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Tubulin aggregation and disaggregation: Mediation by two distinct vinblastine-binding sites

(brain tubulin/polymerization/colchicine/podophyllotoxin)

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ABSTRACT **Rat brain tubulin possesses two distinct binding sites for vinblastine per molecule: a high-affinity site with an affinity constant of $6.2 \times 10^6\ M^{-1}$ and a low-affinity site with an affinity constant of $8 \times 10^4\ M^{-1}$. The high-affinity site is labile, with a $t_{1/2}^{37°}$ of 3.5 hr, is protected by colchicine, and is unaffected by salt, whereas the low-affinity site is stable but is inhibited by salt. Binding to both sites is rapid.**

The high-affinity binding constant of vinblastine to tubulin ($6.2 \times 10^6\ M^{-1}$) corresponds to the half-maximal concentration of vinblastine needed to prevent polymerization of tubulin *in vitro*, whereas the low-affinity binding constant ($8 \times 10^4\ M^{-1}$) corresponds to the half-maximal concentration of vinblastine required to aggregate tubulin. We conclude that vinblastine binding to the high- and low-affinity sites, respectively, accounts for the depolymerization and aggregation behavior of tubulin.

Antimitotic agents such as colchicine, podophyllotoxin, and vinblastine are thought to exert their antimitotic effect, as well as a number of other intracellular actions (1–4), by virtue of their ability to prevent tubulin polymerization to microtubules. For colchicine (5–7) and podophyllotoxin (8, *), affinity constants of these drugs for tubulin are commensurate with the concentrations required to produce the biological effects. However, published affinity constants of vinblastine for tubulin have ranged from 2×10^4 to $8 \times 10^6\ M^{-1}$, and the number of binding sites for vinblastine/molecule of tubulin (molecular weight 110,000) has been stated to vary from 0.5 to 2.0 (6, 9–12).

Vinblastine has a second effect on tubulin that is not shared by colchicine or podophyllotoxin, and which is observable both *in vitro* and *in vivo*. This second effect consists of a gradual aggregation of tubulin monomers from 6 S to 30 S and eventual precipitation (9, 13). *In vivo*, this aggregation is recognized as the reversible formation of macrotubules (340–420 Å diameter instead of 240 Å for normal microtubules), which are possibly formed by the tight coiling of loose helices; these, in turn, are believed to coalesce to form the paracrystalline arrays seen in the cytoplasm of many cells treated with vinblastine (14–16). That these paracrystals are, in fact, composed of tubulin is suggested by the findings that: they bind colchicine (17); they bind fluorescent antitubulin antibodies (18); they can be made *in vitro* from purified tubulin (15, 19); and, when isolated from tissues, paracrystals show an amino acid composition identical to that of purified tubulin (19).

These structural changes occur at higher vinblastine concentrations than those required to disaggregate microtubules and suggested to us that the confusing results on vinblastine binding affinity and stoichiometry might be resolved if one postulated two vinblastine binding sites on tubulin: one, high affinity, whose occupancy is related to microtubule disaggregation and a second site, of lower affinity, related to aggregation and paracrystal formation. In the present study we provide evidence that this is indeed the case.

Abbreviation: Buffer A, 10 mM $MgCl_2$, 0.1 mM GTP, and 10 mM sodium phosphate (pH 6.8).

* F. Cortese, B. Bhattacharyya, and J. Wolff, submitted for publication.

MATERIALS AND METHODS

For polymerization experiments, tubulin was purified from rat brain extracts by three cycles of polymerization and depolymerization, according to the method of Shelanski *et al.* (20). In some experiments we used the column procedure of Weisenberg *et al.* (21) or a procedure that combined both of the methods. After one cycle of polymerization of rat brain tubulin, the protein was depolymerized in buffer A (10 mM $MgCl_2$, 0.1 mM GTP, and 10 mM sodium phosphate, pH 6.8) at 0° for 30 min, applied to a DEAE-cellulose column, and eluted in the usual fashion. This protein yielded a single band in overloaded gels by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (22).

The kinetics of tubule assembly have been studied by turbidimetric measurements at 400 nm as described by Gaskin *et al.* (23) in a temperature-controlled chamber of a Cary spectrophotometer (model 14) at 37°. The polymerization buffer contained 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer (pH 6.4), 1 mM ethylene glycol-*bis*(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 1 mM GTP, and 0.5 mM $MgCl_2$.

Colchicine binding was determined by the DEAE-filter paper method (21) or by fluorescence (5).

Vinblastine Binding Assay. The DEAE-filter paper disc assay for colchicine had to be modified to make it suitable for vinblastine binding. Two DE-81 paper discs (Whatman) were washed with cold buffer A (4°) by mild suction, taking care not to dry the paper. The sample (100 μl) was applied and was absorbed to filters over a period of 1–2 min. The filters were then rinsed four times with 4 ml of cold (4°) buffer A by mild suction. The radioactivity of the filter papers was determined in 10 ml of Hydromix (Yorktown). In all cases, controls were run in the absence of tubulin, and this blank was subtracted from the quantity of vinblastine bound in the presence of tubulin. Binding results of duplicate experiments agreed to within 10%. The concentration of protein was determined by the method of Lowry *et al.* (24), with crystalline bovine albumin as a standard.

GTP (Grade II-S) was obtained from Sigma. Podophyllotoxin was generously supplied by Dr. W. J. Gensler of Boston University. Tritiated colchicine (ring C, [^{3}H]methoxy), a product of New England Nuclear Corp., had specific activity of 18.45 Ci/mmol. [^{3}H]vinblastine was prepared as described (25), and had a specific activity about 19 Ci/mmol and about 95% radiochemical purity. Vinblastine sulfate was a gift of Eli Lilly Laboratories.

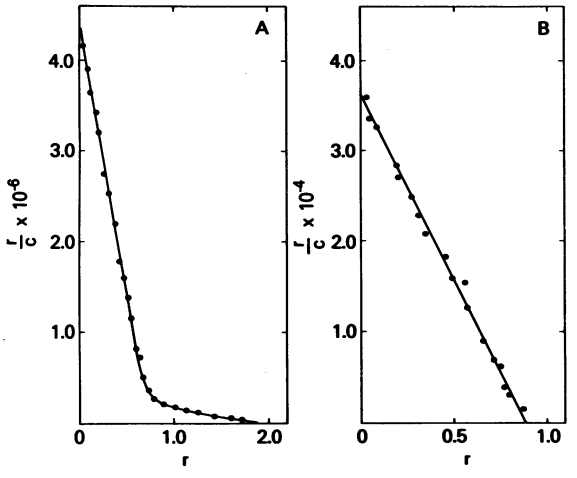

FIG. 1. Scatchard plot of vinblastine binding to rat brain tubulin. Binding of [^{3}H]vinblastine to tubulin was assayed by the DEAE-filter disc method as described in *Materials and Methods*. Incubations were at 37° for 30 min. (A) The reaction mixture contained freshly prepared tubulin (0.2 μM) with increasing concentrations of vinblastine in buffer A containing 0.25 M sucrose (pH 6.8). (B) The aged tubulin solution used in this experiment was devoid of colchicine-binding activity. The incubation mixture contained 0.28 μM tubulin solution in buffer A containing 0.25 M sucrose, with increasing concentrations of [^{3}H]vinblastine starting from 0.1×10^{-7} M to 1×10^{-4} M. Free vinblastine concentrations were determined from the difference between the total and bound ligand.

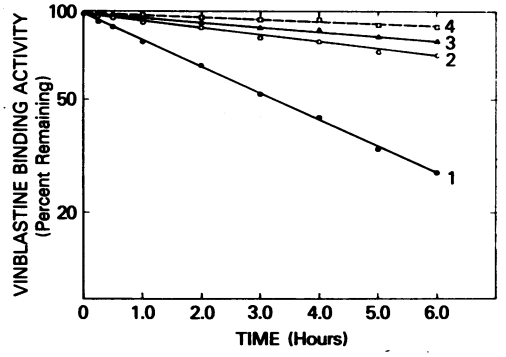

FIG. 2. Effect of preincubation at 37° of rat brain tubulin on vinblastine binding. Tubulin was incubated at 37° for the indicated period and divided into two parts. One part was assayed with 1.2×10^{-7} M [^{3}H]vinblastine for the high-affinity site, and the other part was assayed with 8×10^{-5} M [^{3}H]vinblastine for the low-affinity binding site. In both cases the vinblastine-binding activity was measured for 30 min at 37° at the end of the preincubation period. Conditions for preincubation and binding are: *curve 4*, low-affinity site in buffer A; *curve 3*, high-affinity site in buffer A containing 1×10^{-3} M colchicine; *curve 2*, high-affinity site in buffer A containing 1 M sucrose; and *curve 1*, high-affinity site in buffer A. Since the binding assay with 8×10^{-5} M vinblastine includes both the high- and low-affinity sites, the value for the low-affinity site was corrected for the contribution of high-affinity site.

RESULTS AND DISCUSSION

Affinity constants and binding sites

Preliminary experiments had shown that the binding of vinblastine to tubulin was relatively fast, and in the range of concentrations used (1×10^{-7} to 5×10^{-5} M vinblastine and 1×10^{-6} M tubulin), at 37° less than 15 min were needed to reach equilibrium. In addition, binding was not strongly temperature dependent at 0.5 μM (7), and the number of moles of vinblastine bound per mole of tubulin were 1.35, 1.38, 1.44, and 1.48 at 4°, 10°, 25°, and 37°, respectively, when 4.8×10^{-5} M vinblastine was used. Samples were generally incubated for 30 min at 37° and then analyzed. The affinity of vinblastine for freshly purified rat brain tubulin was determined by analyzing binding values obtained over a wide range of vinblastine concentration by the method of Scatchard, as shown in Fig. 1A. In contrast to previous reports (6, 10, 11), this analysis yielded a nonlinear Scatchard plot with a concavity upward. This suggested the presence of two or more classes of binding sites with different affinities for vinblastine, and results were analyzed on the assumption of two noninteracting sites. Such treatment of the data yielded a high-affinity site with an association constant, K_A, of 6.2×10^6 M^{-1}; 0.74 mole of vinblastine was bound to this site per mole of tubulin dimer (molecular weight 110,000). This value for the affinity constant is in agreement with that reported by Owellen *et al.* (6, 12). Their report of 0.5 mole of vinblastine per mole of tubulin can likely be ascribed to decay of this site with aging (see below) and losses on their filter paper assay.

Also present in tubulin was a second and lower affinity site, which binds vinblastine with an association constant of 8×10^4 M^{-1} and a stoichiometry of 1.84 moles of *total* vinblastine bound per mole of tubulin. Thus each site approaches a stoichiometry of about 1:1. The second constant appears to be similar to that reported by Lee *et al.* (10) (2×10^4 M^{-1}; $n = 2$) and to the half-maximal concentration required to produce fluorescence enhancement of 1-anilino-8-naphthalene sulfonate reported earlier (26). Wilson *et al.* (11) reported an affinity constant of 2×10^5 M^{-1} for vinblastine binding to chick brain tubulin and a stoichiometry of 2 moles of vinblastine per mole of tubulin. The difference in the low-affinity constants reported is not currently understood, but may be due, in part, to the failure to take into account the presence of the high-affinity site.

Stability of the binding sites

Additional differences between these two binding sites for vinblastine could be readily demonstrated. It is well known (25, 27, *) that colchicine binding to tubulin is labile, and that at 37° the colchicine-binding activity of uncomplexed tubulin decays in a first-order manner with a $t_{1/2}$ of 3–5 hr. Vinblastine and sucrose protect this binding activity. We were surprised to find, therefore, that the high-affinity vinblastine site is equally labile despite the fact that it is independent of the colchicine-binding site (6, 27, 28). The protein was pre-incubated at 37° and tested for its high-affinity site with 1.2×10^{-7} M vinblastine and for the lower affinity site with 8×10^{-5} M vinblastine. As shown in the Fig. 2, the high-affinity binding site decayed in a first-order manner with a $t_{1/2}$ of 3.5 hr. In contrast, the lower affinity site did not decay significantly under these incubation conditions (Fig. 2). When tubulin preparations were used that had been stored at 4° until they were entirely devoid of colchicine-binding activity, we found that there was no vinblastine binding to the high-affinity site, whereas the low-affinity vinblastine site persisted with only slightly impaired affinity (4×10^4 M^{-1}) and a stoichiometry of 0.85 mole of vinblastine bound per mole of tubulin (Fig. 1B). The possibility that there are *two* low-affinity binding sites per mole of tubulin, occupancy of which blocks binding by the high-affinity binding site, would seem to be discounted by the use of aged tubulin.

Of considerable interest was the observation that just as vinblastine protects the colchicine-binding site against decay at 37° (27), so does colchicine protect the high-affinity vinblastine-binding site (Fig. 2). Thus, while there appears to be no direct interaction between these ligands at their respective binding sites, site occupation by one ligand protects the other

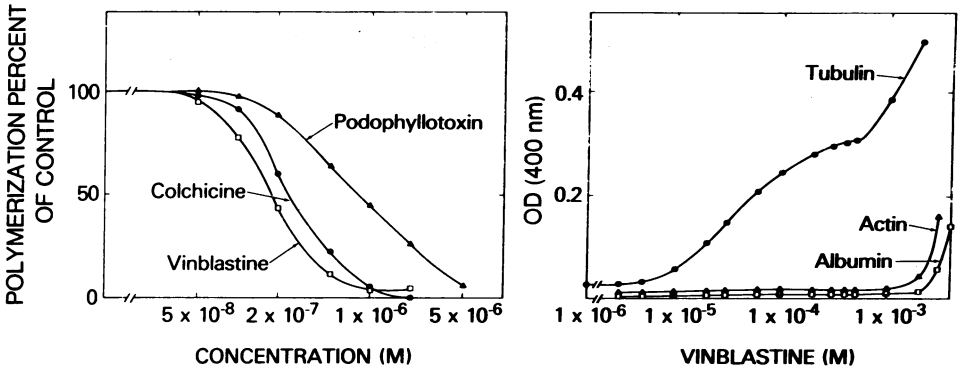

FIG. 3. Effects of antimitotic drugs on tubulin polymerization and aggregation. (Left panel) Aliquots of tubulin (1.8 mg/ml) in polymerization buffer were mixed with different concentrations of vinblastine, colchicine, and podophyllotoxin immediately before incubation at 37°. The polymerization was monitored by optical density at 400 nm in a temperature-controlled recording spectrophotometer. Data are expressed as the maximal plateau level reached without drug as 100%. (Right panel) Tubulin (0.8 mg/ml) in polymerization buffer containing 1 μM vinblastine was titrated with increasing concentrations of vinblastine. After each addition of vinblastine, the solution was incubated for 15 min at 37°, after which the optical density was measured at 400 nm. Similar titrations were carried out with actin (1.2 mg/ml) and bovine serum albumin (2.4 mg/ml).

site from denaturation. Sucrose (1.0 M) also preserves vinblastine binding of the high-affinity site (Fig. 2).

The addition of denaturing agents such as 1% sodium dodecyl sulfate or 8 M urea completely abolished vinblastine binding at both sites in tubulin, indicating the need for the native conformation of tubulin for vinblastine binding.

Ionic strength

Since vinblastine is a cation and since it has been reported that cations strongly influenced the vinblastine-induced aggregation of tubulin (13), effects of ionic strength on vinblastine binding to tubulin was investigated. As shown in Table 1, 0.4 M NaCl, KCl, or $NaNO_3$ had no effect on the binding of vinblastine to the high-affinity site of tubulin. On the other hand, binding of vinblastine to the low-affinity site was decreased by about 40% by each of these salts.† This suggests, on the one hand, that these salt concentrations do not have a major disruptive effect on the protein, and on the other, that vinblastine binding to the low-affinity site has an ionic component.

Correlation of binding to aggregation

In order to test the relation of vinblastine binding to the state of aggregation of tubulin, the effects of this alkaloid on polymerization were studied. As shown by Gaskin *et al.* (23), turbidity is a reliable measure of microtubule assembly that is independent of the mass of the monomer and of the length of the polymer provided this is a rigid rod of a length equal to or greater than the wavelength of the light used (Appendix of ref. 23). To avoid absorption from colchicine, the wavelength for measuring light scattering was increased to 400 nm. Optical density data from a typical polymerization experiment, expressed as percent of the polymerization obtained in the absence of drug versus the logarithm of the concentration of vinblastine, are shown in Fig. 3 (left panel). In this experiment, tubulin solutions were mixed with different concentrations of the drug immediately before incubation and were then incubated in a temperature-controlled chamber of the spectrophotometer at 37°. The half-maximal concentration of vinblastine needed to prevent polymerization in this experiment was 1.8×10^{-7} M, which is very similar to the value reported from experiments *in vivo* (29) for 50% inhibition of mitosis in EBH cells and is almost identical to the reciprocal of high-affinity constant (6.2×10^6 M^{-1}).

Similar experiments were done with colchicine and podophyllotoxin. The half-maximal concentrations for inhibition of polymerization are 2.4×10^{-7} M for colchicine and 8.0×10^{-7} M for podophyllotoxin. For both of these compounds, these half-maximal inhibitory concentrations are in good agreement with their corresponding affinity constants for tubulin, i.e., 3.2×10^6 M^{-1} and 1.8×10^6 M^{-1}, respectively (5, 6, *).

As the concentrations of vinblastine were increased there was a gradual rise in the optical density at 400 nm, which appeared to approach a plateau at about 4×10^{-4} M vinblastine. As can be seen in Fig. 3 (right panel), the half-maximal concentration of vinblastine for aggregation was 2.5×10^{-5} M. This value is similar to the value calculated for 50% occupancy of the low-affinity binding site from the second affinity constant (8×10^4 M^{-1}). It is clear, therefore, that the midpoints of both of these titration curves of Fig. 3 correspond closely to the high- and low-affinity binding sites of tubulin described above. Further increases in vinblastine concentrations led to precipitation of tubulin (at $>1 \times 10^{-3}$ M) and rapid changes in the optical density. This appears to be a quite nonspecific effect, since it is shared by other proteins having acidic isoelectric points, such as serum albumin or rabbit muscle G actin. Unlike tubulin, these two proteins do not appear to progress through a clear-cut stage of oligomer formation.

Table 1. Ionic strength effect on the vinblastine-binding to tubulin

Salt (400 mM)	Inhibition of binding of the control (%)	
	High-affinity site	Low-affinity site
NaCl	0	38
KCl	0	37
$NaNO_3$	0	40

Tubulin (1.1 μM) was incubated in buffer A with [^{3}H]vinblastine with and without salts for 30 minutes at 37°. Vinblastine concentrations were 1.2×10^{-7} M for the measurement of binding at the high-affinity site and 8×10^{-5} M for the low-affinity site.

† This effect was concentration-dependent over the range tested. Higher concentration could not be used because of difficulties with assays that use DEAE-filter paper binding of tubulin.

Table 2. Comparison of high- and low-affinity vinblastine binding sites to tubulin

	High affinity	Low affinity
Affinity constant	(6.2×10^6 M^{-1})	(8×10^4 M^{-1})
Stoichiometry	~1.0	~1.0
$t_{1/2}^{37°}$ for decay	3.5 hr (protected by colchicine)	>25 hr
Salt (400 mM)	No effect	~40% inhibition
Denaturing agents	Binding abolished	Binding abolished
Effect on state of tubulin	Inhibition of polymerization (half-maximal concentration = 1.8×10^{-7} M)	Aggregation (half-maximal concentration = 2.4×10^{-5} M)

CONCLUSIONS

The dual action of vinblastine on the state of aggregation of tubulin can be ascribed to the presence on that protein of two distinct and different binding sites for the alkaloid. The high-affinity site (K_A about 6×10^6 M^{-1}) binds vinblastine at concentrations where antimitotic effects and the disappearance of cytoplasmic microtubules are observed *in vivo*. A lower affinity site (K_A about 8×10^4 M^{-1}) is associated with various aggregation phenomena both *in vitro* and *in vivo* (9, 13, 19). A comparison of the properties of these two sites is given in Table 2. Both sites are specific, and vinblastine effects at these concentrations were not observed with the other acidic proteins examined. On the other hand, the precipitation occurring at $>10^{-3}$ M vinblastine is observed with a variety of proteins. Occupancy of the lower affinity site appears to overcome the blocking of polymerization caused by occupancy of the high-affinity site, without apparently interfering with binding to this site.

Although colchicine and vinblastine do not compete with each other for their respective binding sites, some sort of "long-range" or allosteric interaction between the colchicine and vinblastine sites must occur since occupancy of one site protects the other. Whether the conformational changes in tubulin upon colchicine binding (30) have effects on vinblastine binding, and whether vinblastine will have similar conformational effects remains to be seen.

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