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Action of the *Vinca* Alkaloids Vincristine, Vinblastine, and Desacetyl Vinblastine Amide on Microtubules *in Vitro*[1]

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SUMMARY

The *Vinca* alkaloids differ in their chemotherapeutic effectiveness and their toxicities. To determine whether differences are due to a differential effect on the assembly of tubulin into microtubules, we examined the effects of vincristine, vinblastine, and a newer alkaloid, desacetyl vinblastine amide, on the assembly of bovine brain tubulin *in vitro*.

The three compounds block bovine tubulin polymerization *in vitro* and almost equally effectively at a 1 μM concentration (tubulin, 6.5 μM). At 10 μM, the three alkaloids also interact with preformed microtubules *in vitro*, causing spiral-like distortions of the microtubules. No effect of the alkaloids on polymerization of another fibrous protein, actin, was observed. Thus the differential actions of vinblastine, vincristine, and desacetyl vinblastine amide *in vivo* seem to be based on some biological process other than the reaction with tubulin or the microtubules *per se*.

INTRODUCTION

The *Vinca* alkaloids are widely used in cancer chemotherapy. Microtubular protein, tubulin, has been implicated as a possible target site for these compounds (29). Their oncolytic activity is probably due to the inhibition of formation and possibly to the disruption of the mitotic spindle MT,[3] thereby arresting cell division at metaphase. Although the target molecule for both VCR and VLB appears to be tubulin, their chemotherapeutic effectiveness for different types of malignant cells varies greatly. Whereas VCR is quite effective against Ridgway osteogenic sarcoma and CA755 mammary carcinoma, VLB has no significant effectiveness (24). VCR in combination with other non-*Vinca* drugs is more effective against breast carcinoma, various lymphomas, and lung small cell (oat cell), whereas VLB in this combination is more effective against choriocarcinoma and embryonal carcinoma of the testes (11). The toxic side effects of these drugs may result from their action on tubulin and MT in normal cells. However, the major toxicity of the 2 drugs differs strikingly: the use of VCR is limited chiefly by a neuropathy and of VLB by marrow suppression.

A newer *Vinca* alkaloid, VDS, has shown similar chemotherapeutic behavior to VCR in tests against various experimental cell lines (24). The structure of this compound is presented in Chart 1. Clinical trials are only in the Phase 1 stage, although both neuropathy and marrow suppression may be significant side effects (4).

The question raised in this study was whether or not the biological differences of these 3 alkaloids can be ascribed to differences in their reactivity with the microtubule protein. The comparative effects of the 3 *Vinca* alkaloids on the *in vitro* polymerization of tubulin and on MT formed *in vitro* were determined. It was found that VCR, VLB, and VDS showed only small differences in their ability to prevent tubulin polymerization. Furthermore, all 3 drugs caused distortion in the structure of preformed MT at identical concentrations.

MATERIALS AND METHODS

Beef brain tubulin was purified by the polymerization method of Shelanski *et al.* (23) as described previously (14). The extraction and reassembly buffer was 20 mM [2(*N*-morpholino]ethanesulfonic acid] 70 mM NaCl, 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid, and 0.5 mM $MgCl_2$, pH 6.4. GTP (0.5 mM) was added for polymerization. The protein was purified through 2 polymerization cycles and was stored at −80° in the buffer containing 2 M glycerol. Sodium dodecyl sulfate-gel electrophoresis shows the preparation to be about 90% tubulin. G-actin was extracted from an acetone powder of rabbit muscle by the procedure of Rees and Young (21) and was dialyzed at 4° overnight against 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, and 0.2 mM $CaCl_2$ (pH 7.7) before use. GTP was purchased from Sigma Chemical Company, St. Louis, Mo. VLB, VCR, and VDS were gifts of the Eli Lilly Company, Indianapolis, Ind.

The self-assembly of tubulin was followed by measuring the increase in turbidity using a Gilford 2000 recording spectrophotometer. Polymerization of G-actin was followed by viscosity measurements at 25 ± 0.1° using a Cannon semimicrometer with a flow time for water of 61.6 sec. For electron microscopic examination, samples were negatively stained with 2% uranylacetate.

RESULTS

Inhibition of the self-assembly reaction by the 3 *vinca* alkaloids is shown in Chart 2. In these experiments, the

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[3] The abbreviations used are: MT, microtubules; VCR, vincristine; VLB, vinblastine; VDS, desacetylvinblastine amide (vindesine).

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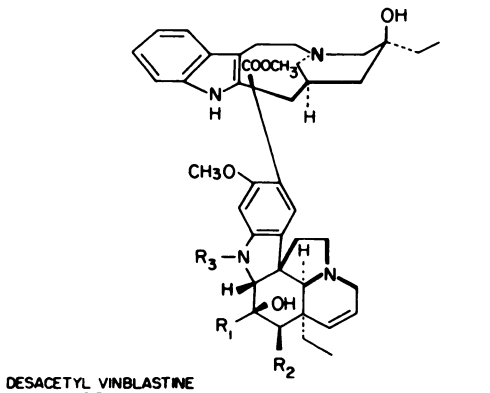

DESACETYL VINBLASTINE AMIDE

desAcVLBAS	VINBLASTINE	VINCRISTINE
$R_1 = CONH_2$	$R_1 = COOCH_3$	$R_1 = COOCH_3$
$R_2 = OH$	$R_2 = OCOCH_3$	$R_2 = OCOCH_3$
$R_3 = CH_3$	$R_3 = CH_3$	$R_3 = CHO$

Chart 1. Chemical structures of VLB, VCR, and VDS.

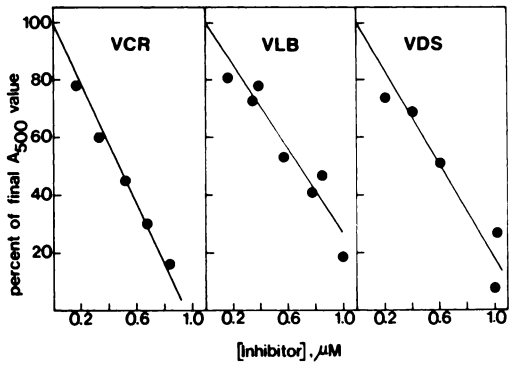

Chart 2. Inhibition of polymerization by the *vinca* compounds. Tubulin (0.8 mg/ml; 6.5 μM) was incubated with the concentrations of the inhibitors shown for 2 min at 37° in 0.5 ml of reassembly buffer. GTP (5 μl of a 50 mM solution) was added and the absorbance at 500 nm was measured. The final absorbance value is plotted as a function of the concentration of inhibitor.

compounds were preincubated with tubulin for 2 min before polymerization was initiated. However, it was found that shorter or longer periods of preincubation did not change the effectiveness of the alkaloids in preventing self-assembly. The results demonstrate that there is little difference in the potency of the drugs in this *in vitro* system. Examination of the data shows VCR to be slightly more effective than the other 2 drugs. All 3 compounds block polymerization almost completely at a concentration of 1 μM when 6.5 μM tubulin was used. (This concentration of tubulin is calculated assuming 90% of the protein to be tubulin.) These results were confirmed by electron microscopic examination. At concentrations slightly above 1 μM, no MT were observed. Ultracentrifuge studies showed that at the molar ratio of *Vinca* alkaloid to tubulin of 0.1, large changes in the size of the sedimenting species occurred. The tubulin preparations contain a 6 and 30 S species in roughly equal proportions (see "Discussion"). In the presence of the *Vinca* alkaloids, the amount of 6 and 30 S decreases dramatically and a new species with a sedimentation constant of >45 S appears. The *in vitro* assembly of porcine brain tubulin prepared in the absence of glycerol (3) displayed a sensitivity to VLB identical to that shown in Chart 2.

Interaction of the 3 *vinca* alkaloids with MT formed *in vitro* was then examined. It has previously been shown by others that both *in vivo* and *in vitro* VLB causes MT to form paracrystals and spiral structures (1, 5, 8, 9, 15, 25). The latter appear to be intermediates between MT and the crystals (25). When the compounds were added after the formation of MT, a small but reproducible decrease in the absorbance did occur (Chart 3). The extent of this decrease was almost identical whether the final concentration of the drug was 1, 10, or 100 μM. However, some differences were observed when these solutions were negatively stained and examined with the electron microscope. MT treated with a 1 μM concentration of the compounds were largely unaffected but a few spiral-like structures were observed. A proliferation of these spirals occurred in the presence of the higher concentrations and few MT were present (Fig. 1). No differences in the abilities of VLB, VCR, or VDS to produce the spirals were noted.

All 3 *vinca* alkaloids apparently have the property to cause the unraveling of the MT. This apparently occurs by the separation of pairs of protofilaments longitudinally, followed by a coiling of these pairs (25). The spiral structures are stable to low temperature and to $CaCl_2$, 2 microtubule depolymerizing agents. This was indicated by the fact that the absorbance was unchanged after these treatments. When self-assembled tubulin was treated with 1 μM concentrations of any of the alkaloids followed by the addition of 1 mM $CaCl_2$ or by cooling to 0° (Chart 3), the absorbance decreased almost to 0. When the same experiment was done using 10 μM of the compounds, the absorbance changed little. As a confirmation, samples were negatively stained and observed with the electron microscope. Spirals were abundant in samples that were incubated at 0° or with 3 mM $CaCl_2$ after treatment with 10 μM drug (Fig. 1). Indeed, concentrations of as high as 8 mM $CaCl_2$ did not disrupt the structures but did appear to cause them to aggregate. The spiral structures, like MT, are sensitive to increases in ionic

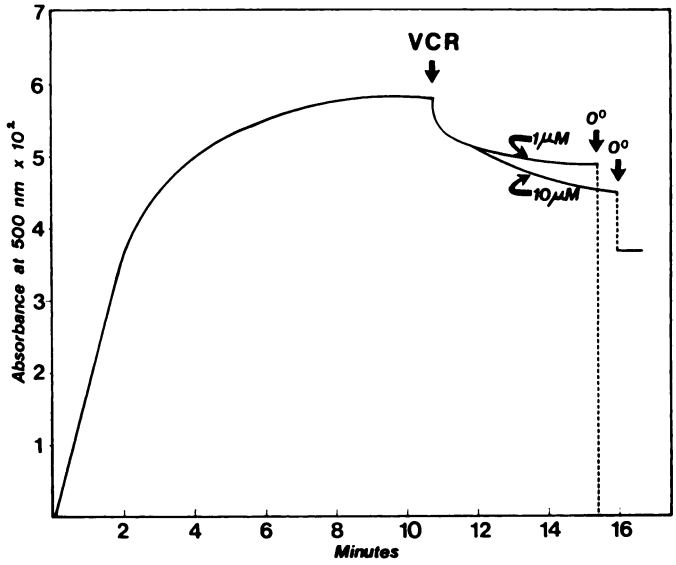

Chart 3. Effect of VCR on MT. Tubulin (1.0 mg/ml; 8 μM) was polymerized in 0.5 ml of reassembly buffer containing 0.5 mM GTP at 37°. *1st arrow*, 5 μl of either a 100 μM or 1 mM VCR solution was added; *2nd arrow*, the sample was cooled to 0° for 5 min and then the absorbance was measured again.

strength. An intermediate NaCl concentration (0.25 M), which is known to depolymerize MT (14), did cause the disassembly of the spirals.

Since it has been shown that VLB can precipitate actin as well as tubulin (30), we examined the possible effects of the *vinca* alkaloids on the conversion of G-actin to F-actin using rabbit muscle actin. As the results in Table 1 demonstrate, these compounds, at fairly high concentrations, exhibited no inhibitory effect on the polymerization of G-actin. Actin filaments formed in the presence of the compounds appear to be normal by electron microscopy.

Table 1

Lack of effect of VLB, VCR, and VDS on G-actin polymerization

Muscle G-actin (36 μM) was incubated in 0.5 ml of 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, and 0.2 mM $CaCl_2$ (pH 7.7) with the *Vinca* alkaloids for 2 min at 25°. KCl (10 μl of a 4 M solution) was then added. After 25 min the viscosity was measured. Two controls of G-actin incubated in the buffer alone and with *Vinca* alkaloids, both without KCl, showed no increase in viscosity in the time period used.

Vinca alkaloids	Concentration (μM)	$\eta_{specific}$
None		1.41
VCR	0.86	1.40
VCR	86	1.53
VDS	117	1.44
VLB	9.8	1.38
VLB	98	1.38

DISCUSSION

The results presented here demonstrate that the *vinca* alkaloids VLB, VCR, and VDS inhibit the self-assembly of brain tubulin with near equal effectiveness. VCR may be about 50% more effective than VCR and VDS (Chart 2).[4] This is consistent with the findings that the 3 compounds inhibit axoplasmic transport with almost equal effectiveness (17) and that there is little difference in the apparent binding constants of VLB, VCR, and desacetylvinblastine to brain tubulin (13, 19, 31). To our knowledge the binding of VDS has not been reported. The literature contains a rather wide range of reported affinity constants for the binding of VLB depending on the source of the tubulin. Apparently 2 moles of VLB are bound per mole of tubulin dimer with affinity constants of 2.3×10^4 M^{-1} (13), 3 to 5×10^5 M^{-1} (31), and 6×10^6 M^{-1} (19), for tubulin from calf brain, chick embryo brain, and porcine brain, respectively. The reasons for these differences are not known. However, it should be pointed out that we find the concentrations of VLB necessary to cause 50% inhibition of the assembly of 6.5 μM beef brain and porcine brain tubulin to be identical (about 0.6 μM). This would imply similar affinities of VLB for tubulin from these 2 sources.

One aspect of these results deserves further comment. We find that the concentration of *Vinca* alkaloids necessary to cause complete inhibition of polymerization is substantially less than the total amount of tubulin present. That is, when the protein concentration is calculated on a molar basis, using 110,000 as the molecular weight, the presence of 1 mole of *Vinca* alkaloid for every 7 moles of tubulin dimer causes essentially complete inhibition (Chart 2). This is difficult to understand simply on the basis of a reaction between tubulin dimer and the alkaloid (dimer + 2 VLB $\rightleftharpoons$ dimer·2 VLB). The apparent disparity can be explained by considering the composition of tubulin preparations obtained by the method used in this study. These preparations contain tubulin as the 6 S dimer and an aggregated form ("rings"). The aggregate is necessary for the polymerization to take place and probably serves as an initiation site (2, 7, 10, 12, 20, 27). The aggregates, usually in the form of a ring, contain tubulin and a small amount of other proteins (6, 16, 22, 26). In our preparations we usually find equal amounts of 6 and 30 S material. Because of differences in molecular weight, the molar concentration of the 30 S component is substantially lower than that of the total tubulin present. If the inhibition of polymerization involves the addition of the *Vinca* compounds to the ends of the aggregates or unfolded rings (7, 12), then a concentration substantially lower than that of the total tubulin would be inhibitory. For example, if the molecular weight of the aggregate is in the order of 1×10^6, at a total protein concentration of 1 mg/ml, the molar concentration of the aggregates would be 0.5 μM.

The concentrations of drugs required for the inhibition of assembly of bovine brain tubulin are much higher than those reported for chick embryo brain tubulin (28). In the latter case, 50% inhibition of the rate of assembly was achieved using a tubulin to VLB ratio of 10^3. The difference between these results and ours could be due to a number of factors. One factor would be the use of tubulin from different sources. Another is the fact that results obtained from rate studies cannot be compared directly with those obtained using extent of reaction. Finally, if the mechanism of inhibition suggested above is valid, the presence of different size aggregates in the tubulin preparation would help explain the different sensitivities to the alkaloids.

It has been proposed that VLB disrupts MT *in vivo* by blocking the polymerization reaction resulting eventually in the disappearance of MT (31). The claim has also been made that the *Vinca* alkaloids do not have a high affinity for MT *per se* (31). To the contrary, the results presented here show that all 3 *Vinca* alkaloids do cause structural changes in the preformed MT at fairly low concentrations. Although a drug to protein molar ratio of about 1 was required for maximum production of the spiral protofilaments, some effect was seen at a ratio of about 0.1. It may be inferred from these observations that the *Vinca* alkaloids have therapeutic effects by interacting with preformed MT in nondividing tumor cells as well as preventing mitotic spindle MT from forming in dividing cells. Similarly, some of their clinical toxicity may be related to damage done to preformed MT, such as in nerve axons.

Although the clinically observed differences between VCR, VLB, and VDS probably cannot be ascribed to their small differences in reactivity with tubulin, some preliminary results in our laboratory indicate that their actions on

[4] After submission of this manuscript, an article by Owellen *et al.* (18) appeared which describes the inhibition of tubulin assembly in crude porcine brain homogenate by a variety of *Vinca* alkaloids. These workers found the relative effectiveness of VCR and VLB to be similar to what we report, although they found VDS to be a little more effective than VCR.

MT in nerve fibers do differ (J. A. Donoso, unpublished data). That is, when isolated vagus nerves are incubated with *Vinca* alkaloids (5×10^{-5} M), VCR affects the MT in unmyelinated axons primarily, whereas VLB affects the MT in myelinated and unmyelinated axons equally. This is in contrast to the greater neurotoxicity of VCR over VLB clinically.

This work indicates that the different oncological and biological actions of the 3 *vinca* alkaloids are not accounted for in their interactions with tubulin or MT as such. This is not to deny that the effect of these compounds on tubulin and MT is not therapeutically important, rather, it raises the possibility of other additional molecular bases for some of their therapeutic effects. For example, it has been shown (30) that VLB, in high concentrations, can precipitate a large number of other acidic proteins, such as actin. Possible interactions with this particular fibrous protein were examined because actin is widespread and abundant in nerve tissue, thereby suggesting another possible area of involvement that could account for the clinical neuropathy. However, no effect by any of the 3 compounds on actin polymerization *in vitro* could be found. Although the polymerization *in vitro* and the normal appearance of the actin filaments in the presence of the *Vinca* alkaloids do not rule out the possibility that actin is involved in the different biological actions of these compounds, they do argue against it.

The results suggest that the differential actions of VLB, VCR, and VDS *in vivo* must be due to some reaction(s) or biological processes other than the direct interaction with tubulin or MT. For example, there may be major differences in transport, absorption, and metabolism of the 3 drugs in different tissues.

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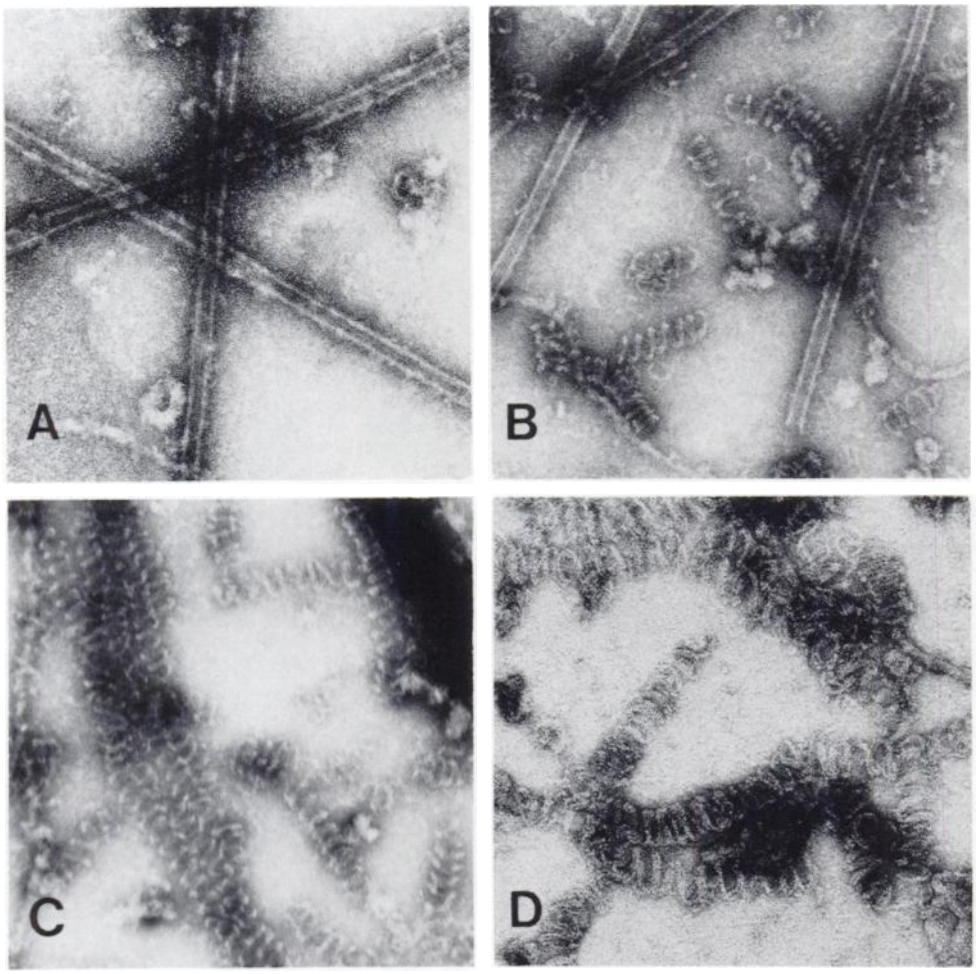

Fig. 1. Electron micrographs of MT formed *in vitro* treated with *Vinca* alkaloids. MT were formed as described in Chart 3 and then treated with VLB for 5 min. *A*, 1 μM VLB; *B*, 10 μM VLB; *C*, 10 μM VLB, then 3 mM $CaCl_2$ for 5 min; *D*, 10 μM VLB, then cooled to 0° for 5 min. Samples were stained with 2% uranyl acetate. × 100,000.