Localization of the Vinblastine-binding Site on β-Tubulin*

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A fluorescent vinblastine derivative, vinblastine-4'-anthranilate, has been shown to inhibit polymerization of rat brain tubulin (IC50 = 4.8 µM). Binding of the drug to tubulin increases fluorescence intensity, causes a small emission blue shift, and has a quantum yield of 0.037. Fluorescence increases as a function of drug concentration, with a high affinity site and an undetermined number of lower affinity sites. Photolabeling, by exciting the fluorescent drug-tubulin complex at the absorption maximum of anthranilic acid, yields a covalent adduct confined to β-tubulin. Its formation is specific in that it is blocked by maytansine or vinblastine. Tryptic hydrolysis identifies a single fluorescent β-peptide coinciding with residues 175-213. The interactions between various ligands at this central portion of β-tubulin are discussed.

Vinblastine (VLB) and its congeners, dimeric indole alkaloids derived from Catharanthus (Vinca) roseus, are highly useful drugs for the treatment of certain malignancies. The bulk of evidence suggests that these effects are mediated by an interaction of these drugs with tubulin and/or microtubules. This interaction may take the form of the following: 1) diminished microtubule dynamics by suppressing dynamic instability at both ends of the microtubule, thus increasing the time spent in the "resting" state (this occurs at a concentration of <1 µM VLB (1)); 2) inhibition of microtubule assembly or promotion of disassembly at intermediate concentrations (2-4); and 3) formation of spirals, tubules with splayed (protofilament) ends, paracrystals, and other aggregates (2, 4, 5-7) at high concentrations. In all cases, normal microtubule function is compromised.

Vinblastine can bind to dimeric tubulin, to microtubule ends, and to certain aggregates formed by self-association of tubulin. This has led to considerable confusion in the literature regarding the affinity constants, which are stated to vary over several orders of magnitude. These values are influenced by the solvent composition, protein concentration, presence or absence of associated proteins, and method of assay (reviewed in Refs. 4 and 7). Certain chemically unrelated compounds such as maytansine or rhizoxin have been shown to bind to the same site in competitive binding assays. There are also low affinity sites. Under the conditions of this study, affinities in the µM range have been considered the appropriate standard of comparison.

Attempts to localize the binding site(s) for VLB to either the α- or β-monomers of tubulin have met with only limited success. Indirect evidence points to β-tubulin. Thus, VLB has major effects on the cross-linking of specific SH groups on β-tubulin with the bifunctional N,N'-ethylene bis(oxyaceticamide): Cys1239-Cys1354 cross-linking is enhanced by VLB, whereas Cys112-Cys120 or Cys122 is inhibited by VLB (8). Similarly, VLB inhibits the hydrolysis of the exchangeable GTP bound to β-tubulin (9, 10). On the other hand, covalent binding to tubulin showed an excess of label on α-tubulin (α/β = 3.2 or 3.1) using either direct photolabeling or a photosensitive adduct (11-13). In both of these studies, however, photolabeling was carried out at low enough wavelengths to permit irradiation of tubulin as well, thus compromising the specificity of the reaction. Addition of a nitro group to the photoactive label linked to the vindoline moiety of vindesine, an active analogue of VLB, circumvented this problem, but labeling still favored α- over β-tubulin.

Because of these uncertainties, we have re-examined VLB binding to tubulin using a photosensitive derivative that absorbs outside the protein wavelength range and that was also as small a substituent as possible because large spacers may lead to erroneous localization in tubulin (12). For this reason, we derivatized VLB with anthranilic acid through an interaction with isatoic anhydride, which could bind to one of the two available OH groups of the drug. This method has been successfully used in adding a fluorescent label to ATP, preserving high affinity for the ATP site (14, 15). The fluorescent adduct of VLB so produced has both a satisfactory antimicrotubule activity and a quantum yield sufficient to permit localization of VLB binding to β-tubulin.

MATERIALS AND METHODS

Vinblastine sulfate and trifluoroacetic acid were from Sigma. Isatoic anhydride was from Janssen Chimica. Exision-grade trypsin was from Calbiochem. HPLC-grade acetonitrile was from Fisher. Rat brain tubulin was prepared as described previously (16, 17). Where indicated, α- and β-tubulins were separated according to Uppuluri et al. (18).

The synthesis of the fluorescent VLB derivative (14, 15), here called Ant-Vin, involves the esterification of the 4′-OH (or 3-OH) group of VLB with isatoic anhydride at pH 9.6. To 2 mg of VLB dissolved in 50% dimethyl sulfoxide and adjusted to pH 9.8 with 0.1 M NaOH, 2 mg of solid isatoic anhydride (recrystallized from ethanol) were added slowly with constant stirring at 38 °C, and the reaction was continued for 2 h, maintaining the pH at 9.6. The reaction mixture was dried and dissolved in methanol for purification in a reverse-phase HPLC column (Vydac C18) using a linear 50-70% methanol gradient in 0.05% aqueous trifluoroacetic acid. Absorbance measurement at 254 nm yielded a peak at 9.8 min, well separated from unreacted VLB or isatoic anhydride. The yield was 68% of the theoretical value. The purified material was dried and characterized by UV, NMR, and mass spectra. The latter yielded a mass of 930 Da (the calculated mass for C32H36N4O14 is 930.1057).

Tubulin (1 mg) was incubated for 30 min at 37 °C with Ant-Vin, with or without the inhibitor VLB or maytansine, and irradiated for 20 min under 2 cm of 20% CuSO4 as described previously (12, 18). Irradiated samples were precipitated overnight with cold acetone in a −20°C freezer and centrifuged in a microcentrifuge at 3°C for 20 min at 11,000 rpm. The pellet was washed once with cold acetone, re centrifuged, and dissolved in Mes assembly buffer by sonication.
Labeled tubulin was extensively hydrolyzed for 24 h with a 1:20 trypsin/tubulin mass ratio at 37 °C in 0.05 M NH₄HCO₃, pH 8.0. In some cases, half again as much trypsin was added after 12 h. Digests were separated by HPLC using a reverse-phase C8 column eluted with a 1–40% gradient of acetonitrile in 0.05% aqueous trifluoroacetic acid at a flow rate of 0.5 ml/min for 90 min. Fractions were dried in a Savant Speedvac concentrator, dissolved in 50% acetonitrile, and assayed for fluorescence. Measurements were carried out in a Perkin-Elmer MFP 66 spectrophotofluorometer in ratio mode (uncorrected). Binding parameters were calculated from fluorescence titration experiments. Tubulin (12.5 μM) was titrated with Ant-Vin (5–160 μM), and the increase in area under the emission curves from 390 to 580 nm was calculated. All areas were corrected for the inner filter effect according to the following relation: 

\[ A_{\text{corrected}} = A_{\text{observed}} \frac{Q_f}{Q_f + 0.5 \cdot Q_{\text{em}}/Q_{\text{ex}}} \]

where \( Q_{\text{em}} \) and \( Q_{\text{ex}} \) are the optical densities at excitation (330 nm) and emission wavelengths, respectively. \( Q_{\text{em}} \) was negligible in these experiments. Bound Ant-Vin was calculated according to the following relation: 

\[ A_{\text{corrected}} = \frac{A_{\text{free}} - A_{\text{bound}}}{A_{\text{free}}} \]

where \( A_{\text{free}} \) is the emission area for free Ant-Vin and \( A_{\text{bound}} \) is the maximum area when all of the drug is bound to tubulin. The latter was determined by titrating a fixed amount of Ant-Vin with excess tubulin and by determining the intercept of a plot of \( 1/A \) versus \( 1/t_{\text{Vlb}} \). Stoichiometry and binding constants were obtained from Scatchard plots.

Polymerization of tubulin was carried out in Mes assembly buffer with 10% dimethyl sulfoxide and 1 mM GTP at 37 °C, measuring light scattering at 400 nm in a thermostatted Cary 219 spectrophotometer. This wavelength was necessitated by the absorption of the ligand at 350 nm.

### RESULTS

Properties of Ant-Vin—The VBL-anthranilate adduct, here called Ant-Vin, yielded a mass spectrum-derived molecular mass of 930 Da, consistent with the addition of a single anthranilate to VLB. The location of the anthranilate could be either on the 3-OH group of the vindoline moiety of VLB or on the 4'-OH group of the carbomethoxyvelbanamine (often incorrectly referred to as the catharanthine) moiety of VLB as shown in Fig. 1. Although not definitively established, we believe that the substitution occurred at the 4'-position for the following reasons. 1) When vindoline is reacted with isatyclic anhydride, the reaction is very slow compared with vinblastine, and the fluorescent spectrum of the product does not resemble the vinblastine product; and 2) a fast atom bombardment mass spectroscopy fragment containing the 4'-OH group is not found after acylation with isatyclic anhydride.

The absorption and emission spectra of Ant-Vin in Mes assembly buffer are depicted in Fig. 2. There are two absorption maxima: one at 265 nm (\( \epsilon = 18,300 \text{ cm}^{-1} \text{ M}^{-1} \)) and the other with a broad peak at \( \sim 320 \text{ nm} (\epsilon = 13,200 \text{ cm}^{-1} \text{ M}^{-1}) \). Excitation spectra with emission set at 445 nm yield peaks at 267 and 327 nm, and excitation at both peaks yields similar emission peaks. The emission maximum occurs at 441 nm (uncorrected) when excited at 330 nm. The relative quantum yield of Ant-Vin in Mes assembly buffer is 0.037 using 0.55 for the quantum yield of quinine in 1 N sulfuric acid as standard.

Polymerization—The effect of Ant-Vin on the polymerization of rat brain tubulin is compared with that of VLB in Fig. 3. Note that light scattering was measured at 400 nm because of absorption by Ant-Vin at 350 nm, the wavelength usually employed. In both cases (Fig. 3, A and B), there is a gradual decrease in the rate and extent of polymerization with increasing concentrations of the drugs. Inhibition is nearly complete at 3 μM VLB and at 15 μM Ant-Vin under these polymerization conditions. These changes are accompanied by a progressive increase in the latent period for polymerization. A potency comparison of the two drugs is depicted in Fig. 3C, which is derived from the data of Fig. 3 (A and B). Ant-Vin is -7.5 times less potent against microtubule assembly than the parent compound, with IC₅₀ = 0.64 μM for VLB and 4.8 μM for Ant-Vin. It is of interest that this IC₅₀ is lower than the binding constant (see below), suggesting that the inhibition of polymerization is substoichiometric.

Binding of Ant-Vin to Tubulin—When Ant-Vin binds to tubulin, an increase in fluorescence intensity is readily observed, which is accompanied by an \(-5 \text{ nm} \) blue shift from 441 to 436 nm (Fig. 4). This is equivalent to a polarity of 25% ethanol in water (Fig. 4, inset). The fluorescence intensity shows a linear dependence on the ethanol concentration (0–50%) and a progressive blue shift in \( \lambda_{\text{max}} \), thus demonstrating sensitivity of the probe to the polarity of the environment.

VLB binding to tubulin is difficult to separate from its effect on tubulin aggregation (7), which, in turn, could influence Ant-Vin binding. To our surprise, under our solvent conditions and at Ant-Vin/tubulin mole ratios up to 25:1, no polymerization occurred as measured by optical density at 400 nm or by 90° light scattering at 400 nm. For this reason, we felt justified in using a simple binding model to analyze Ant-Vin binding to tubulin. As shown in Fig. 5, 12.5 μM tubulin was titrated with increasing Ant-Vin concentrations (5–160 μM). Fluorescence enhancement, as calculated from the area under the curves over a wavelength range of 390–580 nm (corrected for the inner filter effect), is presented as a function of the total Ant-Vin concentration. What appears to be a high affinity, linear portion at low mole ratios is followed by poorly defined lower affinity sites at higher mole ratios. Because it is not known whether the quantum yield for all sites is the same, it is difficult to derive accurate binding data. On the unproved assumption that the quantum yield is the same, we have cal-
culated a rough affinity constant of ∼40 μM for the linear portion. Current evidence favors one high affinity VLB-binding site (4, 7); this and our finding of a single Ant-Vin-labeled peptide (see below) indicate a stoichiometry of ∼0.9 from Scatchard analysis. The latter is consistent with the single β-peptide identified below. Unlike colchicine fluorescence (20), no time dependence of the fluorescence enhancement could be detected over a period of 30 min, i.e. the fluorescence at zero time and 30 min was the same. This suggests that binding is rapid, as has previously been shown for VLB. Under the conditions used for high affinity binding (Ant-Vin/tubulin mole ratio = 1), all of the Ant-Vin binding can be blocked by a 10-fold excess of maytansine, with a return of λmax to the value for free Ant-Vin. At higher Ant-Vin/dimer mole ratios, low affinity binding is seen; this appeared to be nonspecific in that it could not be displaced by maytansine. These sites were not further investigated.

Localization of the Ant-Vin-binding Site—It has previously been shown that photolabeling of tubulin, followed by separation of the α- and β-monomers under denaturing conditions, provides a convenient way to identify the binding monomer. In the present case, the label or its spacer is not so large as to displace the label to the contralateral monomer (12). It is for this reason that we chose anthranilate as the smallest convenient fluorophore. After binding, irradiation, and separation of the monomers, the bulk of the label was located on β-tubulin, with a yield of ∼13%. At mole ratios ≥3, significant label appeared also on α-tubulin. The fluorescence of the isolated and eluted α- and β-monomers was measured after irradiation with and without a 10-fold excess of maytansine, a known competitor at the vinyca site. These results are depicted in Fig. 6. Under conditions where comparisons were made at identical protein concentrations, >90% of the labeling of β-tubulin (curve 3) was blocked by maytansine (curve 4), whereas none of the label in the isolated α-monomer (curve 1) was affected by the presence of maytansine during binding and irradiation (curve 2). This points to the nonspecific nature of Ant-Vin labeling of α-tubulin and the specific labeling of β-tubulin.

Initially, tryptic peptides were prepared from isolated β-monomers. However, the fluorescent peptide could be uniquely identified in hydrolysates of the intact dimer; hence, subsequent studies were carried out without separation of the monomers. Tryptic digests of Ant-Vin-labeled tubulin were analyzed by reverse-phase C18 HPLC. The results are shown in Fig. 7. All peaks were analyzed for fluorescence; two fluores-
Asn-, which corresponds to residues 175–213 of its unlabeled partner, depicted in Fig. 7 (420 nm in the same solvent). This should be compared with a maximum of free Ant-Vin of Vin. The emission maximum occurs at 420 nm; this was determined from an indiffrent unlabeled peptide; the basis for the residual fluorescence present in these curves is unknown. When curve 2 is subtracted from curve 1, yielding curve 3, it is clear that vinblastine successfully competed for the binding site of Ant-Vin. The emission maximum occurs at 420 nm (curves 1 and 3); this should be compared with a maximum of free Ant-Vin of 420 nm in the same solvent.

N-terminal sequencing of the labeled tryptic peptide (*) and its unlabeled partner, depicted in Fig. 7 (inset), yielded the following sequence: Val-Ser-Asp-Thr-Val-Val-Glu-Pro-Tyr-Asn, which corresponds to residues 175–213 of β-tubulin because the next tryptic cut site is between Arg213 and Thr214. The other fluorescent, but nonspecific, peptide region (#) was sequenced, but yielded a mixture of peptides corresponding mainly to sequences derived from α-tubulin. These peptides were not further investigated.

**DISCUSSION**

In this study, we present evidence that a fluorescent derivative of vinblastine, called Ant-Vin, containing a small fluorophore at position 4‘, binds in the central portion of the primary sequence of β-tubulin. Binding increases the fluorescence intensity and is accompanied by a 5-nm blue shift. Steady-state fluorescence studies reveal a single high affinity binding site with a K_d of ~40 μM. There are also low affinity sites whose properties were difficult to quantify because of inner filter effects at the high Ant-Vin concentrations and the uncertainty of their quantum yields. Binding is specific in the sense that being inhibited by maytansine, a vinca site analogue, that blocks the binding of vinblastine (21, 22), and we consider this reasonable evidence that Ant-Vin binds to the vinblastine-binding site. Binding prevents tubulin polymerization, with an IC_{50} of ~5 μM; but, unlike the parent compound, Ant-Vin does not lead to aggregation, as judged by turbidity or 90° light scattering at 400 nm with mole ratios as large as 25:1. (The implications of this finding will be the subject of another report.) UV irradiation at wavelengths >325 nm leads to the formation of a covalent adduct that can be blocked by vinblastine or maytansine. Monomer separation showed the specific label to be confined to β-tubulin. Tryptic digestion, followed by HPLC, yielded a single fluorescent peptide spanning the region β-(175–213). Because of the low yield, the Ant-Vin-binding amino acid could not be identified.

The central region of β-tubulin is also involved in interactions with other ligands as follows. 1) Colchicine binds at β-(214–241) (18); 2) a Taxol derivative binds at β-(217–231) (23); and 3) sulfhydryl groups in this region have been shown to cross-link to other regions of the protein, i.e. Cys^{212}, Cys^{220}, or Cys^{221} and Cys^{229}–Cys^{234} (8). Some of the antimitotic ligands also interact at a second locus. Thus, colchicine also binds at the N terminus of β-tubulin between β-(1–41) (18); Taxol binds in a region near Cys^{212} (23), where GTP also binds (24). It is curious that GTP, one of whose binding sites is at Cys^{212}, is hydrolyzed normally in the presence of Taxol (4, 25), whereas hydrolysis is increased in the presence of colchicine (4) and inhibited by vinblastine. Finally, the A ring of colchicine has recently been shown to bind to Cys^{234} (2). Thus, ligands at the center of β-tubulin span 11 ± 2 Å distances to both the N- and C-terminal regions of the monomer. Surprisingly, and unlike colchicine or Taxol, no additional contribution to the binding site from elsewhere in β-tubulin could be detected. How-

2 E. Hamel, personal communication.
ever, Sawada et al. (26), using an azidodansyl derivative of rhizoxin, a VLB site analogue, located a binding component at \( \beta \)-tubulin (363–379). Possibly, this region may contribute to VLB binding, but was not accessible to Ant-Vin under our conditions.

The above findings imply that some part of the central portion of \( \beta \)-tubulin is accessible to solvent and that the ligands bound there may well affect each other. Such interactions occur, as we shall see below.

1) It has been known for a long time that vinblastine and colchicine do not hinder each other’s binding. The early studies of Wilson et al. (2, 27, 28) have repeatedly demonstrated that, by contrast, vinblastine protects the colchicine-binding site against decay in a number of species. This observation has been confirmed in many laboratories (Refs. 22 and 29–31, among others). A single exception to this consensus (32) remains unexplained. The reverse of this phenomenon has also been shown, i.e., the decay of the high affinity vinblastine-binding site is retarded by colchicine (33), and vinca site compounds increase the specificity of colchicine labeling by increasing the \( \beta \alpha \) labeling ratio (34).

2) Luduena and Roach (8) have shown, using the bifunctional sulfhydryl reagent N,N’-ethylene bis(iodoacetamide) containing a 9-Å spacer, that colchicine site ligands inhibit the formation of the cross-link between \( \text{Cys}^{229} \) and \( \text{Cys}^{354} \), whereas vinca analogues enhance the formation of this cross-link while reattaching cross-linking between \( \text{Cys}^{102} \) or \( \text{Cys}^{211} \). Guanine nucleotides enhance the 239/354 cross-link, while inhibiting the 12/201 or 211 cross-linking reaction. The latter is consistent with the presence of a binding locus for GTP at \( \text{Cys}^{112} \) (24). It seems possible then that the VLB-binding site is near enough \( \text{Cys}^{201} \) and/or \( \text{Cys}^{211} \) to account for the blocking of cross-linking.

3) Taxol binds to tubulin with a stoichiometry of 1/dimer (35). Depending on the location of the photolabel, binding of the derivative occurs either at the N terminus (\( \beta \)-1–31) (23) or near the center of the linear sequence (\( \beta \)-217–231) (36), suggesting that the Taxol-binding site spans the distance between these regions. These regions are near those occupied by colchicine (18) or by vinblastine as shown here, and it is not surprising that both of these drugs inhibit Taxol binding in cells (37) or in vitro (38). Vinblastine is a more effective inhibitor than colchicine. However, in view of the fact that Taxol binds preferentially to polymerized tubulin (39), a trivial explanation may be that VLB- or colchicine-induced conformational changes hinder the capacity of tubulin to polymerize even in the presence of Taxol and hence reduce binding. Once stable microtubules are formed, Taxol binding is no longer significantly inhibited by vinblastine or colchicine (38).

The interactions between the ligands are locally determined at the central portion of the \( \beta \)-monomer or globally or at long range, or both, is difficult to ascertain. However, there are some examples of probable global or long-range effects of these ligands upon each other. Recently, it has been shown that colchicine site occupancy leads to unfolding of an amphipathic helix near residue 390 of \( \beta \)-tubulin as assessed by proteolytic accessibility and that vinblastine site occupancy prevents and/or reverses this unfolding (34, 40). Vinblastine also enhances the fluorescence of anilinonaphthalenesulfonate when bound to tubulin at a high affinity site (41); however, the location of this site is not yet known. Since GTP binding is believed to be confined to the N-terminal portion of \( \beta \)-tubulin, some of the drug effects on GTP hydrolysis must be considered long range with respect to the primary structure. Finally, another long-range effect of vinblastine is the reduced ability of the drug-tubulin complex to interact with antibodies with different epitopes directed against tubulin. This action is similar, but not identical, to that of colchicine because the effects of the two drugs are additive (42). One may conclude then that, while the folding pattern of tubulin is not known, the central portion of the primary sequence is accessible to a number of ligands and must also be in reasonably close proximity to both the N terminus and a portion of the C-terminal half of the \( \beta \)-monomer. Which interactions between these regions of \( \beta \)-tubulin are mediated directly by the ligands and which are long-range conformational effects beyond the span of these ligands remains to be determined.

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