake (pmol Vinca/10^6 cells)		Intracellular concentration (μM)	
	1 μM	0.1 μM	1 μM	0.1 μM
VLB	109	11	100	10
VDS	52	5.2	48	4.8
VCR	43	5.6	39	5.1
VPD	21	~0.7*	19	~0.6*

The results are from one experiment. This experiment was repeated five times with similar results. See Materials and Methods for details.

* These figures are uncertain because they are close to the limit of detection by the method used.

was 1.3 to 1.8 μM. Binding was similar for the four compounds but differences were noted and these differences were consistent with the order of binding affinities determined with bovine brain tubulin. As the data show, the amount of binding was close to what was predicted using the binding constants determined for bovine brain tubulin.

Uptake of the four alkaloids by B16 melanoma cells. The results described above, which indicate that bovine brain and B16 tubulin have a higher affinity for VPD than for VLB, are consistent with the greater effectiveness of the former alkaloid in inhibiting tubulin addition to microtubules *in vitro* [11]. However, they are not consistent with the fact that B16 cells are more sensitive to VLB than to VPD [11]. A possible explanation for this apparent discrepancy is that B16 melanoma cells concentrate VLB to a greater extent than VPD.

To measure uptake of the alkaloids, cells were incubated with 0.1 and 1.0 μM concentrations of the drugs for 90 min. Previous studies demonstrated that this time period was sufficient to achieve maximum uptake of VDS and VCR by B16 cells [21], and, in data not shown here, we found that the same time period was sufficient to achieve maximum uptake for VPD and VLB. HPLC was used to determine the cellular content of the alkaloids (Table 2). Using an intracellular water volume of 1.09 μL/10^6 cells, determined by the use of $[^3H]H_2O$ and ^{14}C-carboxylated inulin, the cellular concentration was calculated. VLB was concentrated about 100-fold by the cells, compared to about 50-fold for VDS and VCR and 20-fold for VPD. In comparing the uptake at the two concentrations, it was apparent that the intracellular concentrations of VLB, VDS and VCR were directly proportional to the external concentrations. This was not the case with VPD in which uptake did not appear to show a direct relationship to the external concentration. However, this is difficult to evaluate from just two data points.

Molecular size of protein–Vinca complex in B16 cells. We attempted to address the question of whether the tubulin–vinca complex in cells exists in the dimer or aggregated forms. In other words, is the aggregation phenomenon induced by Vinca alkaloids restricted to the *in vitro* system? After cells were incubated with 0.1 μM VLB for 90 min, cell extracts were made in either 10 mM phosphate buffer or PEM buffer and placed on a TSK G-3000 SW size exclusion column. In the case of extract in phosphate buffer the radioactivity eluted at the time expected for the tubulin dimer whereas the radioactivity in the extract made in PEM buffer eluted earlier than the dimer peak, indicating that it was an aggregated complex. Unfortunately, these results are difficult to interpret. The PEM buffer could have caused the aggregation of the dimer–alkaloid complex or pre-existing aggregated material may have dissociated in phosphate buffer. It is known that the aggregation induced by Vincas and certain solution variables can be partially reversed by removal of these components [16].

Vinca uptake and cell proliferation inhibition using L cells. In a previous study, we found that L cells were somewhat less sensitive to the Vinca alkaloids than were B16 cells [11]. We investigated this in more detail with VLB and VPD and found that the concentration needed to inhibit proliferation by 50%, in an assay done as the experiment in Fig. 2, was 9 nM for VLB and 190 nM for VPD. When we measured the uptake of VLB and VPD, it was found that the former drug was concentrated 60-fold and the latter, about 10-fold. The cell volume of the L cells was close to that of the B16 cells, 0.9 μL/10^6 cells. Thus, a difference in extent of uptake would explain the difference in sensitivity between B16 cells and L cells, and the differences in uptake and toxicity between VLB and VPD were observed with each cell line.

DISCUSSION

The purpose of this study was to determine the reason for the apparent discrepancy between the known relative tubulin-binding activity of some Vinca alkaloids and their anti-proliferative activity. VPD binds to tubulin with a binding constant which is approximately 4-fold higher than that for VLB and about 2-fold higher than that for VCR and VDS. The relative binding we found for VLB, VCR and VDS was similar to what has been reported previously using purified tubulin and microtubule-associated protein-containing tubulin [24–26]. In addition, the relative effects of VCR, VDS and VLB on microtubule assembly and flux *in vitro* are in agreement with their relative tubulin binding efficiencies [11, 27]. The data in this paper on VPD binding and previous data on the inhibition of tubulin flux by this alkaloid [11] are consistent with a direct relationship between tubulin binding affinity and *in vitro* effects on microtubules. The results of the experiments dealing with binding of the four alkaloids to B16 melanoma cell extract indicate that the binding properties of B16 melanoma tubulin do not differ significantly from those of bovine brain tubulin. Therefore, the differential effects of the Vinca alkaloids on B16 melanoma cell proliferation are not due to differential tubulin binding properties.

The most likely explanation for the fact that VPD is much less effective as an anti-proliferative agent

than the other three alkaloids, in spite of its greater binding affinity to tubulin, is that it is not concentrated in cells to the same extent. This was clearly the case and, in fact, the relative concentrations of the Vinca drugs in the cells after a 90-min incubation corresponded to their ability to inhibit proliferation. It is possible to generalize that differential cellular toxicities among Vinca alkaloids which show only small differences in K_a values for tubulin are due to uptake differences. This suggestion is based on the fact that at the extracellular concentrations of the drugs which inhibit proliferation by 50%, the intracellular concentration is still far lower than the tubulin concentration and all of the alkaloid would be bound to tubulin, assuming the K_a values to be those shown in Fig. 3. In the case of the B16 cells, using the determined cell water volume of 1 pL/cell we calculated a cellular protein concentration of ~200 mg/mL and estimated the total tubulin concentration (3–4% of cellular soluble protein) to be 60–80 μM, or 40–50 μM unpolymerized tubulin assuming 30–40% to be in the polymerized state [5, 28]. The intracellular concentrations of the four alkaloids at the extracellular concentration which produced 50% inhibition of cell proliferation, assuming the same degree of concentration as observed at 1 μM drug, would be VLB, 0.43 μM; VDS, 0.50 μM; VCR, 0.44 μM; and VPD, 1.2 μM. Thus, the presence of about one-tubulin–alkaloid complex molecule per 100 tubulin molecules is sufficient to produce 50% inhibition of proliferation. This figure is not far from that calculated to produce 50% inhibition of the self-assembly of tubulin *in vitro* [8]. The value of 1.2 μM for VPD is somewhat higher than those of the other three compounds and may reflect a non-linear relationship between concentration and uptake at low concentrations, as indicated by the data in Table 2 for 0.1 μM VPD.

The lower cellular toxicity of VPD (4-deoxy-epivincristine) when compared to VCR, which has been observed in other cell lines in addition to the B16 melanoma line [5, 29, 30], could be due to the lack of the hydroxy group on the 4′ carbon or to the epimeric configuration at C4′. Based on a comparison of the toxicities of VLB and the C4′ epimer of VLB, vinrosidine, it would appear to be due to the epimeric configuration since both of these latter compounds have the hydroxy group on the 4′ position. Vinrosidine is far less cytotoxic than VLB [5] but in *in vitro* assays its activity is close to that of VLB [31]. It is likely that decreased uptake of vinrosidine by cells is responsible for its decreased activity in cell culture assays. A possible explanation is the high pK_a of the nitrogen in the piperidine ring of the velbanamine (upper) portion of the molecule. In vinrosidine this pK_a value is 8.8 [32] compared to 7.4 for VLB [33]. As a result, vinrosidine would be protonated at pH 7.4, the cell medium pH, and transport through the plasma membrane would be more difficult. VPD, with a similar epimeric structure, may also have a higher pK_a value when compared to VCR. It should be pointed out, however, that results different from those in cell cultures may be obtained when studying effects on tumors in animals. For example, the antitumor activity of VPD against human rhabdomyosarcoma grown in mice was found to be greater than that of VLB, presumably due to longer retention of VPD by the tumor [34].

In comparing the relative sensitivity of two cell lines, B16 melanoma and L, it was found that the former line was about twice as sensitive as the latter to VLB and about three times as sensitive to VPD. There was a good correlation of this relative sensitivity to the relative degree of concentration of the drugs by the two cell lines. B16 melanoma cells concentrated VLB and VPD twice as much as did the L cell line. In addition, in both cases VLB was concentrated to about 5- to 6-fold greater extent than VPD. However, this simple relationship apparently does not hold in all cases. For example, HeLa cells are about ten times more sensitive to VLB than are B16 cells but HeLa cells concentrate the alkaloid about 50% of the extent of the B16 cells [5].

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REFERENCES

1. Wilson L and Bryan J, Biochemical and pharmacological properties of microtubules. *Adv Cell Mol Biol* **3**: 21–72, 1974.
2. Wilson L, Bamburg JR, Mizel SB, Grisham LM and Creswell KM, Interaction of drugs with microtubule proteins. *Fed Proc* **33**: 158–166, 1974.
3. Creasey WA, Biochemistry of the dimeric Catharanthus alkaloids. In: *The Catharanthus Alkaloids* (Eds. Taylor WI and Farnsworth NR), pp. 209–236. Marcel Dekker, New York, 1975.
4. Wilson L, Anderson K and Chin D, Nonstoichiometric poisoning of microtubule polymerization: A model for the mechanism of action of the Vinca alkaloids, phodophyllotoxin and colchicine. In: *Cell Motility* (Eds. Goldman R, Pollard T and Rosenbaum J), Vol. 3, pp. 1051–1064. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1976.
5. Jordan MA, Thrower D and Wilson L, Mechanism of inhibition of cell proliferation by *Vinca* alkaloids. *Cancer Res* **51**: 2212–2222, 1991.
6. Weisenberg RC and Timasheff SN, Aggregation of microtubule subunit protein. Effects of divalent cations, colchicine and vinblastine. *Biochemistry* **9**: 4110–4116, 1970.
7. Owellen RH, Hartke CA, Dickerson RM and Haines FO, Inhibition of tubulin-microtubule polymerization by drugs of the *Vinca* alkaloid class. *Cancer Res* **36**: 1499–1502, 1976.
8. Wilson L, Jordan MA, Morse A and Margolis RL, Interaction of vinblastine with steady-state microtubules *in vitro*. *J Mol Biol* **159**: 125–149, 1982.
9. Singer WD, Jordan MA, Wilson L and Himes RH, Binding of vinblastine to stabilized microtubules. *Mol Pharmacol* **36**: 366–370, 1989.
10. Warfield RKN and Bouck GB, Microtubule-macrotubule transitions: Intermediates after exposure to the mitotic inhibitor vinblastine. *Science* **186**: 1219–1221, 1974.
11. Jordan MA, Himes RH and Wilson L, Comparison of

the effects of vinblastine, vincristine, vindesine, and vinepidine on microtubule dynamics and cell proliferation *in vitro*. *Cancer Res* **45**: 2741–2747, 1985.
12. Shelanski ML, Gaskin F and Cantor CR, Microtubule assembly in the absence of added nucleotides. *Proc Natl Acad Sci USA* **70**: 765–768, 1973.
13. Algaier J and Himes RH, The effects of dimethyl sulfoxide on the kinetics of tubulin assembly. *Biochim Biophys Acta* **954**, 235–243, 1988.
14. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**: 248–254, 1976.
15. Penefsky HS, Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. *J Biol Chem* **252**: 2891–2899, 1977.
16. Singer WD, Hersh RT and Himes RH, Effect of solution variables on the binding of vinblastine to tubulin. *Biochem Pharmacol* **37**: 2691–2696, 1988.
17. Thimmaiah KN and Sethi VS, Chemical characterization of the degradation products of vinblastine dihydrogen sulfate. *Cancer Res* **45**: 5382–5385, 1985.
18. Scatchard G, The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
19. Rivera-Fillat MP, Pallarés-Trujillo J, Domenech C and Grau-Oliete MR, Comparative uptake, retention and action of vincristine, vinblastine and vindesine on murine leukaemic lymphoblasts sensitive and resistant to vincristine. *Br J Pharmacol* **93**: 902–908, 1988.
20. De Smet M, Van Belle SJP, Seneca V, Storme GA and Massart DL, Determination of vinblastine in MO_4 mouse fibrosarcoma cells by high-performance liquid chromatography. *J Chromatogr* **416**: 375–381, 1987.
21. Donoso JA and Himes RH, The action of two *Vinca* alkaloids on B16 melanoma *in vitro*. *Cancer Biochem Biophys* **7**: 133–145, 1984.
22. Na GC and Timasheff SN, Interaction of vinblastine with calf brain tubulin: Multiple equilibria. *Biochemistry* **25**: 6214–6222, 1986.
23. Na GC and Timasheff SN, Interaction of vinblastine with calf brain tubulin: Effects of magnesium ions. *Biochemistry* **25**: 6222–6228, 1986.
24. Lee JC, Harrison D and Timasheff SN, Interaction of vinblastine with calf brain microtubule protein. *J Biol Chem* **250**: 9276–9280, 1975.
25. Prakash V and Timasheff SN. The interaction of vincristine with calf brain tubulin. *J Biol Chem* **258**: 1689–1697, 1983.
26. Conrad RA, Cullinan GJ, Gerzon K and Poore GA, Structure–activity relationships of dimeric *Catharanthus* alkaloids. 2. Experimental antitumor activities of N-substituted deacetylvinblastine amide (vindesine sulfates). *J Med Chem* **22**: 391–400, 1979.
27. Wilson L, Morse ANC and Bryan J, Characterization of acetyl-^{3}H-labeled vinblastine binding to vinblastine-tubulin crystals. *J Mol Biol* **121**: 255–268, 1978.
28. Minotti AM, Barlow SB and Cabral F, Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin. *J Biol Chem* **266**: 3987–3994, 1991.
29. Thompson GL, Boder GB, Bromer WW, Grindley GB and Poore GA, LY119863, a novel potent vinca analog with unique biological properties. *Proc Am Assoc Cancer Res* **23**: 201, 1982.
30. Boder GB, Bromer WW, Poore GA, Thompson GL and Williams DC, Comparative cellular responses to semisynthetic and natural vinca alkaloids. *Proc Am Assoc Cancer Res* **23**: 201, 1982.
31. Potier P, Guénard D and Zavala F, Résultats récents dans le domaine des alcaloïdes antitumoraux du groupe de la vinblastine. Etudes biochemiques. *CR Soc Biol* **173**: 414–424, 1979.
32. Svoboda GH, Alkaloids of vinca rosea (*Catharanthus roseus*). IX. Extraction and characterization of leurosidine and leurocristine. *Lloydia* **24**: 173–178, 1961.
33. Neuss N, Gorman M, Svoboda GH, Maciak G and Beer CT, Vinca alkaloids. III. Characterization of leurosine and vincaleukoblastine, new alkaloids from *Vinca rosea* Linn. *J Am Chem Soc* **81**: 4754–4755, 1959.
34. Mullin K, Houghton PJ, Houghton JA and Horowitz ME, Studies with 4′-deoxyepivincristine (vinepidine), a semisynthetic Vinca alkaloid. *Biochem Pharmacol* **34**: 1975–1979, 1985.