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Inhibition of Tubulin-Microtubule Polymerization by Drugs of the *Vinca* Alkaloid Class[1]

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SUMMARY

A series of *Vinca* alkaloids were found to block polymerization of crude tubulin extracts of porcine brain in a dose-dependent manner. This appears to be a specific effect occurring at low concentrations of drug. The concentration of vinblastine that prevents polymerization by 50% was 4.3×10^{-7} mole/liter for a tubulin concentration of 3.0 mg/ml, and this concentration is consistent with levels achieved *in vivo* following routine pharmacological doses in humans.

INTRODUCTION

It has been proposed that CLC[3] and the *Vinca* alkaloids, VLB and VCR cause their *in vivo* cytolytic effects by interaction with tubulin, the soluble 120,000-dalton protein component of microtubules (10, 14, 16, 20, 21). Recently, several investigators have reported the *in vitro* assembly of soluble tubulin into distinctive microtubular forms, confirmed by electron microscopy, and readily monitored by measuring viscosity changes in the solution (2, 8, 11). Both CLC and the *Vinca* alkaloids reportedly prevent this polymerization (11, 21).

As a continuation of our studies of the *Vinca* alkaloids and their biochemical interaction with cellular components, particularly tubulin, we chose to determine quantitatively the ability of these alkaloids to prevent the polymerization of tubulin, as measured by viscometry. We know from earlier studies that the binding of VLB and VCR to microtubular proteins occurs very rapidly (<7.5 min) and that this process will occur at 0°, as well as at 37° (14, 16). Thus, study of the inhibition of the polymerization process by the *Vinca* alkaloids should provide information concerning the number of protein sites that must be blocked to disrupt microtubular assembly. In addition, we have compared the ability of the various drugs to inhibit tubulin polymerization.

MATERIALS AND METHODS

Fresh pig brain, obtained from a local distributor (Esskay, Inc., Baltimore, Md.) within 3 hr of slaughter, was homogenized, 1.5 part by weight of brain cortex with 1.0 part by volume of 100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid):1.0 mM ethyleneglyol bis(β-aminoethyl ether)-*N,N'*-tetracetic acid buffer by 10 strokes in a motor-driven glass-Teflon homogenizer. This homogenate was then centrifuged at 20,000 × *g* for 30 min. To the supernatant was added 2.5 mM GTP, and this solution was used immediately in each of the experiments. Protein concentrations were determined by the method of Lowry *et al.* (9) (standardized against crystalline bovine serum albumin).

Oswald capillary viscometers, with a working volume of approximately 0.6 ml, were held in a large water bath, regulated at 37.0 ± 0.1°. Each viscometer was separately calibrated against the above buffer with stopwatches calibrated to 0.1 sec. For determination of the inhibitory effect of each drug on the polymerization of the crude tubulin solution, 6 simultaneous determinations were performed, 1 control of the solution alone with 5 with varying drug concentrations. The data were expressed as specific viscosity.

Binding of tritium-labeled CLC to the protein preparations was used to determine the amount of tubulin present by means of a Scatchard plot, as reported earlier (3, 14, 16, 19, 20). The K_a's were measured as the slope of this line, and the intercept of the abscissa provided an estimate of the amount of tubulin present, when corrected for the extent of binding [60% of maximum after 3 hr of incubation (16)]. [^{3}H]CLC binding has been shown by others to be specific for tubulin, and thus the data afford a reasonable measure of the percentage of tubulin in a protein mixture (19, 21).

The *Vinca* alkaloids, VLB, VCR, vinleurosine, vinrosidine, vinglycinate (7), desacetylvinblastine hydrazide (5, 6), desacetylvinblastine hydroxyethyl amide (6), desacetylvinblastine amide (vindesine) (5, 6, 18), catharanthine, and vindoline were graciously prepared and provided by the Eli Lilly Company, Indianapolis, Ind. Desacetylvinblastine was prepared as reported by Hargrove (7). Dithiothreitol, cyclic 3′:5′-AMP, and cyclic 3′:5′-GMP were purchased from Sigma Chemical Company, St. Louis, Mo., while triethylamine was purchased from Eastman Kodak, Rochester, N. Y.

RESULTS AND DISCUSSION

Other investigators (8, 11) have determined that polymerization of tubulin can be specifically monitored by the very large changes in specific viscosity that occur during formation of long strands of microtubules. This phenomenon has been confirmed by electron microscopy. The appearance of long intact microtubules correlated only with large changes

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[3] The abbreviations used are: CLC, colchicine; VLB, vinblastine; VCR, vincristine; ID_{50}, concentration of drug corresponding to 50% inhibition of the polymerization process.

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in specific viscosity ($\Delta \eta > 0.6$), and simple denaturation or other physical changes would not produce this effect. In our hands, the polymerization process proceeded very rapidly (*cf.* Ref. 8), with a rapid rise in specific viscosity beginning as soon as the cold solution was placed in the viscometer and rising to very high levels of specific viscosity (η = 1.0 to 1.6). Light microscopic examination using polarized optics revealed marked linear strands of birefringence in the solution only after a rise in viscosity. Control solutions at 0° or of denatured tubulin afforded no linear birefringence pattern. The ability to monitor tubulin polymerization by light microscopic linear birefringence and its correlation with electron microscopic linear birefringence and its correlation with electron microscopic observations have been reported by others (17). To obtain reproducible polymerization with high specific viscosities, we discovered that the GTP must be added just prior to the polymerization experiments.

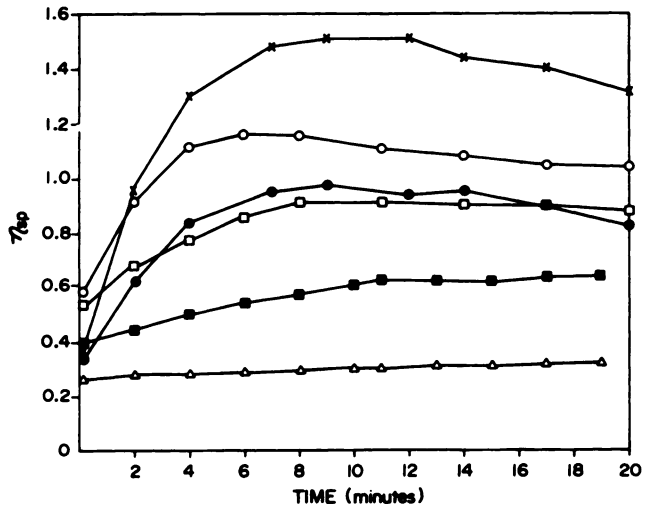

Chart 1. Time course of tubulin polymerization and its inhibition by VLB. To a solution of crude porcine tubulin (16.6 mg/ml) was added VLB to the final concentrations (mole/liter) noted below; the specific viscosity was determined in 0.6-ml Oswald viscometers. ×, control; ○, 5×10^{-8}; ●, 1×10^{-7}; □, 5×10^{-7}; ■, 1×10^{-6}; △, 5×10^{-5}.

By addition of varying concentrations of each of the different drugs, the maximum obtainable specific viscosity was decreased in an orderly progression (Chart 1). For each run, we determined the change in specific viscosity of the control, related this to the specific viscosity of the completely inhibiting concentration, and expressed the specific viscosity at each concentration of drug as a percentage of the control value. The values were plotted on probability paper, and the ID_{50} value was calculated. The data for each of the drugs are listed in Table 1. Because there was variability among the data that was paralleled, in general, by variations in the protein concentration, we next divided the ID_{50} by the protein concentration of that particular preparation to obtain the ratio ω. We found that these values were better correlated, and this also appears in Table 1.

From these data, we see that all except 2 of the dimeric *Vinca* derivatives block polymerization by 50% at 1.5 to 6.0 $\times 10^{-8}$ mole/g. The 2 exceptions were vinleurosine and vinrosidine, which required 10.7×10^{-8} and 21.4×10^{-8} mole/g, respectively.

To test whether the blockade of tubulin polymerization was dependent upon the intact dimeric *Vinca* structures, we studied vindoline, the bottom half of several of the dimeric alkaloids, and catharanthine, a compound related to the top half of these alkaloids. For both compounds, the ID_{50}'s were considerably above the values obtained with the dimers. In addition, neither drug is biologically active, even at very high doses.

That the effects seen in blocking the polymerization process were not due simply to the presence of a tertiary amine salt was shown by the relative ineffectiveness of triethyla-

Table 1

Effect of Vinca alkaloids and CLC upon tubulin polymerization

Drug	$ID_{50} \times 10^{-7}$ moles/liter	S.E.	$\omega \times 10^{-8}$ moles/g	S.E.	% inhibition
CLC	5.2	1.50	3.8	0.50	
VCR	3.2	0.50	2.6	0.30	
VLB	4.3	1.60	2.9	0.50	
Desacetylvinblastine amide[a]	2.3		1.5		
Desacetylvinblastine hydrazide[a]	2.6		1.6		
Desacetylvinblastine hydroxyethyl amide[a]	3.5		2.1		
Vinglycinate	5.0	0.00	3.6	0.10	
Desacetylvinblastine	6.0	0.05	6.0	1.70	
Vinleurosine	9.4	1.00	10.7	1.00	
Vinrosidine	16.7	2.20	21.4	5.60	
Catharanthine	~80		~62		
Vindoline	37		28		
Ca^{2+}	5500		3300		
Triethylamine-HCl	~4000		~2400		
Mn^{2+} (1×10^{-3} mole/liter)					46
Dithiothreitol (1×10^{-5} mole/liter)					69
Cyclic 3′:5′-AMP (1×10^{-5} mole/liter)					84
Cyclic 3′:5′-GMP (1×10^{-5} mole/liter)					112
Melatonin (1×10^{-3} mole/liter)					78

[a] Single run only.

mine-HCl to block the process. Calcium ion will inhibit polymerization, but only at a concentration of about 0.1 mM (10), and in our data ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid necessitated a slightly higher concentration to afford a free Ca^{2+} concentration approaching 0.1 mM. Most interesting was the observation that Mn^{2+} ion will block polymerization at about 1 mM, and we assume that its action is similar to that of Ca^{2+} ion. Dithiothreitol and cyclic 3':5'-AMP had only a moderate inhibitory effect on the polymerization process at 10^{-5} mole/liter, while cyclic 3':5'-GMP at this concentration, if anything, augmented the process. Melatonin, a compound recently found to inhibit the binding of CLC to tubulin (22), showed some moderate reduction in polymerization at a concentration of 10^{-3} mole/liter. This corresponds to a level where there is 60% inhibition of CLC binding to tubulin (21).

Each of the protein solutions used in the viscometry studies was analyzed for tubulin, using the binding of CLC as an assay. Scatchard plots of the data provided a measure of the K_a and the percentage of the protein that was tubulin (14, 16, 19, 21). The average for the K_a's obtained was 2.17 ± 0.20 × 10^6 liters/mole, and the average amount of tubulin present was 20.4 ± 2.2%.

Several values have been reported in the literature for the K_a and stoichiometry of binding of VLB to tubulin. In our own work we measured a value of 5 to 6 × 10^6 liters/mole with 1 mole of VLB binding to 2 moles of porcine tubulin (14, 16). Wilson has reported a K_a of 1.2 × 10^5 liters/mole for chick tubulin and, from the Scatchard curve giving this value, had a molar VLB:tubulin ratio of 1:2 (20). He reports, however, that use of an equilibrium column gave a different stoichiometry, with 2 moles of VLB bound per mole of tubulin. He offers that the VLB:tubulin complex is easily washed off the DEAE paper, affording a low value for the extent of VLB binding using this method. Since the equilibrium column uses a different technique, no K_a was measured for these conditions. We have recently resolved the K_a value differences in the DEAE paper assay by discovering that a simple variation in the washing technique used provides data compatible with both values, depending upon the conditions (unpublished data). We suspect that the differences reflect both an elution of VLB:tubulin complex off of the paper and the presence of 2 different binding sites, but further work is needed and the stoichiometry of this methodology has still to be worked out. At present, we consider the 2 K_a values as valid, and we suggest that they represent the known extremes.

Calculations were carried out on the basis of a tubulin concentration of 3.0 mg/ml (20% of 15-mg/ml crude concentration) and the VLB ID_{50} value of 4.3 × 10^{-7} mole/liter. We have assumed that the best measure of the molarity of VLB:tubulin is the 1:1 ratio found by Bryan (4) for VLB-induced crystals. When a 1:1 stoichiometry for the VLB:tubulin interaction was assumed, the percentages of drug and protein bound, respectively, for each K_a value were: K_a, 6.0 × 10^6 liters/mole, tubulin = 1.71%, VLB = 99.3%; K_a, 1.2 × 10^5 liters/mole, tubulin = 1.29%, VLB = 74.7%. Although the K_a values themselves differ by a factor of 50-fold, it appears to make little difference in the amount of tubulin that we calculate must be bound to block polymerization. Stoichiometries of 2:1 VLB:tubulin or 2:1 tubulin:VLB gave values of VLB bound of less than 0.4% and of tubulin bound of less than 0.0064%. We suspect that these latter values are far too low for practical considerations, especially in light of the known behavior of CLC (CLC:tubulin, 1:1) where <4% of the tubulin is bound when polymerization is completely blocked (11). Values of about 1.5% tubulin bound by VLB this appear to be reasonable in accounting for the blockage of tubulin in polymerization.

An important correlation is the fact that human *in vivo* blood levels of VLB are in the range of 1.0 to 3.0 × 10^{-7} mole/liters (12, 13, 15), and that the tissue levels [suggested by animal data (1)] are probably severalfold higher. In addition, tubulin concentrations in the cytosol of dividing cells are considerably lower than 3.0 mg/ml (3). Significant then is the blockade of tubulin polymerization in a concentration range that is consistent with that found or expected as an intracellular level following normal pharmacological dosing.

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