Photoaffinity Labeling of Tubulin Subunits with a Photoactive Analogue of Vinblastine

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Received June 11, 1986; Revised Manuscript Received August 18, 1986

ABSTRACT: A photoactive, radioactive analogue of vinblastine, *N*-(*p*-azido[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine ([^{3}H]NABV), was used to localize the *Vinca* alkaloid binding site(s) on calf brain tubulin after establishing that its in vitro interactions with tubulin were comparable to those of vinblastine. Microtubule assembly was inhibited by 50% with 2 μM NABV or vinblastine. At higher drug concentrations, NABV and vinblastine both induced tubulin aggregation, and both drugs inhibited tubulin-dependent GTP hydrolysis. Vinblastine and NABV inhibited each other's binding to tubulin, but the binding of neither drug was inhibited by colchicine. Two classes of binding sites for NABV and vinblastine were found on calf brain tubulin. High-affinity sites had apparent K_D values of 4.2 and 0.54 μM for NABV and vinblastine, respectively, whereas the low-affinity binding sites showed apparent K_D values of 26 and 14 μM for NABV and vinblastine, respectively. Mixtures of tubulin and [^{3}H]NABV were irradiated at 302 nm and analyzed for incorporation of radioactivity into protein. Photolabeling of both the α- and β-subunits of tubulin with increasing concentrations of [^{3}H]NABV exhibited a biphasic pattern characteristic of specific and nonspecific reactions. Nonspecific labeling was determined in the presence of excess vinblastine. Saturable specific covalent incorporation into both subunits of tubulin was observed, with an α:β ratio of 3:2 and maximum saturable incorporation of 0.086 and 0.056 mol of [^{3}H]NABV/mol of α-tubulin and β-tubulin, respectively. Such photolabeling of the tubulin subunits will permit precise localization of *Vinca* alkaloid binding sites, including identification of the amino acid residues involved, an essential requirement for understanding the interactions of these drugs with tubulin.

The antineoplastic action of *Vinca* alkaloids in cells has been ascribed, at least in part, to microtubular function. These drugs are known to bind to the tubulin dimer and disrupt microtubules in cells (George et al., 1965) and in vitro (Bryan, 1972; Owellen et al., 1976; Himes et al., 1976). At relatively low concentrations, they inhibit the assembly of tubulin into microtubules (Owellen et al., 1976; Himes et al., 1976 Wilson et al., 1982; Jordan et al., 1985) and at higher concentrations cause tubulin aggregation (Bhattacharyya & Wolff, 1976; Manfredi & Horwitz, 1984), with some workers describing two or more binding sites for *Vinca* alkaloids on tubulin (Lee et al., 1975; Wilson, 1975; Wilson et al., 1975; Bhattacharyya & Wolff, 1976). The localization of these drug binding sites is essential for analyzing the effects of the *Vinca* alkaloids on the assembly and disassembly of microtubules and the characterization of mutants of tubulin with altered affinities for these agents.

Photoaffinity labeling is a powerful tool which has been used extensively for identification of acceptor molecules in heterogeneous mixtures and in the selective labeling of receptor sites in biological systems (Baley & Knowles, 1977; Chowdhry & Westheimer, 1979). Using a photoactive, radioactive analogue of vinblastine, *N*-(*p*-azido[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine ([^{3}H]NABV),[1] we were recently able to identify *Vinca* alkaloid specific polypeptides in calf brain (Safa & Felsted, 1987) and in sensitive and multidrug-resistant tumor cells (Safa et al., 1986). The analogue was pharmacologically active in P388 murine leukemia cells (Safa et al., 1985) and induced ultrastructural alterations identical with those observed for cells treated with other *Vinca* alkaloids (Krishan, 1970; Paintrand & Pignot, 1983; Fan, 1985). In the present report, we determined that the in vitro interactions of tubulin with NABV and with vinblastine are essentially identical, and we have therefore used NABV to localize the binding site(s) of *Vinca* alkaloids on calf brain tubulin.

Materials and Methods

Materials. Vinblastine sulfate and maytansine were obtained from the Natural Products Branch, National Cancer Institute, Bethesda, MD; [^{3}H]vinblastine (10 Ci/mmol) and [^{3}H]colchicine (4.9 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL); *N*-hydroxysuccinimidyl 4-azido[3,5-^{3}H]benzoate (50.6 Ci/mmol) was from New England Nuclear (Boston, MA); *N*-hydroxysuccinimidyl 4-azidobenzoate was from Pierce Chemical Co. (Rockford, IL); and podophyllotoxin was from Aldrich Chemical Co. (Milwaukee, WI). Electrophoretically homogeneous calf brain tubulin and heat-treated MAPs were prepared as described previously (Hamel & Lin, 1981, 1984). [8-^{14}C]GTP was obtained from Moravek Biochemicals (Brea, CA) and nonradiolabeled GTP from Sigma Chemical Co. (St. Louis, MO), and both nucleotide preparations were repurified by triethylammonium bicarbonate gradient chromatography on

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[1] Abbreviations: [^{3}H]NABV, *N*-(*p*-azido[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

DEAE-Sephadex A-25. All drugs were dissolved in dimethyl sulfoxide.

Synthesis. The procedures for preparation of deacetylvinblastine monohydrazide, deacetylvinblastine acid azide, and *N*-(β-aminoethyl)vindesine were similar to those described for preparation of vindesine (Barnett et al., 1978) and a number of deacetylvinblastine acid azide N-substituted analogues (Conrad et al., 1979).

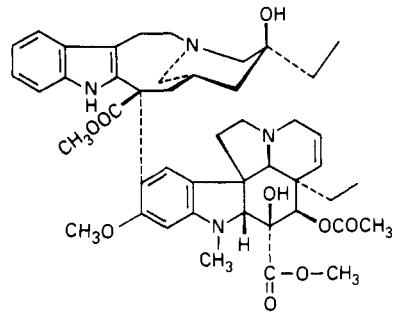
The photoactive analogue of vinblastine, *N*-(*p*-azidobenzoyl)-*N'*-(β-aminoethyl)vindesine (NABV), was synthesized by dissolving 5 μmol of *N*-(β-aminoethyl)vindesine in 1 mL of chloroform. To this was added 10 μmol of *N*-hydroxysuccinimidyl 4-azidobenzoate. The reaction mixture was kept at 4 °C overnight. The product was purified by high-performance liquid chromatography (HPLC). The detailed purification and chemical characterization of NABV have been presented elsewhere (Safa & Felsted, 1987). *N*-(*p*-Azido[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine ([^{3}H]NABV) (Figure 1) was prepared by *N*-azidobenzoylation of 0.5 μmol of *N*-(β-aminoethyl)vindesine in 500 μL of chloroform with 5 nmol of *N*-hydroxysuccinimidyl 4-azido[3,5-^{3}H]benzoate at 4 °C, with purification as for the corresponding nonradioactive compound. NABV and [^{3}H]NABV had identical mobilities on HPLC and thin-layer chromatography, and both preparations appeared to be homogeneous.

Photolabeling with [^{3}H]NABV and SDS–Polyacrylamide Gel Electrophoresis. Tubulin samples (0.3 mg/mL) in 50 μL of a buffer solution containing 0.1 M Mes (pH 6.7 with NaOH), 1 mM EGTA, 0.5 mM $MgCl_2$, and 4% dimethyl sulfoxide (solution A) were incubated with increasing concentrations (0–24 μM) of [^{3}H]NABV (0.1–0.2 Ci/mmol) in poly(vinyl chloride) V microtiter wells (Dynatech Labs, Inc., Alexandria, VA) for 15 min at 25 °C before photolysis. Photoactivation was performed for 20 min by irradiation with a short-wavelength (302 nm) ultraviolet 15-W self-filtering lamp (Model xx-15, Ultra-Violet Products, Inc., San Gabriel, CA) placed 3.4 cm above the photolabeling mixture. As a control experiment, brain tubulin was irradiated in the presence of 2 μM [^{3}H]vinblastine.

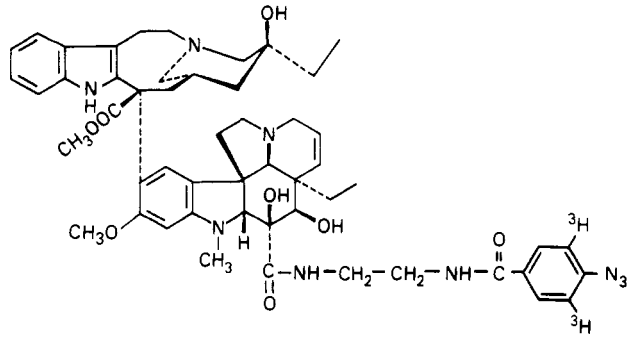
Photolabeled samples were immediately dialyzed against a solution containing 2.5 mM Tris–glycine (pH 8.3), 0.1% SDS, 10% glycerol, and 0.5 mM dithiothreitol in a microdialysis system (Bethesda Research Laboratories, Inc., Gaithersberg, MD). The α- and β-tubulin monomers were separated by slab gel electrophoresis according to the procedure of Stephens (1975). Following fixation and staining of the gel in 0.25% Coomassie brilliant blue, 45% methanol, and 10% acetic acid, either the lanes of stained protein corresponding to each sample slot were cut and sliced into 1.0-mm slices or tubulin bands were cut from the gel and incubated with 30% H_2O_2 in a closed scintillation vial at 100 °C for 1 h to solubilize the gel slice, with radioactivity subsequently quantitated.

When tubulin was incubated with [^{3}H]NABV in the absence of ultraviolet light for 30 min, no radioactivity was found in subsequent SDS–PAGE gel slices above the ambient background level, except for unbound [^{3}H]NABV which migrated near the tracking dye front. Similar results were obtained after photoactivation of [^{3}H]NABV in the absence of tubulin, which was later added prior to performing SDS–PAGE and gel slice analysis. Additionally, under our standard photolabeling conditions, no radioactivity in calf brain tubulin subunits was detected using [^{3}H]vinblastine.

Other Methods. GTP hydrolysis was measured by thin-layer chromatography on poly(ethylenimine)–cellulose and autoradiography as described elsewhere (Hamel et al., 1983).



VINBLASTINE



N-(p-AZIDO-[3,5- ^{3}H] BENZOYL)-N'-β-AMINOETHYLVINDESINE

FIGURE 1: Chemical structures of vinblastine and the photoactive derivative of vinblastine, *N*-(*p*-azido[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine.

Turbidimetry (Gaskin et al., 1974) was used to follow microtubule assembly and tubulin aggregation in a Gilford Model 250 recording spectrophotometer equipped with a Gilford Thermoset electronic temperature control unit. In assembly experiments, base lines were established at 0 °C, and tubulin polymerization was initiated by setting the temperature control unit at 37 °C. Cold reversibility was evaluated by resetting the temperature control unit at 0 °C, with the reaction followed until the drop in turbidity was complete. Measurement of the binding of radiolabeled drugs to tubulin and electron microscopy were performed as described elsewhere (Batra et al., 1986).

Results and Discussion

Comparison of NABV and Vinblastine Interactions with Tubulin. We have previously demonstrated that NABV and vinblastine (chemical structures presented in Figure 1) have nearly identical cytotoxic and morphological effects on cells in tissue culture, including formation of tubulin paracrystals (Safa et al., 1985). Since a number of workers, however, have argued that the toxic effects of the *Vinca* alkaloids on cells may only be partially related to their interference with microtubule function (Gout et al., 1984; Plagemann, 1970; Howard et al., 1980), it was also important to compare the photoactive analogue to vinblastine in its interactions with tubulin in vitro.

NABV and vinblastine had nearly identical effects on microtubule assembly dependent on MAPs (Figure 2A), progressively inhibiting the cold-reversible reaction until complete inhibition occurred at 4 μM drug. At higher concentrations (Figure 2B), both drugs induced the formation of cold-stable aggregates, but NABV was almost twice as potent as vinblastine in this reaction. The aggregation reaction was characterized by very high turbidity readings, and as the drug concentration increased, a significant reaction occurred even at 0 °C.

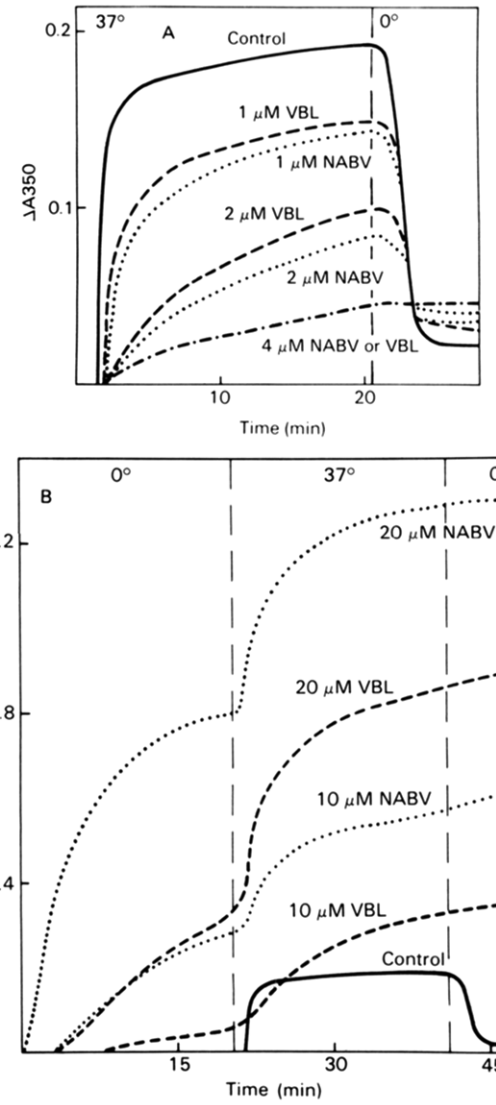


FIGURE 2: Comparison of the effects of NABV and vinblastine on microtubule assembly (A) and the formation of tubulin aggregates (B). Each 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.5 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 1.0 mM GTP, and drugs as indicated (final concentration of dimethyl sulfoxide was 1%). At the times indicated by the vertical dashed lines, the electronic temperature controller was set at the indicated temperatures (temperatures of the reaction mixtures rose at approximately 0.1 °C/min and fell at approximately 0.5 °C/min).

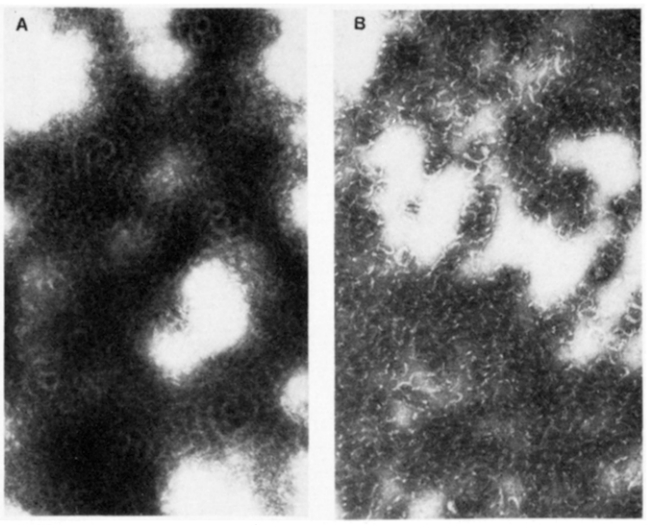


FIGURE 3: Morphology of tubulin aggregates induced by NABV at 0 (A) and 37 °C (B). The 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.5 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 1.0 mM GTP, and 20 μM NABV (final concentration of dimethyl sulfoxide was 2%). The reaction was followed spectrophotometrically, with temperature maintained by the electronic temperature controller. Negatively stained specimens (0.5% uranyl acetate) were prepared after 30 min at 0 °C (A) and an additional 20 min at 37 °C (B). Magnification 56000×.

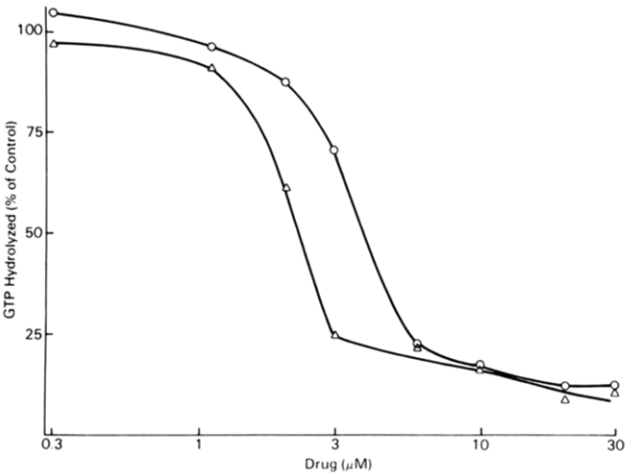


FIGURE 4: Comparison of the effects of NABV and vinblastine on tubulin-dependent GTP hydrolysis. Each 50-μL reaction mixture contained 1.5 mg/mL tubulin, 0.5 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 0.2 mM [8-^{14}C]GTP, and the indicated drug concentration (final concentration of dimethyl sulfoxide was 1%). After 30 min at 37 °C, an aliquot of each reaction mixture was added to 25% acetic acid to stop GTP hydrolysis, and the amount of [8-^{14}C]GDP formed was quantitated. (○) Vinblastine; (△) NABV.

Even though a more extensive reaction occurred with NABV than vinblastine, the aggregates induced by the two drugs were morphologically identical, consisting of multiple rings and tightly coiled spirals (Figure 3 presents the structures formed with NABV). The aggregates formed at 0 °C seemed less densely packed than those at 37 °C. Samples prepared from 0 °C reaction mixtures had clusters of rings with a rosette-like appearance (Figure 3A) which were much less prominent at 37 °C (Figure 3B) with both drugs.

NABV and vinblastine also had similar effects on glutamate-induced polymerization of purified tubulin, in the absence of MAPs (data not presented). Both drugs inhibited cold-reversible polymerization at lower concentrations, while at higher concentrations they induced the formation of cold-stable aggregates. They thus interact with tubulin rather than MAPs.

Tubulin polymerization both with MAPs (David-Pfeuty et al., 1977) and in glutamate (Hamel & Lin, 1981) is associated with GTP hydrolysis, and vinblastine inhibits these GTPase reactions. Under both conditions, the effect of NABV is similar to that of vinblastine at all concentrations examined, as demonstrated in Figure 4 for MAP-dependent GTP hydrolysis.

We next examined the direct binding of [^{3}H]NABV and [^{3}H]vinblastine to tubulin. The preliminary studies presented in Table I indicate that both drugs bind in a similar manner to tubulin. At equimolar concentrations, they are comparable in inhibiting each other's binding to the protein, while maytansine potently inhibits the binding of [^{3}H]NABV as well as [^{3}H]vinblastine to tubulin. Colchicine does not inhibit the binding of either radiolabeled compound, nor do vinblastine and NABV inhibit the binding of radiolabeled colchicine to tubulin. These findings are consistent with previously described drug–tubulin interactions (Bryan, 1972; Owellen et al., 1972, 1974; Wilson, 1975; Mandelbaum-Shavit et al., 1976).

Table I: NABV Inhibits Vinblastine but Not Colchicine Binding to Tubulin

potential inhibitor added	% of control ± standard deviation: binding of vinblastine[a]	binding of NABV[a]	binding of colchicine[b]
10 μM vinblastine		69 ± 14.6	
100 μM vinblastine		1.2 ± 2.1	97.6 ± 3.8
10 μM NABV	58.6 ± 1.3		
100 μM NABV	15.9 ± 1.5		97.4 ± 2.9
10 μM maytansine	16.8 ± 1.1	22.1 ± 1.9	
100 μM colchicine	130 ± 1.5	174.4 ± 21.2	
100 μM podophyllotoxin			0.53 ± 0.7

[a] Binding of [^{3}H]vinblastine and [^{3}H]NABV to tubulin was measured by using the gel filtration syringe column procedure [see Batra et al. (1986)]. Reaction mixtures (0.7 mL) contained 0.5 mg/mL tubulin, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 10 μM [^{3}H]vinblastine or [^{3}H]NABV, and the inhibiting drugs at the indicated concentrations. Incubation was at room temperature for 15 min. Triplicate 0.2-mL aliquots from each reaction mixture were placed on syringe columns, and the amount of radioactivity and protein in the filtrates obtained by centrifugation were determined. [b] Binding of [^{3}H]colchicine to tubulin was measured by filtration of reaction mixtures through DEAE-cellulose filters [see Batra et al. (1986)]. Triplicate 0.1-mL reaction mixtures contained 0.1 mg/mL tubulin, 5 μM [^{3}H]colchicine, 1.0 M monosodium glutamate (pH 6.6), and 0.1 mM GTP and were incubated at 37 °C for 1 h prior to filtration.

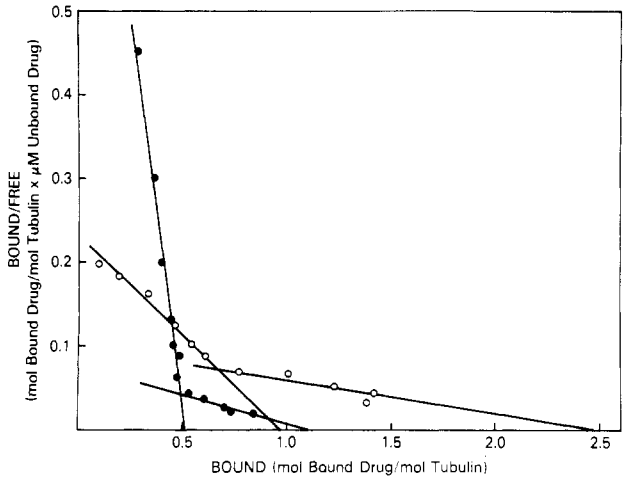


FIGURE 5: Scatchard analysis of the binding of [^{3}H]NABV or [^{3}H]vinblastine to tubulin. Each 0.9-mL reaction mixture contained 0.5 mg/mL tubulin, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, and the indicated concentration of [^{3}H]NABV (○) or [^{3}H]vinblastine (●) (final concentration of dimethyl sulfoxide was 1%). The highest drug concentration examined ws 50 μM. Incubation was for 15 min at room temperature. Quadruplicate 0.2-mL aliquots of each reaction mixture were placed on 1-mL syringe columns of Sephadex G-50 (superfine) [see Batra et al. (1986) for further experimental details], and protein and radioactivity in the filtrates obtained following centrifugation were determined.

Colchicine, moreover, appeared to enhance the binding of both vinblastine and NABV to tubulin. This may represent stabilization of tubulin by colchicine [cf. Bhattacharyya & Wolff (1976)], or, alternatively, colchicine may reduce dissociation of *Vinca* alkaloids from tubulin during centrifugal gel filtration.

A more detailed binding study did indicate minor differences in the binding of NABV and vinblastine to tubulin. Scatchard (1949) analysis was performed on these data (Figure 5). There were two classes of binding sites for both drugs (Bhattacharyya & Wolff, 1976), with vinblastine having apparently higher affinities than NABV at each class of sites. The K_D values derived from the slopes presented in Figure 5 were 0.54 μM ($K_A = 1.8 \times 10^6$ M^{-1}) and 14 μM ($K_A = 7.1 \times 10^4$ M^{-1}) for vinblastine and 4.2 μM ($K_A = 2.4 \times 10^5$ M^{-1}) and 26 μM ($K_A = 3.9 \times 10^4$ M^{-1}) for NABV. The actual

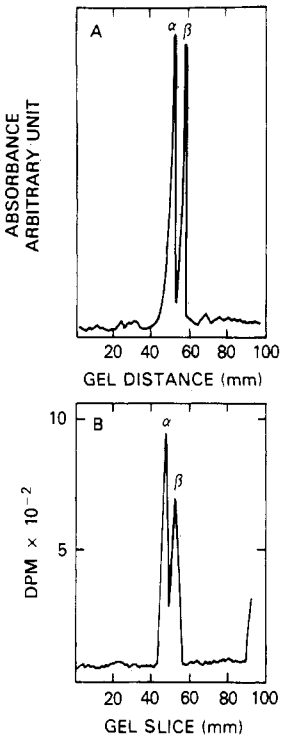


FIGURE 6: SDS–PAGE and densitometry tracing of Coomassie brilliant blue stained (A) and radioactive SDS–PAGE profile (B) of purified tubulin. Following photolabeling of tubulin (0.3 mg/mL in solution A) with 50 nM [^{3}H]NABV (50.6 Ci/mmol), 5 μg of photolabeled tubulin was subjected to SDS–PAGE. The densitometry tracing and radioactivity in 1-mm gel slices were compared.

stoichiometry of binding of NABV to tubulin in this experiment was 0.61 mol of drug bound/mol of tubulin at 10 μM [^{3}H]NABV and 1.4 mol/mol at 50 μM [^{3}H]NABV.

These values seem inconsistent with the nearly identical effects of the two drugs on microtubule assembly and GTP hydrolysis (Figures 2 and 4). It is possible, however, that the two drugs dissociate at different relative rates from tubulin during the analytical procedure, which requires retention of drug by protein during filtration (for these studies were not performed under equilibrium conditions), or that the MAPs required for the assembly and GTPase assays altered the affinities of the drugs for tubulin [other laboratories (Donoso et al., 1979; Luduena et al., 1984) have demonstrated that MAPs alter *Vinca* alkaloid-induced aggregation of tubulin].

In short, the studies described above, together with the previously reported findings in intact cells, demonstrate that NABV is an excellent photoactive analogue of vinblastine. Like vinblastine, NABV at lower concentrations inhibits cold-reversible microtubule assembly and at higher concentrations induces the formation of cold-stable ring and spiral aggregates. Both agents inhibit tubulin-dependent GTP hydrolysis and bind to two types of sites on tubulin. We can thus anticipate that photoactivation of NABV bound to tubulin should result in the formation of a covalent drug–protein cross-link at the *Vinca* alkaloid binding site(s) of tubulin.

Photolabeling of Tubulin with [^{3}H]NABV. The photolabeling profile was obtained by ultraviolet irradiation of mixtures of 50 nM [^{3}H]NABV and 0.3 mg/mL purified tubulin followed by SDS–PAGE and comparison of the radioactivity in 1-mm gel slices relative to the densitometric pattern of the tubulin subunits (Figure 6). Under these conditions, the photoactive probe was incorporated into both α- and β-subunits of tubulin in a ratio of 3:2. Identical radioactivity profiles were obtained after preincubation of [^{3}H]NABV with purified tu-

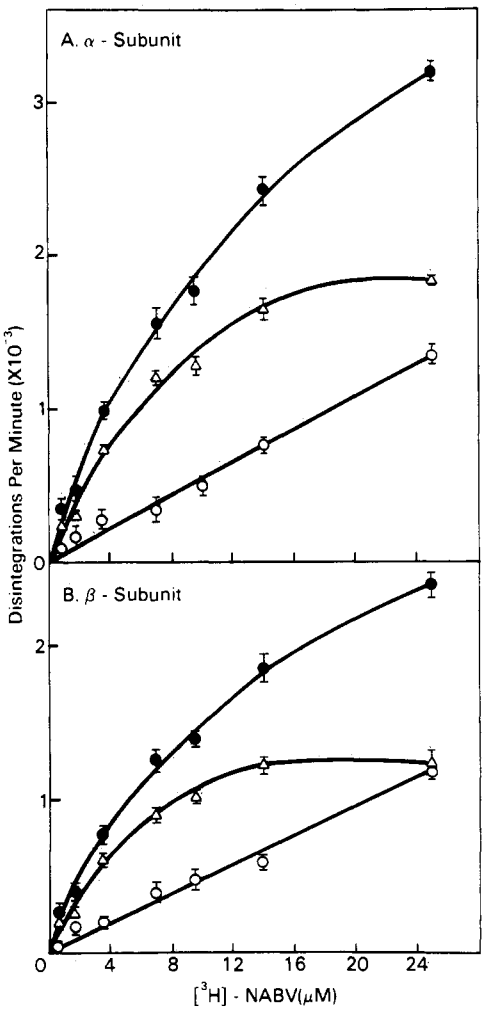


FIGURE 7: Photolabeling of purified tubulin (0.3 mg/mL in solution A) with increasing [^{3}H]NABV concentrations (0.2 Ci/mmol). Tubulin and different concentrations of [^{3}H]NABV with and without 100 μM vinblastine were photolabeled for 20 min, and 5 μg of the photolabeled tubulin was subjected to SDS–PAGE. The subtraction of the radioactivity incorporated in the presence (○) of vinblastine from the radioactivity incorporated in the absence (●) of vinblastine represents the net specific incorporation (Δ). Each point represents the mean integrated radioactivity (n = 3) minus the average base-line radioactivity ± standard deviation.

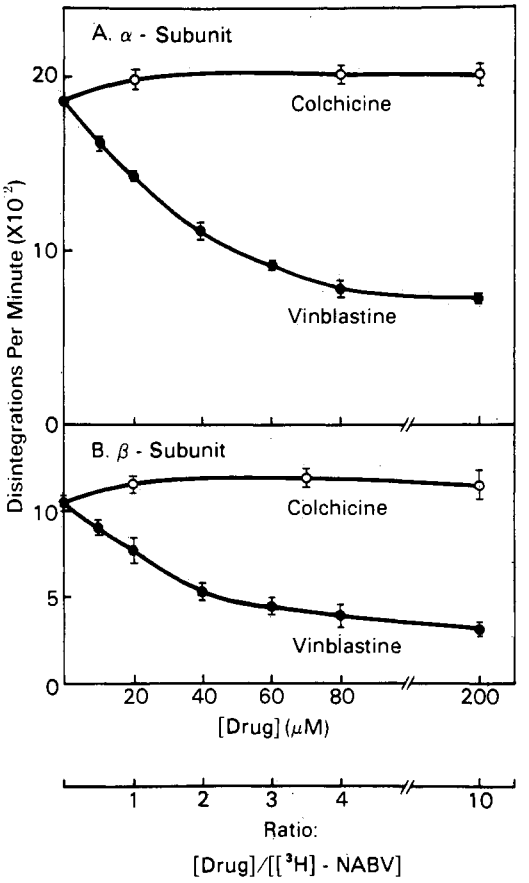


FIGURE 8: Photoaffinity labeling of tubulin (0.3 mg/mL in solution A) with 20 μM [^{3}H]NABV (0.1 Ci/mmol) in the presence of increasing concentrations (0–200 μM) of vinblastine (●) and colchicine (○). The photolabeled tubulin (5 μg) was subjected to SDS–PAGE. Each point represents the mean integrated radioactivity (n = 3) minus the average base-line radioactivity ± standard deviation.

bulin for 1, 30, or 60 min followed by photolabeling at 25 or 37 °C for 20 min.

Photolabeling specificity was analyzed by labeling tubulin with increasing concentrations of [^{3}H]NABV (0–24 μM) in both the absence and presence of excess vinblastine. In the absence of vinblastine, the amount of label found in both α- and β-subunits exhibited a biphasic increase in the amount of radioactivity characteristic of mixed specific and nonspecific photolabeling (Figure 7). In the presence of excess vinblastine, specific *Vinca* alkaloid photolabeling was blocked, and the incorporation of radioactivity into both polypeptides increased linearly with a slope parallel to the terminal nonspecific linear portion of the biphasic curves. The specific photolabeling was obtained by subtracting the nonspecific linear curve from the mixed profile. After correction for nonspecific labeling, α- and β-subunits exhibited half-maximal saturation photolabeling at 6 and 4 μM [^{3}H]NABV, respectively. Maximum saturable incorporation of 0.083 and 0.056 mol of [^{3}H]NABV/mol of tubulin α- and β-subunits, respectively, was observed. The low stoichiometry of covalent binding despite efficient total binding of NABV to tubulin (see above) is typical in photoaffinity labeling experiments, which usually yield efficiencies of less than 10% (Cavalla & Neff, 1985).

At a fixed [^{3}H]NABV concentration (20 μM), increasing concentrations of vinblastine gradually reduced the specific labeling to a limiting minimum of about 70% for both α- and β-subunits of tubulin (Figure 8). The portion of radioactive labeling not blocked corresponds to nonspecific incorporation and agrees closely with the percent of total labeling ascribed to nonspecific labeling at saturating concentrations for both α- and β-subunits of tubulin (30–35%) (Figure 8). In contrast, increasing concentrations of colchicine had negligible effects on photolabeling of either subunit of tubulin.

These results thus suggest either two distinct binding sites for the *Vinca* alkaloids, one on each tubulin subunit, or a single site shared by the two subunits. While some workers have reported two or more binding sites for *Vinca* alkaloids on tubulin (Lee et al., 1975; Wilson, 1975; Wilson et al., 1975; Bhattacharyya & Wolff, 1976) [including one report which argued that inhibition of polymerization resulted from vinblastine's binding to a high-affinity binding site while aggregation resulted from the drug's binding to a low-affinity site (Bhattacharyya & Wolff, 1976)], we are unable at present to choose unambiguously between these two alternatives. The maximum amount of NABV specifically and covalently bound to tubulin so far found has been substoichiometric (about 0.14 mol of drug/mol of tubulin). The 3:2 ratio of covalent binding to α- and β-tubulin has, however, been observed under every reaction condition and at all tubulin and NABV concentrations examined (with both [tubulin]:[NABV] >>1 and <<1). Moreover, Grammbitter et al. (1985) have prepared a different

photoactive vinblastine analogue [3-[[[2-amino(4-azido-2-nitrophenyl)ethyl]amino]carbonyl]-3-de(methoxycarbamyl)-vincaleukoblastine], and they, too, found covalent labeling of α- and β-tubulin in approximately a 2:1 ratio. It thus appears most likely that a single high-affinity *Vinca* alkaloid site is being photolabeled and that this site is shared by the α- and β-tubulin subunits.

There are, however, other possible explanations for our observations. For example, a half of the site reactivity (or flip-flop) process, in which binding on one of two equivalent sites excludes binding on the other, could produce drug–protein cross-links on both subunits. Alternatively, there may be two or more sites with low stoichiometries of binding, with the α-subunit site binding NABV more efficiently than the β-subunit site. This could result from the existence of multiple variant tubulin dimers as a consequence of the microheterogeneity of both tubulin subunits (George et al., 1981). Consequently, one type of tubulin dimer could bind NABV primarily on α-tubulin while another could bind the drug primarily on the β-subunit. A more trivial explanation is that the length of the side arm of NABV may allow a covalent bond to form with both subunits even though the *Vinca* alkaloid binding site(s) is (are) located on only one of them (presumably α-tubulin).

ACKNOWLEDGMENTS

We thank T. Januszewski for performing the electron microscopy.

Registry No. [^{3}H]NABV, 102976-55-6; NABV, 103974-27-2; vinblastine, 865-21-4; colchicine, 64-86-8.

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