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Mechanism of Interaction of Vinca Alkaloids with Tubulin: Catharanthine and Vindoline†

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ABSTRACT: The interactions of the vinca alkaloid drugs catharanthine and vindoline with tubulin have been investigated and compared with those of vinblastine and vincristine. Both drugs were found to be less effective in bringing about the inhibition of tubulin self-assembly into microtubules than vincristine and vinblastine, the drug to protein molar ratio required being 3 orders of magnitude greater. An analytical ultracentrifuge study has shown that catharanthine can induce the self-association of tubulin into linear indefinite polymers with an efficacy that is 75% that of vinblastine or vincristine, the intrinsic dimerization constant for the liganded protein being $K_2 \simeq 1 \times 10^5$ M^{-1}. The effect of vindoline was marginally detectable. Binding studies of catharanthine using the gel batch and fluorescence perturbation techniques showed a polymerization-linked binding of one catharanthine molecule per tubulin α-β dimer with a binding constant of $(2.8 \pm 0.4) \times 10^3$ M^{-1}. For vindoline, binding to tubulin was marginally detectable by fluorescence spectroscopy, although addition of vindoline to tubulin did generate a difference spectrum. It was concluded that the binding of vinblastine/vincristine to tubulin and its consequences are determined by the interaction of the indole part of catharanthine with tubulin, the role of vindoline being that of an anchor.

The two major vinca alkaloids from the plant *Catharanthus roseus*, vinblastine (VBL)[1] (structure Ia, Chart I) and vincristine (VCR)[1] (structure Ib), are well-known effective mitosis-arresting reagents that are widely used in the treatment of neoplastic diseases (Dustin, 1978). Structurally these two drugs are "dimeric" molecules, as they comprise two domains, vindoline (VDN)[1] (lower half of VBL/VCR structure) and rearranged catharanthine (CTN)[1] (structure II). Both vincristine and vinblastine induce tubulin to self-associate in vitro

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[1] Abbreviations: VBL, vinblastine; VCR, vincristine, CTN, catharanthine; VDN, vindoline; GTP, guanosine 5′-triphosphate; PG buffer, 10^{-2} M sodium phosphate, 1×10^{-4} M GTP, pH 7.0; PMG buffer, PG buffer to which is added 5×10^{-3} M $MgCl_2$.

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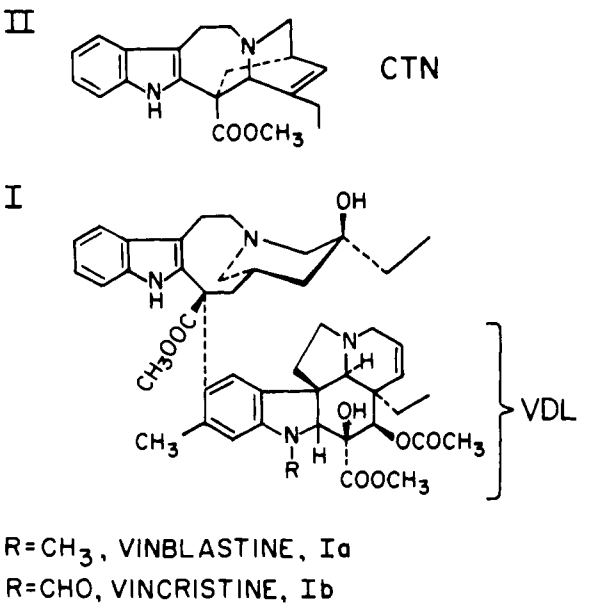
Chart I



into linear polymers, which lead to the formation of paracrystals in vivo (Shelanski & Wisniewski, 1969; Schochet et al., 1968) and in vitro from pure tubulin (Na & Timasheff, 1982). This self-association process has been characterized quantitatively (Na & Timasheff, 1980a,b, 1986; Prakash & Timasheff, 1983, 1985). Both drugs inhibit the self-assembly of tubulin into microtubules and cause their dissolution at substoichiometric concentrations (Schochet et al., 1968; Shelanski & Wisniewski, 1969). Their extensive clinical use has motivated their synthesis by the coupling of CTN with VDN (Taylor, 1957; Kutney et al., 1979a,b; Potier, 1980; Kuehne et al., 1986). CTN has been shown to stimulate the release of amylase from pancreatic fragments and also to cause extensive degranulation of the pancreatic acinar cells with accumulation of membrane material in the Golgi region (Williams, 1978). Clinically it has been shown that CTN has only one-thousandth of the activity of either VBL or VCR as an antimitotic agent, expressed as 50% mitotic arrest in HeLa cells (Wilson et al., 1974). CTN is totally ineffective in precipitating tubulin and was not an effective competitor of vinblastine-induced tubulin precipitation (Ventilla et al., 1972; Wilson et al., 1974). The VDN moiety has been little studied. The LD_{50} dose determined for anti P-388 activity was >800 mg/kg for both CTN and VDL, compared to values for VBL and VCR of 17.2 and 5.3 mg/kg, respectively (Owellen et al., 1977). Even though both pieces of VBL have been found to be much weaker in their biological action than the whole molecule, it seemed of interest to investigate their contributions individually on a molecular level to the interactions that impart to VBL/VCR their biochemical and liganding properties. The results of such a study are reported in this paper.

Materials and Methods

All chemicals (unless stated otherwise) were of reagent grade. Ammonium sulfate and sucrose, ultrapure grade, were obtained from Schwarz/Mann. Guanosine 5′-triphosphate (GTP)[1] type II-S and tryptophan ethyl ester were from Sigma Chemical Co. Ultrapure guanidine hydrochloride was purchased from Heico Co. and purified as reported elsewhere (Prakash & Timasheff, 1983). Catharanthine hydrochloride, vindoline, and vinblastine were gifts from Eli-Lilly and Co. through the courtesy of Dr. Koert Gerzon and of Dr. Pierre Potier, CNRS, Gif-sur-Yvette, France. They were used without further purification. Calf brains were brought in ice from the local slaughter house and used within an hour after sacrificing.

Preparation of CTN and VDN Solutions. Since both drugs have a limited solubility in pH 7.0, 0.01 M sodium phosphate buffer (PG),[1] a known amount of the drug was dissolved in 0.2 mL of dimethyl sulfoxide and sonicated for 15 min, after which the volume was made up to 2 mL and the solution was kept at 4 °C overnight. It was then centrifuged at 10000*g* for 30 min and the concentrations of CTN and VDN were obtained spectrophotometrically. The extinction coefficients in pH 7.0, 0.01 M sodium phosphate buffer were found to be 6915 ± 120 at 289 nm and 8090 ± 140 at 281 nm for CTN and 4625 ± 105 at 301 nm and 6760 ± 115 at 249 nm for VDN. The solutions were stored for not more than 5 days at 4 °C in brown bottles wrapped in aluminum foil and black tape.

Tubulin. Tubulin was isolated from calf brains by the method of Weisenberg et al. (1968) and Weisenberg and Timasheff (1970), as modified by Lee et al. (1973). $MgCl_2$ was used at a concentration of 0.5 mM throughout the preparation. In the DEAE–Sephadex step the protein adsorbed to the resin was separated from the suspension by passing it through a sintered glass funnel instead of by centrifugation. Tubulin prepared in this manner was stored in 1 M sucrose–PMG–buffer (10^{-2} M sodium phosphate, 5×10^{-3} M $MgCl_2$, and 1×10^{-4} M GTP, pH 7.0)[1] under liquid nitrogen until needed (Frigon & Lee, 1972). For each experiment, the tubulin was freed from sucrose and brought to equilibrium with the pertinent buffer on a dry and then a wet Sephadex G-25 column (Lee et al., 1973). The tubulin concentration was determined spectrophotometrically at 275 nm in 6 M guanidine hydrochloride after spinning at 12000*g* for 30 min. The value of the absorptivity used was 1.03 L/(g cm) (Na & Timasheff, 1981).

Self-Assembly of Tubulin. Tubulin equilibrated with the assembly buffer (pH 7.0, 10^{-2} M sodium phosphate, 10^{-4} GTP, 8×10^{-3} M $MgCl_2$, 3.4 M glycerol) was transferred to a thermostated cuvette maintained at 10 °C in a Cary 118 spectrophotometer. With the protein in the cuvette, the base line was measured at 350 nm, and water at 37 °C was then allowed to flow through the cell jacket. The increase in absorbance was monitored at 350 nm (Gaskin et al., 1974; Lee & Timasheff, 1975). For inhibition measurements, aliquots of concentrated stock solutions of VBL, CTN, or VDN were pipetted into the protein solution, such that the desired final concentration was obtained with minimum dilution of the protein solution.

Determination of Ligand Binding to Tubulin. The binding of CTN to tubulin was measured by the batch gel filtration technique (Fasella et al., 1965; Pearlman & Crepy, 1967; Hirose & Kano, 1971; Na & Timasheff, 1986). Forty milligrams of dried Bio-Gel P-100 was weighed into each 12 × 75 mm borosilicate culture tube to which was added 0.55 g of the experimental PG buffer. The tube was tightly closed with parafilm and the gel was allowed to swell for 24 h at room temperature. At the end of the swelling, 0.2 g of PG buffer, containing a known concentration of CTN, was added and the gel was mixed gently. To this was added 0.2 g of tubulin stock solution that had been preequilibrated with the same buffer. The entire mixture was incubated at 20 °C with gentle shaking for 90 min. After the incubation, the gel was allowed to settle to the bottom of the test tube and aliquots were carefully taken from the supernatant for the spectrophotometric determination of the ligand concentration. The data were corrected for the

fraction of CTN adsorbed to the gel matrix, which remained constant at 0.02% over the CTN concentration range used (between 1×10^{-4} and 3×10^{-3} M). Controls were performed as described by Na and Timasheff (1986). The internal, or excluded, volume of the gel was determined by using Blue Dextran 2000. With complete exclusion of the Blue Dextran 2000 from the internal phase of the gel assumed, the internal volume per unit weight of the gel, V_i, was calculated from (Na & Timasheff, 1986)

$$A_e/A = V_t/V_e \quad (1)$$

$$V_t = V_e + V_i \quad (2)$$

$$\bar{V}_i = V_i/g \quad (3)$$

where A and A_e are the absorbance at 260 nm of Blue Dextran 2000 in the stock solution and the external phase, respectively, V_e is the external volume, V_i is the internal volume, and g is the weight of the gel. $\bar{V}_i$ had a value of 9.02 ± 0.12 mL/g for the Bio-Gel P-100 used. The concentration of the ligand in the internal phase of the gel (CTN_i) was calculated from

$$[CTN_i] = \{(CTN) - [CTN_e]V_e\}/V_i \quad (4)$$

where (CTN) is the total amount of CTN added and $[CTN_e]$ is the concentration of CTN in the external volume. The concentration of CTN bound to tubulin, $[CTN_b]$, is then given by

$$[CTN_b] = [CTN_e] - [CNT_f] \quad (5)$$

In the spectrofluorimetric measurements, relative fluorescence intensities were obtained with a Hitachi Perkin-Elmer MPF-3 spectrofluorometer equipped with a thermostated chamber maintained at 20 °C with a Neslab temperature bath. PG buffer, pH 7.0, was used in all of the fluorescence experiments. The protein concentration was 1×10^{-6} M. Any internal filter effect was monitored and corrected for empirically by measuring the change in the fluorescence intensity of a solution of freshly prepared tryptophan ethyl ester equivalent to the tubulin concentration in the presence of the respective ligands. The relative fluorescence yield, which is the ratio of the fluorescence intensity of the tubulin sample to that of the corresponding tryptophan standard, was used throughout the calculation (Pesce et al., 1971). The association constant, K_a, was calculated from the above data by the method of Lehrer and Fasman (1966)

$$K_a = (\beta/1 - \beta)[1/(L_f)] \quad (6)$$

where $\beta = (Fl = Fl_p)/(Fl_{pl} - Fl_p)$ and $(L_f) = (L) - \beta(C)$. The quantities Fl, Fl_p, and Fl_{pl} are the relative fluorescence yields of the experimental mixture, the unliganded protein, and the fully liganded protein, respectively; C is the molar concentration of ligand binding sites; (L_f) and (L) are the concentrations of free ligand and total ligand present, respectively. Fl_{pl} can be determined by plotting $(Fl - Fl_p$ versus $1/(L)$ and extrapolating to $1/(L) = 0$. The association constant, K_a, can then be determined from the slope of a plot of $\beta/(1 - \beta)$ versus (L_f).

Spectra and Difference Spectra. The direct spectra and difference spectra were recorded on a Perkin-Elmer lambda 3B spectrophotometer at 20 °C. Difference spectra were obtained by using 1-cm square tandem cells from Quara cell, New York. A protein concentration of 9.1×10^{-6} M was used in all the experiments unless stated otherwise. Proper base-line corrections were applied for all the spectra. Spectra were generally recorded in the range 350–230 nm. Repeated scans were obtained by varying experimental parameters, such as position of the cell, mixing time, etc., and no significant differences were seen between the scans; two or three scans were always obtained for both the direct and the difference spectra.

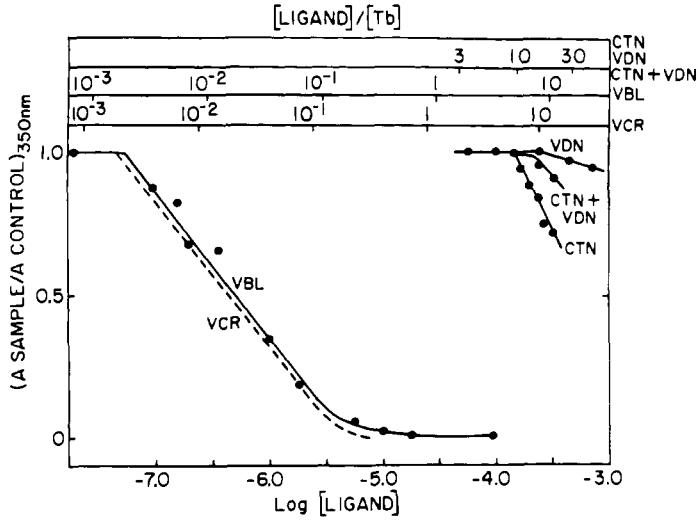


FIGURE 1: Effect of vinca alkaloids on the self-assembly of tubulin. The solvent was assembly buffer (0.01 M sodium phosphate, pH 7.0, 8 mM $MgCl_2$, 1×10^{-4} M GTP, and 3.4 M glycerol). The protein concentrations were 2.9×10^{-5} M for VBL, 2.45×10^{-5} M for VCR, and 2.2×10^{-5} M for CTN and VDN. The ligands were added to the protein dissolved in assembly buffer, the mixture was stirred well, and the assembly was monitored at 350 nm at 37 °C. The absorbance of the protein solution without ligand was normalized to 1.0 and the fraction of turbidity was expressed as the optical density in the presence of each of the ligands or a combination thereof relative to the control. This is plotted as a function of ligand concentration and the ratio [ligand]/[Tb] is shown with different scales for each of the ligands. The dashed line represents the previous VCR data reproduced from Prakash and Timasheff (1983).

Velocity Sedimentation. Velocity sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with an RTIC unit, an electronic speed control system, and a photoelectric scanner assembly. Generally the experiments were performed at 20 °C and at 60000 rpm, using a Kel-F coated aluminum double-sector center piece with quartz windows or sapphire windows when using the scanner. The optics of the system were aligned according to the procedure of Dyson (1970) and Richards et al. (1971a,b). The schlieren patterns were recorded on Kodak Metallographic or Kodak Type II-G spectroscopic plates. The plates were read on a Nikon Model 6C microcomparator equipped with an Elk Model 9200 precision digital positioner and an electronic digital display. The $\bar{s}_{20,w}$ values were calculated according to the standard procedure (Schachman, 1959). The percentage composition of the different peaks was determined by area measurements either on enlarged tracings or with a Du Pont curve resolver and had an error of 1.5%. Johnston-Ogston corrections were not applied to any of the calculations. Fresh tubulin was used in each experiment.

Results

Microtubule Assembly. The in vitro interaction between CTN or VDN and pure tubulin was probed first by the effect of the drugs on the assembly of the protein into microtubules. The results are shown in Figure 1, where they are compared with the effects of VBL and VCR. As can be seen, CTN did not have any effect on the polymerization of tubulin up to a concentration of 1.6×10^{-4} M, which is equivalent to a CTN to protein ratio of approximately 7. On the other hand, VDN did not have any effect on the polymerization up to a concentration of 5×10^{-4} M, which is equivalent to a VDN to protein ratio of approximately 23. Above these drug con-

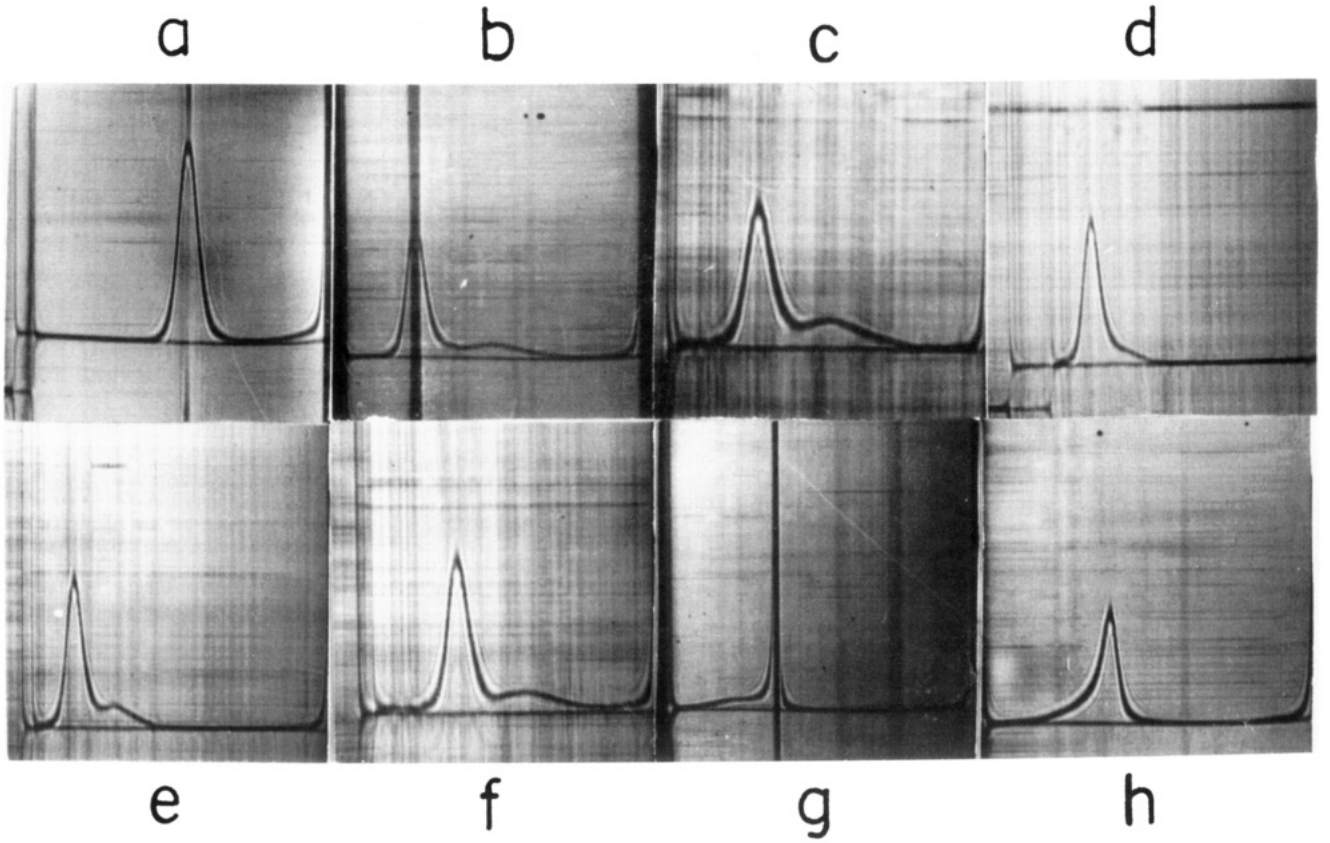


FIGURE 2: Representative sedimentation velocity patterns of tubulin in the presence of various concentrations of CTN, VDN, and a mixture of CTN and VDN. For comparison the sedimentation velocity patterns of tubulin in the presence of VBL and VCR are also shown. All runs were performed at 20 °C, unless stated otherwise, at a speed of 60 000 rpm. The photographs were obtained at a bar angle of 70°, unless stated otherwise. The time of the photograph was generally 40 min after reaching two-thirds of the maximum speed. A protein concentration of 11.1 mg/mL was used in all of the experiments unless stated otherwise. The drug concentration given is for free (unbound) drug. (a) Pure tubulin (bar angle 60°). Tubulin in the presence of (b) 1.5×10^{-4} M CTN, (c) 6.8×10^{-4} M CTN, (d) 3×10^{-4} M VDN, (e) 5×10^{-4} M VDN, (f) 0.75×10^{-4} M CTN and 0.75×10^{-4} M VDN (12.8 mg/mL protein concentration), (g) 1.25×10^{-4} M VCR, and (h) 1.25×10^{-4} M VBL (8.2 mg/mL protein concentration). All the experiments were completed within 3 h from taking the tubulin out of liquid nitrogen.

centrations, microtubule inhibition was observed, 3.2×10^{-4} M CTN giving a 30% reduction of the protein turbidity at 37 °C. With VDN, only marginal inhibition (a 5% decrease in turbidity) was induced by 6×10^{-4} M ligand. An equimolar mixture of these two drugs to a total drug concentration of 3×10^{-4} M led to a 10% decrease in turbidity, i.e., to the level of inhibition induced by 1.5×10^{-4} M CTN alone. Both VBL and VCR inhibited assembly already at 1×10^{-7} M, i.e., at [VCR] and [VBL] to [Tb] ratios of $(3.5–4) \times 10^{-3}$, and reached completion at ligand concentrations of 5×10^{-6} M for VCR and 1×10^{-5} M for VBL. Parallel to the inhibition, the time needed to attain the plateau increased progressively. Even though VCR appears to be slightly more effective in bringing about the inhibition of tubulin assembly than VBL, both act substoichiometrically, while CTN and VDN inhibit only at large excess levels.

Effect of Catharanthine and Vindoline on the Self-Association of Tubulin. The effects of CTN and VDN on the sedimentation behavior of tubulin were examined in PG buffer at free drug concentrations between 1×10^{-5} and 6.8×10^{-4} M CTN and between 5×10^{-5} and 5×10^{-4} M VDN, respectively, at tubulin concentrations between 1 and 16 mg/mL. Higher drug concentrations could not be used due to limited solubility and availability of material. The results are shown in Figures 2 and 3. In PG buffer, in the absence of drugs, tubulin exists as a stable dimer of 108 000 molecular weight and sediments as a single symmetrical peak with $s^\circ_{20,w} = 5.8$S (Figure 2a) and a linear concentration dependence, $s = s^\circ(1 - gc)$, with a value of g, the hydrodynamic nonideality constant, of 0.018 mL/mg (Frigon & Timasheff, 1975). As shown in Figure 2, addition of the drugs induced bimodality in the patterns. CTN at 1×10^{-5} M had no observable effect on the sedimentation pattern. As the concentration of CTN was increased, however, bimodality set in (frames b and c), with the $s_{20,w}$ values of the fast moving peak varying between 9 and 13 as a function of protein and drug concentrations. With VDN the effect observed was much smaller and a weak bimodality set in only at higher concentrations of the drug (frames d and e). When a mixture of 7.5×10^{-5} M CTN and 7.5×10^{-5} M VDN was used, the sedimentation pattern was that of an average between patterns obtained with the two drugs independently (frame f). At similar concentrations, VCR and VBL generated patterns characterized by a hypersharp fast-moving peak with a small trailing edge (frames g and h). These are typical of the ligand-induced isodesmic polymerization of tubulin in the presence of these drugs (Na & Timasheff, 1980a,b; Prakash & Timasheff, 1985).

For the quantitative evaluation of these results, the weight-average sedimentation coefficients at different ligand concentrations were calculated and plotted as a function of protein concentration. As shown in Figure 3, in the presence of CTN, the weight-average sedimentation coefficient of tubulin increased hyperbolically with increasing protein concentration in a manner similar to that observed in the presence of VBL (Na & Timasheff, 1980a) or VCR (Prakash & Timasheff, 1985), whose protein concentration dependencies are plotted for comparison at similar drug concentrations to those used with CTN. It is evident that the self-association observed

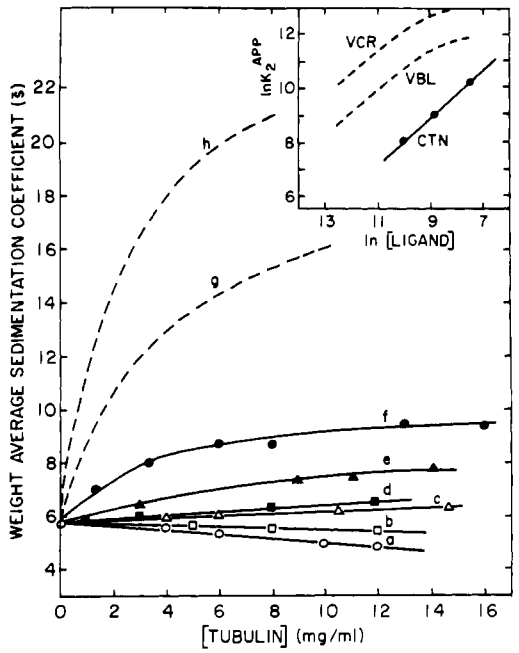


FIGURE 3: Weight-average sedimentation coefficients ($\bar{s}_{20,w}$) of tubulin determined as a function of protein concentration. Tubulin was equilibrated with different concentrations of ligands: (a) PG only; (b) 5×10^{-4} M VDN; (c) 5×10^{-5} M CTN; (d) 0.75×10^{-4} M each CTN + VDN; (e) 1.5×10^{-4} M CTN; (f) 6.8×10^{-4} M CTN. The solid lines are fittings of the experimental data to the isodesmic, indefinite, self-association model for the protein. Inset: Wyman plot of the apparent dimerization constants for the CTN-induced self-association of tubulin. The VBL (g, 2×10^{-4} M) and VCR (h, 1.5×10^{-4} M) data shown are reproduced from Na and Timasheff (1980a) and Prakash and Timasheff (1985), respectively.

in the presence of CTN is much less extensive than that in the presence of VBL/VCR. For example, at 10 mg/mL protein, the $\bar{s}_{20,w}$ value is 6.1 S in the presence of 5×10^{-5} M CTN. At higher CTN concentrations, the values were 7.5 and 9.2 S for 1.5×10^{-4} M and 6.8×10^{-4} M CTN, respectively. In the presence of 5×10^{-4} M VDN, the $s_{20,w}$ versus tubulin concentration slope was negative, indicating a much weaker effect on tubulin aggregation. In contrast, the values of $s_{20,w}$ in 1.5×10^{-4} M VCR and 1.0×10^{-4} M VBL were 21.5 and 16 S, respectively. A comparison of the shapes of the sedimentation patterns, as well as of the $s_{20,w}$ versus tubulin concentration plots at the various CTN concentrations, to those previously observed for VBL (Na & Timasheff, 1980a) and VCR (Prakash & Timasheff, 1985) strongly suggested that the self-association induced by CTN is of the same mode as that induced by VBL and VCR, namely, a stepwise indefinite polymerization. Furthermore, photoelectric scanner examination showed the proper ligand depletion across the rapid peak. Therefore, the present data seem to indicate that, while at identical ligand concentrations the tubulin polymerization inducing capacity of CTN is much weaker than those of VCR or VBL, it nevertheless can lead to the same self-assembly process. VDL was found to be much less effective and it is not possible to say that the aggregation seen is of a specific nature. An equimolar mixture of CTN and VDN at a total free drug concentration of 1.5×10^{-4} M had a $\bar{s}_{20,w}$ of 6.4 S at 10 mg/mL tubulin, which is only slightly above that of 5×10^{-5} M CTN. Therefore, there is no cooperative action of the two halves of the VBL molecule when these exist as independent kinetic units.

The CTN results were curve-fitted to the same isodesmic, indefinite self-association mode as VBL results (Na & Timasheff, 1981). The apparent dimerization constants, K_2^{app}, derived from this analysis are listed in Table I, along with the apparent standard free energy changes, $\Delta G°_{app}$. For CTN, K_2^{app} attained a value of 3×10^4 M^{-1} at 6.8×10^{-4} M CTN. For VDN, at 5×10^{-4} M, the estimated value of K_2^{app} was $<1 \times 10^3$ M^{-1}. The values of K_2^{app} for VBL and VCR, also listed in Table I, were 15 and 30 times higher than that for CTN. Analysis of the data of Figure 3 and Table I in terms of the linkage relations described by Na and Timasheff (1980b) and with the use of the Wyman (1964) plot of ln K_2^{app} versus ln [n], where [n] is the concentration of free ligand, is shown as the inset in Figure 3. This plot indicates that the equilibrium constant increases with increasing CTN concentration in a manner similar to that of the tubulin–VBL and tubulin–VCR systems, suggesting that the ligand-mediated pathway is kinetically open, similar to what had been found for VBL (Na & Timasheff, 1980a,b) and VCR (Prakash & Timasheff, 1983, 1985). The inset of Figure 3 clearly demonstrates that at any given concentration of free ligand the effectiveness of CTN is reduced by at least 1 order of magnitude in K_2^{app} relative to VBL/VCR.

Table I: Dependence of the Self-Association of Tubulin on Catharanthine Concentration

$[CTN_f]$ (mol/L)	K_2^{app} (L/mol)	$\Delta G°_{app}$ (kcal/mol)
5×10^{-5}	3×10^3	−4.67
1.5×10^{-4}	8×10^3 [a]	−5.24
6.8×10^{-4}	3×10^4	−6.01
7.5×10^{-5} (+7.5×10^{-5} VDN)	4×10^3	−4.83

[a] At 1.5×10^{-4} M, the K_2^{app} values for VBL and VCR were 1.2×10^5 and 2.9×10^5 M^{-1}, respectively (Na & Timasheff, 1980b; Prakash & Timasheff, 1985).

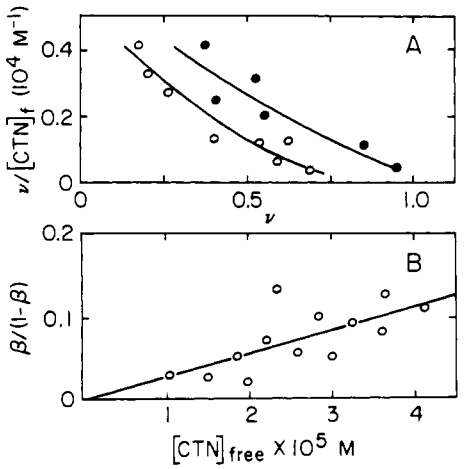


FIGURE 4: (A) Scatchard plots of the binding of CTN to calf brain tubulin in PG buffer at 20 °C. The protein concentrations were 1.4×10^{-5} M (○) and 2.8×10^{-5} M (●). (B) Fluorimetric titration of tubulin with CTN in PG buffer at pH 7.0 and 20 °C. The protein concentration was 1×10^{-6} M. The straight line drawn through the points is a least-squares fit. The apparent binding constant was obtained from the slope of this line.

Binding of CTN to Tubulin. The binding of CTN to tubulin was examined by batch gel filtration and fluorescence. Figure 4a shows the Scatchard plots measured by batch gel filtration at 1.4×10^{-5} and 2.8×10^{-5} M tubulin. The points, which indicate weak interaction, tend toward one CTN binding site on the tubulin molecule. The scatter reflects the small difference between total and free ligand in the solution, which is a consequence of the weak binding. This clear evidence of the dependence of the binding isotherms on protein concentration, with binding increasing at higher protein concentration, is consistent with the sedimentation velocity results that the

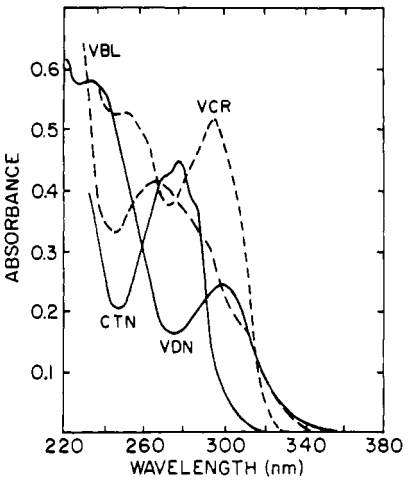


FIGURE 5: Ultraviolet absorption spectra of VBL, VCR, CTN, and VDN in PG buffer. The concentrations have been adjusted arbitrarily so that no overlapping of spectra takes place.

liganding of CTN to tubulin induces protein self-association. No binding of VDL could be detected by this technique under the same conditions.

Using the binding stoichiometry of $n = 1$, the binding of CTN was further examined by the protein fluorescence quenching technique (Lehrer & Fasman, 1966). Tubulin has a fluorescence emission maximum at 328 nm when excited at 275 nm (Andreu & Timasheff, 1982). Addition of CTN to a solution of tubulin resulted in the quenching of the fluorescence. A protein concentration of 1.0×10^{-6} M, which is well below the level of detectable self-association, was used in all the experiments and proper corrections were made for the inner filter effects. The data were analyzed according to eq 6 by using a 1:1 binding stoichiometry of CTN to tubulin. A plot of $\beta/(1 - \beta)$ versus (CTN_f) is shown in Figure 4B. From the slope of the straight line an apparent binding constant, K_1^{app}, of $(2.8 \pm 0.4) \times 10^3$ M^{-1} was obtained in PG buffer, resulting in a standard free energy of binding of −4.6 kcal/mol. Similar experiments with VDN gave only marginal quenching. From the highly scattered points obtained, K_1^{app} for VDN could be estimated as $\leq 5 \times 10^2$ M^{-1}, if it is assumed that the binding is specific.

To probe the nature of interactions between tubulin and the drugs, difference spectra of tubulin with CTN or VDN were obtained and compared with those of the two drugs in a medium of reduced dielectric constant. For purposes of comparison, the spectra of VBL, VCR, CTN, and VDN in PG buffer are shown in Figure 5. Figure 6 shows the difference spectra of CTN at 5×10^{-5} and 1×10^{-4} M after mixing with tubulin in PG buffer. At both CTN concentrations, the difference spectrum is characterized by two small peaks at 294 and 285 nm with minor shoulders. Doubling of the CTN concentration essentially doubled the intensities of the peaks without changing their positions. These spectra were strikingly similar to those obtained for the tubulin–VBL system (Lee et al., 1975), which shows difference bands at 298 and 288 nm. Comparison of these spectra with the difference spectrum of CTN in 50% ethanol versus water (Figure 6B) shows that, just as for VBL, the two bands are due to perturbations of CTN transitions by transfer from an aqueous medium to one of lower polarity. In the case of the VDN–tubulin system, no difference spectrum was observed at 5×10^{-5} M VDN (Figure 7). At the higher concentration of 1×10^{-4} M VDN, a difference spectrum developed with a negative peak at 312 nm and positive peaks at 254 and 281 nm. At 3×10^{-4} M VDN, positive peaks were at 258, 267, and 290 nm with a

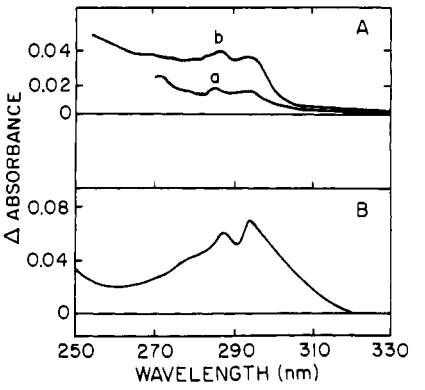


FIGURE 6: Difference spectra of the tubulin–CTN complex in PG buffer, pH 7.0, and CTN alone in 50% ethanol. (A) (a) Tubulin (9×10^{-6} M) in the presence of 5×10^{-5} M CTN versus the same concentrations of tubulin and CTN in buffer; (b) same as (a) in the presence of 1×10^{-4} M CTN. (B) Difference spectrum of 6×10^{-5} M CTN in 50% ethanol versus 6×10^{-5} M CTN in water.

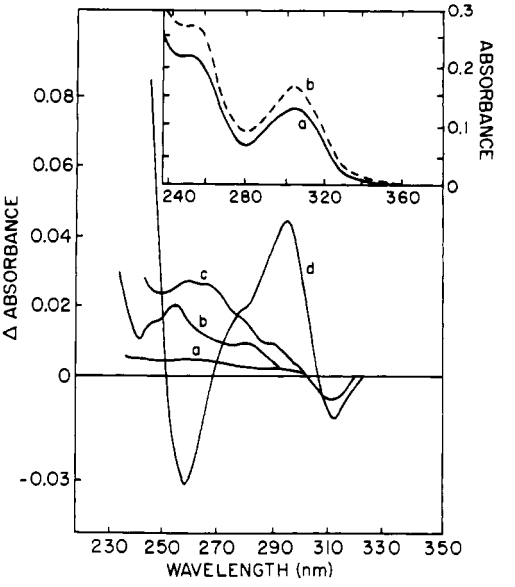


FIGURE 7: Difference spectra of tubulin–VDN and VDN alone in 50% ethanol. The protein concentration was 9×10^{-6} M in PG buffer, pH 7.0. (a) Tubulin in the presence of 5×10^{-5} M VDN versus the same concentrations of tubulin and VDN; (b) tubulin in the presence of 1×10^{-4} M VDN versus the same concentrations of tubulin and VDN; (c) tubulin in the presence 3×10^{-4} M VDN versus the same concentrations of tubulin and VDN; (d) 1×10^{-4} M VDN in 50% ethanol versus 1×10^{-4} M VDN in water. Inset: Ultraviolet spectra of (a) 1×10^{-4} M VDN in water and (b) 1×10^{-4} M VDN in 50% ethanol.

shoulder at 281 nm. Comparison with the difference spectrum between VDN in 50% ethanol and water, which is similar to that of VCR in the same solvents (Prakash & Timasheff, 1983), identified the negative peak at 312 nm as due to perturbation of a drug transition on transfer to an environment of lower polarity, while the peak at 294 nm and shoulder at 278 nm in pure VDN indicate that the spectral features seen in the tubulin–VDN spectrum can be attributed to drug transitions. The similarity of the peaks at 290 and 281 nm in the tubulin–VDN difference spectrum to those seen with CTN may reflect features of the indole spectrum. The lower wavelength region most probably reflects a summation of protein and drug spectral perturbations.

Discussion

The present study has shown that, of the two moieties of the VBL molecule, CTN and VDN, CTN is vastly more active

by itself both in inhibiting the self-assembly of tubulin into microtubules and in inducing the polymerization of tubulin into linear structures, which, in the case of VBL, lead to the formation of paracrystals.

The binding of both CTN and VDN to tubulin is weak. In the case of VDN it could be detected marginally by fluorescence titration, with a maximal binding constant of 5×10^2 M^{-1}. Comparison of the difference spectra in 5×10^{-5} and 3×10^{-4} M VDN, however, provides definite evidence of tubulin–VDN contacts at the higher drug concentration, while no difference spectrum was generated at the lower VDN concentration. This, in itself, does not indicate that the binding is specific. Catharanthine, on the other hand, has a measurable binding, most consistent with one binding site, just as VBL, with an intrinsic binding constant of $K_1 = (2.8 \pm 0.4) \times 10^3$ M^{-1}. Comparison of the binding strength of VBL with those of its two moieties shows that a covalent link between the two is required for the relatively strong bindings of VBL and VCR, since an equimolar mixture of the two moieties gave binding results intermediate between the two individually. What are the consequences of the covalent linkage? In the case of colchicine it had been identified as the source of its very strong binding to tubulin due to the gain of the cratic free energy change (Andreu & Timasheff, 1982). For the vinca drugs the same is not true. VBL and VCR both bind to tubulin with a standard free energy change, $\Delta G°$, of −6.3 kcal mol^{-1} under our experimental conditions. For CTN, $\Delta G°$ is −4.6 kcal mol^{-1}. For VDN, $\Delta G°$ may be estimated as no stronger than −1.8 kcal mol^{-1}. The sum of the two moieties, if the binding of VDN is specific, should contain, therefore, a maximal binding capacity of $\Delta G° = \sim -6.4$ kcal mol^{-1}, which is essentially that of the dimeric drugs. This, however, does not take into account the covalent linkage between the two moieties, which must contribute another −2.5 kcal mol^{-1} of cratic standard free energy. The maximal binding strengths of VBL and VCR to tubulin could be expected to be, therefore, on the order of $\Delta G° = -8.9$ kcal mol^{-1}, which corresponds to $K_1 = 4.3 \times 10^6$ M^{-1}, or essentially half that of colchicine, but which is 2 orders of magnitude larger than the known intrinsic binding constants of VBL and VCR. Therefore, contrary to the case of colchicine, in which the full contributions of its parts are translated into binding to tubulin, in the case of the vinca alkaloids, as much as 2 kcal mol^{-1} of standard free energy are used to overcome unfavorable events linked to the binding process, such as the freezing of any possible free rotation between the two parts of VBL/VCR and the induction of strain into the drug or/and tubulin molecules.

In the vinca alkaloid induced self-association of tubulin, CTN induces in tubulin a self-association reaction that gives the same velocity sedimentation pattern of protein and ligand concentration dependence as VBL and VCR. For the drug-mediated mechanism of self-association that describes the results in the presence of VBL and VCR (Na & Timasheff, 1980b; Prakash & Timasheff, 1985), the intrinsic association constant, K_2, for the addition of each liganded α-β tubulin to the growing polymer is related to K_2^{app}, within close approximation, by

$$K_2 = K_2^{app}(1 + 1/K_1[A])^2 \quad (7)$$

where [A] is the concentration of free ligand in equilibrium with the polymerizing protein. For CTN, $K_1 = 2.8 \times 10^3$ M^{-1} gives $K_2 \simeq 1 \times 10^5$ M^{-1}, or $\Delta G_2° = -6.7$ kcal mol^{-1}. Thus, the standard free energy change for the addition of each tubulin–CTN complex to the growing polymeric chain is weaker than that of tubulin–VBL and tubulin–VCR addition by only 0.3 and 0.7 kcal mol^{-1}, respectively. Therefore, the much lower degree of polymerization in the presence of CTN than with VBL/VCR at identical free drug concentration observed experimentally (see Figure 3), which at first glance might seem inconsistent with the similarity of the self-association constants, is seen to reflect not a difference in ability to polymerize but the greater difference (1.7 kcal mol^{-1}) in the binding affinities of the drugs to tubulin. In the presence of VDN, polymerization of tubulin was marginally detectable and a mixture of the two halves gave no enhancement over the effect of CTN alone. It is clear, therefore, that CTN contains the information necessary for inducing in tubulin the conformational state required for the protein to self-assemble into the linear structures that result eventually in the well-known spirals and paracrystals.

What is the role of the two moieties of VBL/VCR in imparting to tubulin the properties linked to binding? Since CTN binds much more strongly to tubulin than VDN and by itself induces the self-association in the proper mode, it should be assigned the role of the moiety that determines what properties will be imparted to tubulin. This is consistent with the suggestion of Wilson et al. (1974) that the biological activity of the vinca alkaloids resides in the catharanthine portion of the molecule. The role of VDN must then be the anchoring of the bidentate ligand. Its weak interaction with tubulin provides sufficient additional free energy to raise the biochemical activity of the dimeric molecules to the level at which it becomes readily measurable in the laboratory and useful clinically. The net thermodynamic effect of covalently linking VDN to CTN is essentially to add −1.7 kcal mol^{-1} of binding free energy. Thermodynamically, CTN has 75% of the binding strength of VBL and VCR. In terms of equilibrium constants, this manifests itself in a decrease by 1 order of magnitude, with the consequence that, in order to measure the binding of CTN in the laboratory, it becomes necessary to push techniques to the limit with the consequent scatter of data. Yet, is it correct to call a standard free energy change of −6.3 kcal/mol strong and one of −4.6 kcal/mol weak?

The single property in which the action of CTN appears to be qualitatively different from that of VBL/VCR is the inhibition of tubulin self-assembly into microtubules. Both VBL and VCR inhibit microtubule formation substoichiometrically, 50% reduction of turbidity occurring typically at a 50–60 molar ratio of tubulin to drug in our assembly buffer. In the case of CTN, this ratio is on the order of 35 drug molecules to one tubulin, or just reversed. Substoichiometric inhibition requires, therefore, the covalent linkage of VDN to CTN. The close to 2000-fold difference in the drug to tubulin molar ratio cannot be accounted for just in terms of the weaker binding of CTN to tubulin. The inhibition of microtubule formation by VBL is regarded to proceed by a mechanism similar to that of colchicine, namely, by the formation of a tubulin–VBL complex at the end of a growing microtubule and consequent blocking of self-assembly (Wilson et al., 1976; Margolis et al., 1980). Wilson et al. (1982), in fact, have shown that the inhibition of microtubule growth can take place through the direct binding of vinblastine to a microtubule end. CTN does not seem capable of such action, since its concentration required for inhibition of microtubule growth would correspond to a binding constant to microtubule ends on the order of 2.5×10^2 M^{-1}, or 1 order of magnitude lower than the binding to free tubulin. Addition of VDN does not change the situation. The current observation that, in order to inhibit microtubules, CTN must be present at a 1000-fold higher ratio to tubulin than VBL is fully consistent with earlier observations (Owellen et al., 1976, 1977; Wilson et al., 1976). In in vivo

studies, Wilson et al. (1974) have found that mitosis in HeLa cells is inhibited by VBL 1000-fold more strongly than by CTN (ID_{50} of 7.5×10^{-8} M versus 5×10^{-5} M), while VDN is inactive.

A further question that needs to be addressed is the identification of the structural features of the vinca alkaloids that impart to them their interaction properties. As shown above, both the binding of the drug and induction of tubulin polymerization are modulated by the CTN moiety of VBL/VCR. Comparing the structure of CTN with its counterpart when incorporated into VBL shows that the two are identical only in the indole part and half of the middle homopiperidine ring in CTN and octamethyleneimine in VBL (compare structures I and II). This would, then, seem to be the best structural candidate for binding into the CTN binding subsite on tubulin. The rest of the structure differs greatly sterically between CTN and CTN when it is part of the VBL molecule. This conclusion is consistent with both the difference spectra and the chemical modification studies. The difference spectra generated by the binding of VBL and CTN to tubulin are characterized by bands in the indole region of absorption (298 and 288 nm for VBL, 294 and 285 nm for CTN). The corresponding difference spectrum of VDN is different from that of VBL. In chemical modification studies, Potier (1980) has synthesized a number of VBL analogues, which led to the demonstration (Potier et al., 1979) that the ester group in the middle ring of CTN within VBL is required for activity, although these studies failed to observe the induction of self-association by CTN. Modifications in the region of the piperidine ring seemed to have little effect (Potier et al., 1979). Other modifications, in particular in the VDN moiety, led to a complex pattern of changes in clinical activity (Gerzon, 1980), which are difficult to relate to the present physicochemical studies on the pure protein.

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