Vinca Site Agents Induce Structural Changes in Tubulin Different from and Antagonistic to Changes Induced by Colchicine Site Agents

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ABSTRACT: Vinca site agents are antimicrotubule compounds that bind to the same site on tubulin as do the vinca alkaloids. These include agents that induce the formation of nonmicrotubule oligomers of tubulin (vinblastine and vincristine) and agents that do not (maytansine and rhizoxin). All of these quench the fluorescence of tubulin upon binding. Quenching preferentially affects the red side of tubulin emission, likely affecting more than one solvent-exposed tryptophan. Similar quenching is observed upon binding to either tubulin or tubulin−colchicine. None of these agents induces the local unfolding of the amphipathic helix in the carboxyl terminal region of β-tubulin that colchicine and other colchicine site ligands do. All four vinca site agents inhibit this unfolding in tubulin−colchicine complexes without displacing colchicine. Both groups of vinca site agents enhance the chymotryptic cleavage of β-tubulin after Tyr-281. Both groups of vinca site agents increase the β-tubulin specificity of photolabeling with colchicine, and both groups inhibit colchicine-stimulated GTP hydrolysis. All of these effects common to both groups of vinca site agents are interpreted as due to vinca site occupancy alone. The vinca alkaloids differ from maytansine and rhizoxin by causing a large enhancement of chymotryptic cleavage of β and a large inhibition of typtic cleavage of α, after Arg-339. These effects are interpreted as due to vinca induced oligomerization of tubulin. It is argued that the common binding site for the vinca site agents is located on β-tubulin, close to the helix that is disrupted by colchicine.

Microtubules are the main structural component of the mitotic apparatus, and therefore tubulin, the main structural component of microtubules, is a principal target of therapeutic and environmental antimitotic agents. The mechanism by which these agents interfere with microtubule function has been a focus of interest for some time. It is clear that these agents do not all inhibit microtubule function by the same mechanism nor do they all bind to the same site on the tubulin dimer. However, they may be grouped into families based on the ability to bind to one or another of a fairly small number of binding sites.

These anti-tubulin agents may be broadly classified as those that favor microtubule polymerization and those that inhibit polymerization. The first of these groups is represented by a single family of compounds: taxol and its congeners [reviewed by Kingston (1991)]. The second group, agents that inhibit polymerization, is quite large and consists of a number of families of compounds which have distinct binding sites on the tubulin molecule and presumably distinct mechanisms of action. Two principal binding sites are refered to by their defining binding agents: the colchicine site and the vinca site. Agents with a wide variety of chemical structures are known to bind at each site, but there is no overlap in the sets of binding agents. Binding at each site is independent of the other site, as indicated by the failure of each set to competitively inhibit the binding of agents from the other set.

Colchicine is the classic paradigm of a tubulin binding antimitotic agent. The binding of colchicine defines the site on tubulin shared by a number of other agents. These include a number of colchicinoids [reviewed by Hastie (1991)], compounds of related structure such as podophyllotoxin, combretastatin, and steganacin [reviewed by Sackett (1993)], benzimidazole derivatives [reviewed by Lacey (1988)], and others. All agents that bind at the colchicine binding site share the property of inhibiting tubulin polymerization, even as they differ in their chemical structure. Colchicine site agents tend to be smaller than vinca site agents. Indeed combretastatin A-4, the smallest known antitubulin agent (also one of the most potent), binds at the colchicine site. A variety of data indicate that the colchicine site is located on β tubulin: analysis of resistant mutants [reviewed by Cabral and Barlow (1992)], direct photoaffinity labeling (Wolff et al., 1991; Uppuluri et al., 1993), linkage of binding and guanine nucleotide exchange (Shearwin & Timasheff, 1994), disruption of -SH cross-linking [reviewed by Luduena and Roach (1991)], and changes in proteolysis (Sackett & Varma, 1993). The colchicine site is near the α/β interface as indicated by photodimer formation (Wolff et al., 1992) and by photolabeling of α induced by structural changes in the probe and/or altered solution condirtions (Floyd et al., 1989; Lin et al., 1989; Wolff et al., 1991).

The antimitotic vinca alkaloids define the second tubulin binding site. The principal representatives of this alkaloid family are vinblastine and vincristine, though many others are known [reviewed by Himes (1991)]. A number of other compounds are known to bind at the vinca site based on their ability to inhibit the binding of vinblastine or vincristine to tubulin. Many of these agents are quite different from

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vinblastine chemically. These agents include maytansine and ansamytocin, rhizoxin, and others [reviewed by Hamel (1992)]. Competetive binding indicates that maytansine, rhizoxin, and the vinca alkaloids all share the same (or at least overlapping) binding sites [Bai et al., 1990; Bhattacharyya & Wolff, 1977; Mandelbaum-Shavit et al., 1977; Takahashi et al., 1987; discussed in Hamel (1992)].

The location of the vinca site on the tubulin dimer is not established. On the one hand photolabeling with radioactive vinblastine labels α preferentially, though both subunits are labeled (α/β ratio = 1.5−3) (Safa et al., 1987; Wolff et al., 1991). On the other hand, binding of vinca site agents inhibits guanine nucleotide exchange (Bai et al., 1990; Huang et al., 1985), known to occur on β (Geahlen & Haley, 1977; Nath et al., 1985). Vinca site agents also affect the specific crosslinking of sulfhydryls on β-tubulin, although vinca alkaloids but not maytansine affect alkylation of sulfhydryls of α [Luduena et al., 1982; reviewed by Luduena and Roach (1991)]. Additionally, the status of residue β100 controls sensitivity to rhizoxin: tubulins with Asn at this position confer sensitivity to rhizoxin, while mutation of this residue to ile results in resistance to rhizoxin (Takahashi et al., 1990). Protein extracts from these rhizoxin-resistant mutants showed greatly reduced binding of labeled rhizoxin (Takahashi et al., 1989). Photoaffinity labeling of tubulin with an azidodansyl derivative of rhizoxin labeled a β-tubulin peptide containing residues 363−379 (Sawada et al., 1993). It may be that the location of the common binding site is on β-tubulin, but, like the colchicine site, it is near the α/β interface, allowing for photolabeling of both subunits to occur [discussed in Hamel (1992)].

Inhibition of polymerization by colchicine or vinca site agents is presumably due to structural changes induced in tubulin by binding site occupancy. Structural changes induced by colchicine binding have been demonstrated by spectral changes (Andreu & Timasheff, 1982), changes in -SH reactivity and cross-linking (Luduena & Roach, 1991), and immunological reactivity (Morgan & Spooner, 1983). Colchicine-induced changes have been identified as the partial unfolding of an amphipathic helix in β, around residue 390 (Sackett & Varma, 1993).

A structural change induced in tubulin by vinca site agents has also been demonstrated by quenching of tubulin fluorescence (Lee et al., 1975; Prakash & Timasheff, 1983), by changes in sulfhydryl reactivity and cross-linking (Luduena & Roach, 1991), and by immunological reactivity (Morgan & Spooner, 1983). Binding of vinblastine or vincristine results in self-association of the tubulin−drug complex in a non-MT form [David-Pfeuty et al., 1979; Fujiwara & Tilney, 1975; Na & Timasheff, 1986a,b; Warfield & Bouck, 1974; Weisenberg & Timasheff, 1970; reviewed by Himes (1991)]. Other vinca site agents such as maytansine or rhizoxin do not result in self-association [Bhattacharyya & Wolff, 1977; Takahashi et al., 1987; discussed in Hamel (1992)], demonstrating that binding of different agents to this site can have different structural results, also shown by differing effects on tubulin sulfhydryl alkylation (Luduena & Roach, 1981).

In order to further explore the structural changes induced in tubulin by vinca site agents, we have examined the spectral properties of tubulin−drug complexes and the patterns of proteolysis of tubulin with bound vinca site agents: vinblastine, vincristine, maytansine, and rhizoxin. We identify changes in tubulin fluorescence common to all vinca site agents examined. We also show changes in proteolysis common to all agents examined as well as changes that allow grouping of vinca site agents into two classes. In addition, we have examined the results of occupying both colchicine and vinca sites at the same time by the resulting effects on fluorescence and proteolysis as well as on photolabeling with colchicine and GTPase properties of doubly liganded complexes. We describe the antagonism between the structural changes induced by colchicine site and by vinca site agents.

MATERIALS AND METHODS

Materials. Microtubule proteins were purified from rat brains by temperature-dependent polymerization and depolymerization as described previously (Sackett et al., 1991). Tubulin was purified from this material by selective polymerization (Sackett & Varma, 1993). Purified tubulin was diluted to 25 g/L in Mes assembly buffer [0.1 M morpholinoethanesulfonic acid, 1 mM $MgCl_2$, 1 mM ethylene glycol bis-(β-aminoethyl ether), pH 6.9], drop frozen in and stored in liquid nitrogen. [γ-^{32}P]GTP (25 Ci/mmol; 1 Ci = 37 GBq) and [ring C, *methoxy*-^{3}H]colchicine (73.4 Ci/mmol) were obtained from New England Nuclear. Maytansine and rhizoxin were obtained from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. Steganacin and combretastatin were the kind gift of Dr. Ernest Hamel and were originally obtained from Dr. J. P. Robin and Dr. G. R. Pettit, respectively. Podophyllotoxin, 99%, was obtained from Aldrich Chemical Co., Milwaukee, WI. Colchicine, vinblastine, vincristine, phenylmethanesulfonyl fluoride, and leupeptin were obtained from Sigma Chemical Co., St. Louis, MO. Chymotrypsin and trypsin (TPCK-treated) were obtained from Worthington. For electrophoresis, the detergent used was sodium lauryl sulfate from Sigma Chemical Co. (Sackett & Wolff, 1986). All other materials were reagent grade.

Spectra. All absorbance data were collected with a Cary Model 219 spectrophotometer. Fluorescence excitation and emission spectra were obtained using a Perkin-Elmer MPF-66 spectrofluorometer in ratio mode. Microcells with a sample volume of 250 μL and a path length of 0.5 cm (NSG Precision Cells, Farmingdale NY) were used for most assays. Excitation slits were set to 2 nm and emission slits to 5 nm. Data were collected, and preliminary spectral analysis was performed using software (PECLS-III) from Perkin-Elmer. All spectra were collected with samples having final optical densities (0.5 cm) less than 0.1 at maximum absorbance of added compound and were corrected for inner filter effects according to

$$F_{corr} = F_{obs}[10^{((OD_{ex}+OD_{em})/2)}]$$

where OD_{ex} and OD_{em} refer to the optical density (0.5 cm) of the sample in the 0.5 cm path length cuvet (Lakowitz, 1983). Correction for inner filter effects was validated using *N*-acetyltryptophanamide in Mes buffer.

Samples designed to assess the drug-induced quenching of tubulin fluorescence were prepared in more than one step if colchicine was present. This procedure was designed to minimize the final optical density due to free colchicine while producing tubulin essentially saturated with colchicine. First, tubulin at high concentration (250 μM) was incubated for 30 min at 30 °C in the presence of 450 μM colchicine in order to make the tubulin−colchicine complex. The sample

was then diluted 50-fold in Mes assembly buffer, vinca site drugs were added as indicated (addition volume was ≤1% of sample volume), and the sample was incubated for a further 10 min before spectra were collected. Typical final concentrations were: 5 μM tubulin, 9 μM colchicine, and 8 μM vinca site agent. Differences from this procedure are noted in the relevant figure legends.

The emission of *N*-acetyltryptophanamide was used to evaluate the difference in fluorescence emission produced by vinca site agents. Solutions were prepared in Mes assembly buffer such that the absorbance at 295 nm was the same as that of the tubulin sample. This required a 55 μM solution of *N*-acetyltryptophanamide to equal a 5 μM solution of tubulin. The resulting emission spectrum was then numerically scaled by division to equal that of a desired concentration of *N*-acetyltryptophanamide.

Proteolysis. Digestion of tubulin and tubulin−drug complexes with trypsin and chymotrypsin was performed in Mes assembly buffer, essentially as described by Sackett and Varma (1993). Briefly, tubulin (10−15 μM) was preincubated for 30 min with drug additions as indicated to form tubulin−drug complexes. Trypsin or chymotrypsin was then added (1−40, w/w to tubulin), and digestion was allowed to proceed for 30 min on ice or as noted in the figure legends. Proteolysis was stopped by addition of 0.2 mM phenylmethanesulfonylfluoride for chymotrypsin or 0.01 mM leupeptin for trypsin. Samples were then prepared for electrophoresis.

Esterolytic Assays. Drug effects on proteases were evaluated by assays of esterolytic activity. The activity of chymotrypsin was assayed by cleavage of 0.1 mM succinyl-alanylalanylpropylphenylalanyl-*p*-nitroanilide, and activity of trypsin was assayed by cleavage of 0.5 mM benzoylarginyl-*p*-nitroanilide, both reactions being monitored by increase in absorbance at 400 nm.

Electrophoresis. Samples were separated by sodium dodecyl sulfate (SDS) gel electrophoresis (Sackett, 1989; Sackett et al., 1991). Coomassie blue R stained gels were scanned with a Microtek 600G flatbed scanner, operated at 200 dpi resolution and controlled by Photostyler software (Aldus Corp., Seattle, WA). Integration of band intensity was performed using Quantiscan software (Biosoft, Ferguson, MO).

Photolabeling. Direct photolabeling of tubulin with [^{3}H]-colchicine was performed essentially as described by Wolff et al. (1991), using conditions found to enhance specific labeling of β-tubulin. [^{3}H]Colchicine was dried in microcentrifuge tubes under nitrogen. Tubulin, 10 μM in Mes assembly buffer, was added to the tube and mixed gently to redissolve the colchicine. Cold colchicine was added to a final concentration of 10 μM and the mixture incubated for 15 min at 37 °C in the dark. Vincristine or maytansine was added to the indicated tubes to 15 μM, and all tubes were incubated for 5 min at room temperature. Samples were irradiated for 5 min at 4 °C with a high-pressure mercury lamp operated at 85−95 W with output filtered through 2 cm of 20% $CuSO_4 \cdot 5H_2O$. Samples were diluted in SDS loading solution, boiled, and separated by gel electrophoresis. Following staining and destaining, gels were photographed, each lane was cut into fractions from top to bottom, each strip was dissolved in 300 μL of 30% H_2O_2 at 65 °C, and radioactivity was determined by addition of scintillation fluid (Packard UltimaGold) and counting to <2% error.

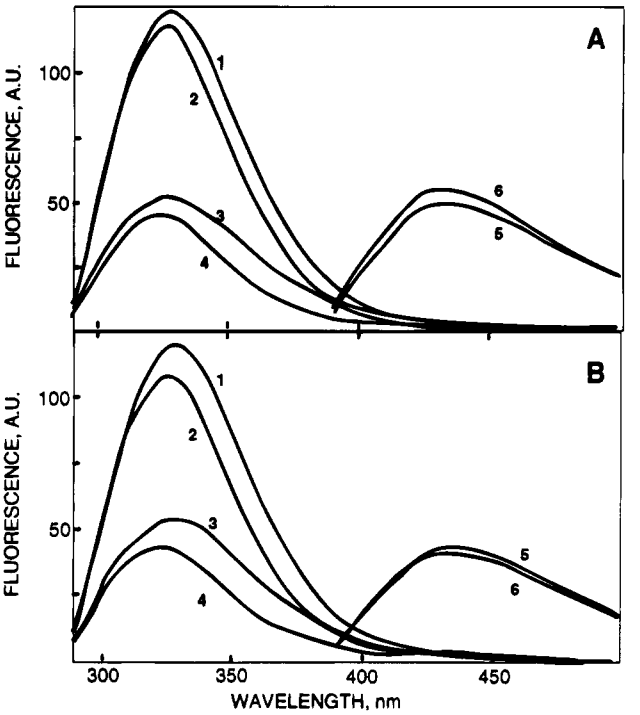

FIGURE 1: Fluorescence emission of tubulin and quenching by bound drugs. The fluorescence of tubulin (excitation 280 nm, emission 290−500 nm, spectra 1−4) and tubulin-bound colchicine (excitation 350 nm, emission 390−500 nm, spectra 5 and 6) are shown. In both panels A and B, spectra 1−4 are tubulin fluorescence: 1 is tubulin in buffer alone, 2 is tubulin plus a vinca site agent, 3 is tubulin plus colchicine, and 4 is tubulin plus colchicine plus vinca site agent. Spectra 5 and 6 are colchicine emission spectra, multiplied by 30: 5 is tubulin plus colchicine (same sample as 3, excited at 350 nm) and 6 is tubulin plus vinca site agent. In panel A the vinca site agent is vincristine; in panel B it is maytansine. Samples were prepared as described in Materials and Methods, and the final concentrations are 5 μM tubulin, 9 μM colchicine, and 8 μM vincristine or maytansine. All spectra are corrected for inner filter effects as described in Materials and Methods.

GTPase Assay. The effect of various drugs on tubulin-mediated hydrolysis of GTP was assessed at room temperature of about 24 °C essentially as described by Davis et al. (1993). Briefly, tubulin samples (10−15 μM) were prepared in Mes buffer with drug additions as noted (typically 100 μM). Samples were preincubated at 30 °C for 30 min and removed to room temperature, and [^{32}P]GTP (100−200 Ci M^{-1}) was added to 50−100 μM. After timed intervals, 10−20 μL samples were quenched with 400 μL of 6% (v/v) acetic acid, 2.5 mM KH_2PO_4 and 10% (w/v) Norit A (prewashed several times in the same solution). After mixing and a 5 min incubation, the samples were centrifuged at full speed in a microcentrifuge (≈12000*g*) for 3 min. The top 200 μL sample was then carefully removed for scintillation counting.

Analytical Ultracentrifugation. Sedimentation velocity studies were performed with a Beckman Ultima Model XLA analytical ultracentrifuge using dual sector cells. Data were analyzed using software provided by Beckman Instruments.

RESULTS

Binding of vinca site agents alters the structure of tubulin. This is reflected in the quenching of tubulin fluorescence by vincristine and maytansine, shown in Figure 1, panels A and B, respectively. Tubulin fluorescence is characterized

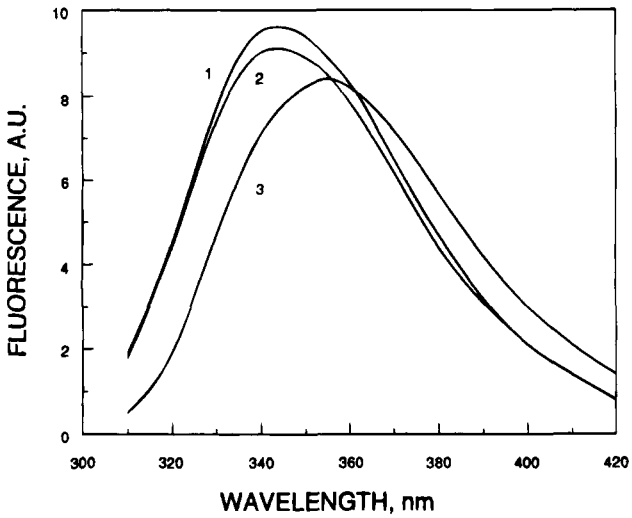

FIGURE 2: Spectra of the tryptophan emission quenched by vinca site agents. The tryptophan emission spectra (excitation 295 nm, emission 300−400 nm) were collected from samples containing 5 μM tubulin alone or with the addition of 8 μM maytansine or 10 μM vincristine. Corrected spectra of the tubulin−drug complexes were subtracted from that of tubulin alone, and the resulting difference spectra are presented. The unperturbed tubulin emission spectrum had maximal intensity of 44.5 at 330 nm (not shown). Spectrum 1 is tubulin minus tubulin−vincristine; 2 is tubulin minus tubulin−maytansine. Spectrum 3 is the emission spectrum of 15 μM *N*-acetyltryptophanamide, collected under the same conditions (see Materials and Methods). The wavelength of maximum intensity for spectra 1 and 2 is about 345 nm (in replicates the range is 345−349 nm). The wavelength of maximum intensity for spectrum 3 is 355−356 nm.

by a peak intensity around 328 nm (spectrum 1 in Figure 1A,B). The emission intensity is reduced by addition of vincristine (spectrum 2 in panel A) and by maytansine (spectrum 2 in panel B), as well as by colchicine (spectrum 3 in panels A and B). Addition of vinca site agents to the tubulin−colchicine complex causes additional quenching of fluorescence beyond that caused by colchicine (spectrum 4 in panels A and B). As expected, binding of vinca site agents does not alter colchicine binding, as reflected in unchanged colchicine fluorescence (compare spectra 5 and 6 in panels A and B). There are several characteristics of the quenching by vinca site agents that should be noted.

The decrease in fluorescence is not uniform. Quenching of fluorescence is more pronounced on the lower energy, red side of the spectrum. The difference is shown more clearly in Figure 2. Tryptophan-selective emission spectra were collected for tubulin with and without vinca site agents, and the resulting difference spectra are presented. The difference between the emission spectra of tubulin with and without maytansine or vincristine has a shape similar to the emission spectrum of free tryptophan, but with an emission maximum at 345−349 nm, while *N*-acetyltryptophanamide shows an emission maximum at 355−356 nm under these conditions. Curve 3 in Figure 2 is the emission spectrum of 15 μM *N*-acetyltryptophanamide, which allows the emission lost from tubulin (5 μM) due to drug binding to be compared to the emission expected from three solvent-exposed tryptophans/dimer. These results indicate that the structural changes induced by vinca site agents affect more surface-exposed tryptophans than buried ones. Similar results were obtained with vinblastine and rhizoxin, although the rhizoxin case is complicated by its more complex absorbance spectrum (data not shown). Since the spectral asymmetry is characteristic of the quenching seen by maytansine (Figures 1B and 2) as well as by vincristine (Figures 1A and 2), this effect is not due to formation of the polymer induced by vincristine.

Tubulin fluorescence is quenched less by binding of vinca site agents than it is by binding of colchicine (Figure 1A,B, spectrum 3). Nonetheless, addition of either vincristine or maytansine to the tubulin−colchicine complex results in further quenching of tubulin fluorescence beyond that produced by colchicine alone. This additional quenching also preferentially affects the red side of the emission spectrum, as seen when vinca site agents are added to tubulin alone. Colchicine quenching of tryptophan-selective tubulin fluorescence is much broader and actually quenches the *blue* side of the emission slightly more (data not shown). The difference between tryptophan selective emission from tubulin−colchicine and tubulin−colchicine−maytansine is very similar to that shown in Figure 2 in terms of: spectral shape, wavelength maximum (345 nm), and area (data not shown). This indicates the loss upon maytansine binding of essentially the same emission from tubulin and tubulin−colchicine, although the fractional loss is greater from tubulin−colchicine. Thus, the changes responsible for the vinca site agent-induced quenching of fluorescence are different from, and independent of, those responsible for the colchicine site-induced quench. It is well known that binding at the two sites are independent events, in terms of competitive binding, and this is demonstrated here by the fact that the fluorescence of bound colchicine is not altered by binding of either vincristine or maytansine (spectra 5 and 6, Figure 1A,B).

The quenching due to the binding of vinca site agents is concentration-dependent and results in 15−20% quench of integrated tryptophan fluorescence (excitation at 295 nm; emission at 305−450 nm) at saturation. Analysis of this dependence may be used to yield an apparent binding constant. Analysis of titrations of tubulin fluorescence emission (at 360 nm) by added vincristine or maytansine yield apparent K_d values of of 3.5 and 0.8 μM, respectively (data not shown).

The quenching of fluorescence caused by the binding of colchicine is significantly due to resonance energy transfer from excited tryptophans to bound colchicine (Andreu & Timasheff, 1982; Sackett, manuscript in preparation). This is unlikely to be the explanation for the quenching of tubulin fluorescence caused by binding of vincristine or maytansine, as demonstrated in Figure 3. Resonance energy transfer requires an overlap between the fluorescence emission spectrum of the donor (here tubulin) and the acceptor (colchicine, vincristine, or maytansine). Figure 3A shows the absorbance spectrum of tubulin in Mes buffer (spectrum 1) and the fluorescence emission spectrum of tubulin when excited at 280 nm (spectrum 2), approximately the peak of absorbance. Figure 3B shows the absorbance spectra of colchicine (1), vincristine (2), and maytansine (3). The significant overlap between tubulin emission and colchicine absorbance is clear. Clearly, the overlap with the absorbance of vincristine is much less than colchicine, and the overlap with maytansine absorabance is minimal. Yet maytansine and vincristine cause comparable quenching of tubulin tryptophan emission (Figure 2). Furthermore, spectral overlap between tubulin fluorescence and the absorbance of vincristine or maytansine is greatest on the blue side of tubulin emission, yet the quenching observed on binding is

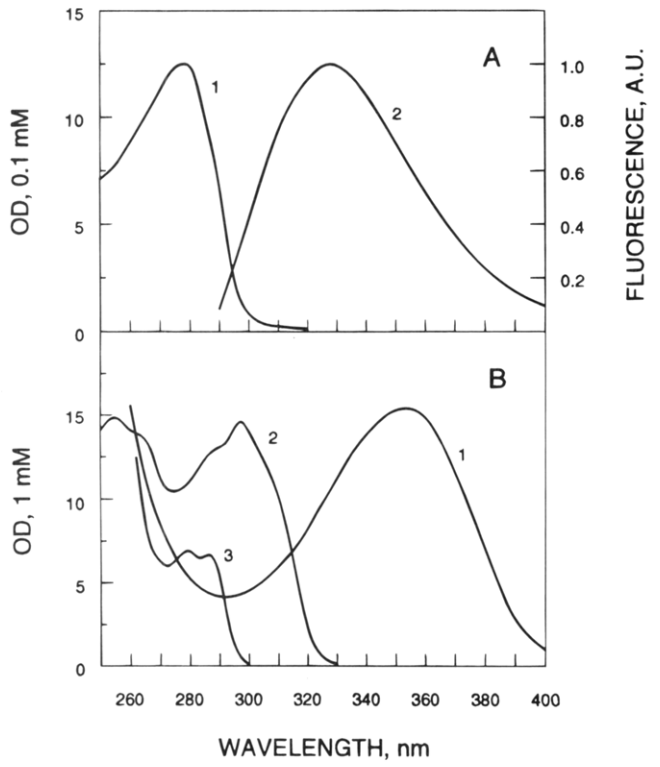

FIGURE 3: Spectra of tubulin and tubulin-binding drugs. (A) (1) The absorbance spectrum of tubulin is shown, scaled for a 0.1 mM solution (equivalent to the molar absorptivity $\epsilon \times 10^{-4}$). (2) The fluorescence emission spectrum (excitation at 280 nm) is shown, scaled to a peak intensity of 1. (B) The absorbance spectra of colchicine (1), vincristine (2), and maytansine (3) are shown. The spectra are presented as the absorbance of a 1 mM solution (equivalent to the molar absorptivity $\epsilon \times 10^{-3}$).

maximal on the red side of the spectrum. Therefore, quenching of tubulin fluorescence by vinca site agents is unlikely to be due to energy transfer and must reflect structural changes induced by binding.

The structural changes brought about by vinca site occupancy might be demonstrated by the patterns of proteolysis of the different tubulin−drug complexes. The structure of native tubulin presents only a few regions susceptible to proteolysis [reviewed by Sackett (1995)]. Trypsin cleaves α-tubulin after Arg-339, producing a large amino terminal fragment, αN, and a smaller carboxyl terminal fragment, αC. Chymotrypsin cleaves β-tubulin after Tyr-281, producing a larger βN and smaller βC fragment (Sackett & Wolff, 1986). Colchicine binding to tubulin induces local unfolding of a region of β-tubulin around Arg-390, and this allows a novel chymotryptic cleavage, resulting in a new band ("β-col") on an SDS[1] gel (Sackett & Varma, 1993). This result is due to occupancy of the colchicine site and not due to some property unique to colchicine, as is shown in Figure 4. Other colchicine site agents, such as the bicyclic, readily reversible agent MTPT, as well as less structurally related agents such as podophyllotoxin, steganacin, and combretastatin (Sackett, 1993) all induce the novel chymotryptic cleavage. As will be seen, there are differences in some structural changes produced by different vinca site agents, but all colchicine site agents induce the production of β-col. Vinca site agents do not induce this cleavage,

[1] Abbreviations: MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone; SDS, sodium dodecyl sulfate; β-col, the proteolytic fragment of β-tubulin induced by colchicine binding.

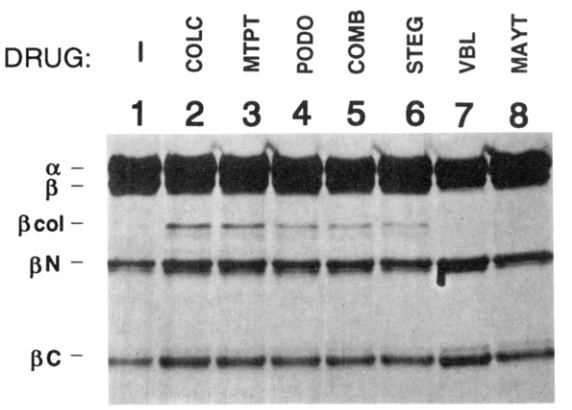

FIGURE 4: Proteolysis of tubulin with bound colchicine site agents and vinca site agents. Tubulin was digested with chymotrypsin following preincubation with several colchicine site (lanes 2−6) and vinca site (lanes 7,8) agents. Additions were as follows: 1, no drug; 2, colchicine; 3, MTPT; 4, podophyllotoxin; 5, combretastatin A-4; 6, steganacin; 7, vinblastine; 8, maytansine. Tubulin, 10 μM, was preincubated for 30 min at 30 °C with 100 μM drug. Chymotrypsin was added at a ratio of 1:50 (w/w to tubulin). Digestion proceeded for 30 min on ice and was stopped by addition of PMSF. Markers by the side of the gel indicate the positions of intact α- and β-tubulin, the usual chymotryptic cleavage products βN and βC, and the colchicine site-induced cleavage β-col.

indicating that the structural changes induced by vinca site occupancy do not result in unfolding of the helix around β-390. The absence of this cleavage product is demonstrated for vinblastine and maytansine in Figure 4, lanes 7 and 8, respectively. The same results have been observed for vincristine and rhizoxin and are discussed below. Inhibition of this cleavage is not due to a direct inhibition of chymotrypsin by vinblastine or maytansine, since the cleavage observed in the absence of drug (which occurs after β-Tyr-281 and yields the fragments βN and βC) is still observed. Furthermore, esterolytic activity of trypsin and chymotrypsin was found to be unaffected even by 100 μM vinblastine (data not shown).

The unfolding induced by colchicine site occupancy may be detected by the enhancement of a minor tryptic cleavage of β-tubulin as well as by the appearance of the novel chymotryptic cleavage (Sackett & Varma, 1993). This minor tryptic cleavage allows the effects of vinca site occupancy on the β-390 region to be evaluated for enhancing or inhibiting effects. The experiment in Figure 4 suggests that vinblastine and maytansine are unlikely to enhance the minor tryptic cleavage since they fail to induce the novel chymotryptic cleavage. Figure 5 demonstrates this for vincristine and rhizoxin as well (panel A, lanes 3 and 6). The results in panel B (lanes 3−6) demonstrate that all of these agents also suppress, rather than enhance, the minor tryptic cleavage that is observed here in the absence of any colchicine site drug and that is enhanced by colchicine site occupancy. These results indicate that all of these vinca site agents not only fail to induce *unfolding* around β-390 (which is induced by all colchicine site agents), they appear to tighten the *folding* of this region, eliminating accessibility of this site to proteolysis by trypsin. Since binding of all four agents has this result, this structural change is due to site occupancy alone and not due to the oligomerization of tubulin induced by vinca alkaloid binding. Sedimentation velocity studies confirmed that vincristine did and maytansine did not induce oligomerization of tubulin under these conditions (data not shown).

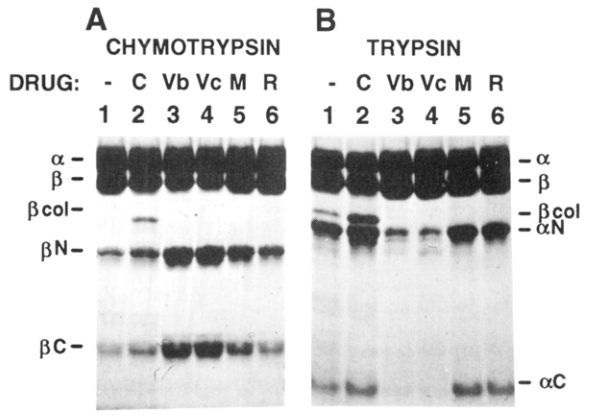

FIGURE 5: Proteolysis of tubulin by trypsin and chymotrypsin in the presence of vinca site agents. Tubulin was digested with (A) chymotrypsin or (B) trypsin following preincubation with 1, no drug; 2, colchicine; 3, vinblastine; 4, vincristine; 5, maytansine; or 6, rhizoxin. Tubulin was 10 μM, colchicine was 100 μM, and all vinca site agents were 50 μM. The markers on the side of the gel indicate the positions of intact α- and β-tubulin, the tryptic cleavage products αN and αC, the chymotryptic cleavage products βN and βC, and the colchicine-site related cleavage produced by trypsin and chymotrypsin, β-col. Densitometry of the gel in panel A gave relative yields for βN (control, lane = 1) of 1.7 (colchicine), 3.4 (vinblastine), 3.3 (vincristine), 2.2 (maytansine), and 1.5 (rhizoxin).

Structural changes induced by vinca site binding are not restricted to the β-390 region affected by colchicine. Clearly other changes are found in the patterns of proteolysis in addition to the lack of cleavage producing βcol. In Figure 5A it is clear that all vinca site agents enhance the chymotryptic cleavage after β-Tyr-281 that produces βN and βC (compare βN in lane 1 with lanes 3−6; quantitative comparisons are given in the Figure legend). Vinblastine and vincristine enhance this cleavage more than do maytansine or rhizoxin and maytansine consistently enhances cleavage slightly more than rhizoxin. This enhancement is more pronounced in Mes assembly buffer than in phosphate buffer (0.01 M, pH 7), both containing 1 mM $MgCl_2$ (data not shown, but see Figure 6 and discussion). A small enhancement is also seen with colchicine, about equal to that seen with rhizoxin and again more pronounced in Mes buffer than in phosphate buffer. In Figure 5B it is clear that vinblastine and vincristine significantly inhibit the tryptic cleavage after α-Arg-339 that yields αN and αC. Inhibition of this cleavage is not produced by maytansine or rhizoxin. This result was found in both Mes assembly buffer and phosphate buffer (not shown). Thus, all four vinca site agents induce the same effects on β-tubulin: they enhance chymotryptic cleavage producing βN and βC; they fail to produce β-col cleavage by chymotrypsin; and they inhibit even its minor cleavage by trypsin. But they differ in their effects on α-tubulin: vincristine and vinblastine inhibit tryptic cleavage producing αN and αC, but maytansine and rhizoxin do not.

The ability of vinca site agents to inhibit the minor tryptic cleavage around β-390 (producing the β-col fragment) seen in the absence of colchicine site drugs allows a different question to be addressed: What happens when both the colchicine site and the vinca site are occupied? Do the structural changes in tubulin induced by binding at one site predominate over the other? or does the doubly liganded tubulin assume a conformation different from either of the singly liganded states? One approach to this question is presented in Figure 6. In this experiment, tubulin−colchicine

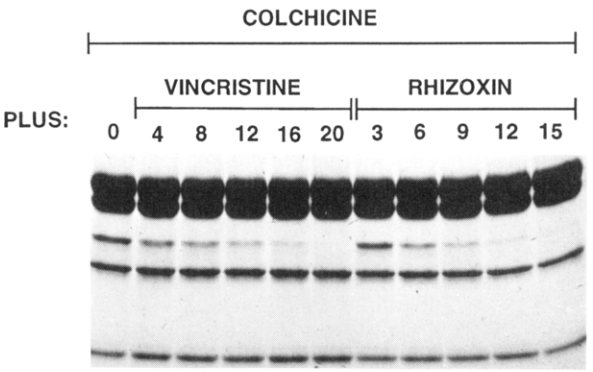

FIGURE 6: Suppression of colchicine-induced cleavage by vinca site agents. Tubulin, preincubated 30 min at 30 °C with colchicine, was further incubated 5 min either alone or with the addition of various concentrations of vincristine or rhizoxin. Chymotryptic digestion was then performed as in Figure 4. The concentration of tubulin was 15 μM, colchicine was 100 μM, and vincristine and rhizoxin concentrations (μM) are indicated above each lane.

complex is digested with chymotrypsin. In the absence of added drugs, the digestion produces the expected bands βN, βC, and the colchicine-induced band, β-col. If various concentrations of vincristine or rhizoxin are added prior to digestion, the pattern changes. A clear decrease in the yield of β-col is observed as the concentration of either drug is increased, suggesting that the binding of either rhizoxin or vincristine results in a reversal of the unfolding around β-390 induced by the binding of colchicine. Rhizoxin is more potent in this effect than is vincristine, consistent with the greater tubulin binding affinity of rhizoxin than vincristine. However, it appears that binding of one mole of either vinca site drug per mole of tubulin reverses the unfolding and prevents chymotryptic production of β-col. This can be seen in Figure 6, where drug concentrations slightly greater than stoichiometric to tubulin result in complete suppression of β-col. Similar results are obtained with vinblastine and maytansine (results not shown). This reversal is not due to displacement of colchicine, since it has already been shown that these conditions do not result in loss of bound colchicine (Figure 1). The inhibition of cleavage is also not due to inhibition of chymotrypsin. This was established using esterolytic assays with small molecule substrates (see Materials and Methods; data not shown). Thus, vinca site binding results in loss of proteolytic accessibility around β-390 regardless of the presence or absence of bound colchicine site agent.

Inhibition of cleavage yielding β-col in the experiment in Figure 6 is not accompanied by significant enhancement of cleavage yielding βN and βC as would be expected from Figure 5. This is not because the substrate was tubulin in Figure 5 and is tubulin−colchicine in Figure 6 since control digests yielded similar enhancement of production of βN and βC by all four vinca site agents in the presence or absence of colchicine (data not shown). Rather the explanation is that the experiment in Figure 6 was carried out in phosphate buffer (0.01 M phosphate, 1 mM $MgCl_2$, pH 7) rather than in Mes assembly buffer as used elsewhere. In phosphate buffer the enhancement of βN and βC production by any of the vinca site agents is less than in Mes assembly buffer. The significance of this is not clear. Inhibition of cleavage near β-390, however, is observed by vinca site agents in phosphate buffer as well as in Mes assembly buffer.

The data of Figure 1 demonstrate that vinca site occupancy does not result in the loss of fluorescence due to bound

Table 1: Vinca Site Agents Increase the Specificity of Photolabeling of β-Tubulin by [^{3}H]Colchicine[a]

drug	β/α ratio[b]	α[c]	β[c]
colchicine	7.6 ± 1.4	1	1
colchicine + maytansine	16.1 ± 3.5	0.64 ± 0.06	1.36 ± 0.26
colchicine + vincristine	10.1 ± 1.7	0.71 ± 0.13	0.93 ± 0.22

[a] Tubulin, 10 μM in Mes assembly buffer, was incubated with 10 μM [^{3}H]colchicine (25 Ci/mmol) for 15 min at 37 °C. Aliquots were taken and supplemented with no addition or 15 μM maytansine or 15 μM vincristine and incubated for 5 min at RT. Photo-cross-linking of the colchicine to the tubulin followed by separation of α from β by SDS gel electrophoresis and quantitation by liquid scintillation counting was done as described in Materials and Methods. Values given are mean ± SD for four replicates of each. Each replicate was a separate binding reaction, separately irradiated, electrophoresed, and quantitated. [b] Radioactivity (^{3}H dpm) in β-tubulin band divided by that in α-tubulin band in the same lane in the same gel. [c] Radioactivity (^{3}H dpm) in α- or β-tubulin compared to that found in the matched control (colchicine alone).

colchicine, but this does not exclude the possibility of some structural influence at the colchicine site by vinca site occupancy. Indeed, it has long been known that vinblastine stabilizes the colchicine site against time-dependent loss (Wilson, 1970). However, this effect could be due to a general stabilization of the protein, not involving specific effects on the locus of colchicine binding. Stabilization of colchicine binding is not observed with maytansine or rhizoxin (Hamel, 1992). To investigate possible vinca site ligand-induced structural changes at or near the colchicine site, photolabeling of tubulin with [^{3}H]colchicine was carried out in the presence and absence of bound vinca site ligand. Photolabeling requries production of covalent bond(s) between colchicine and nearby protein functional groups and is therefore presumed to be senstitive to small perturbations that alter the separation distance between the two potential bonding partners. The data in Table 1 show that the presence of vinca site agents decreases the efficiency of labeling of α and can increase it for β, resulting in a near doubling in the β/α ratio for maytansine. Thus, vinca site ligands induce conformational changes in tubulin that alter the detailed spatial array of protein groups around the colchicine site. This effect is not dependent on the oligomerization induced by vinca alkaloids, since it is produced by binding of maytansine, indeed to a somewhat greater extent than by vincristine.

Are there other ways to assess the structural results of doubly liganding tubulin? It is known that colchicine and some, though not all, other colchicine site ligands stimulate tubulin GTPase activity, but vinca site ligands uniformly inhibit GTPase activity [reviewed by Hamel (1990)]. What happens if tubulin contains ligands in both sites? This is addressed in Figure 7. The hydrolysis of GTP by tubulin (a) is clearly enhanced by colchicine (b) and slightly inhibited by maytansine (c). If tubulin contains both colchicine and maytansine, the resulting activity is not different from that observed with maytansine alone (d). Here again, the doubly liganded tubulin has characteristics dictated by occupancy of the vinca site, regardless of the presence of bound colchicine.

The inhibition of colchicine-enhanced GTPase activity shown in Figure 7 was found in the presence of super-stoichiometric concentrations of maytansine. The concentration dependence of this inhibition is demonstrated in Figure 8. In the type of experiment in Figure 8, GTPase activity

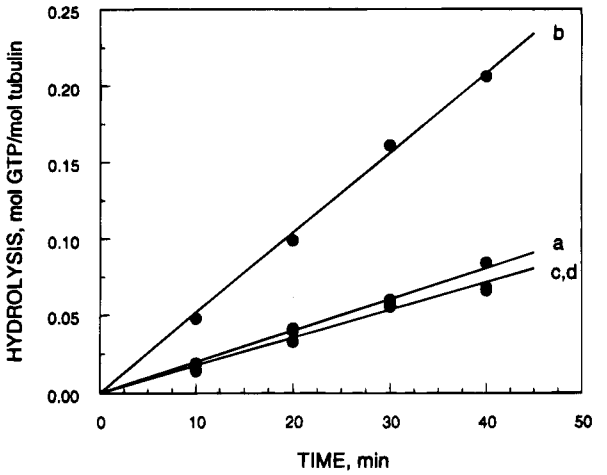

FIGURE 7: GTP hydrolysis by tubulin in the presence of colchicine and maytansine. Tubulin, 10 μM, was preincubated with (a) no additions, (b) colchicine, 100 μM, (c) maytansine, 50 μM, or (d) colchicine, 100 μM, and maytansine, 50 μM. At time = 0, [^{32}P]-GTP was added, and incubation continued. At various times, aliquots of 10 μL were removed, and GTP hydrolysis was assayed as described in Materials and Methods. The difference between the data points for c and d was less than the size of the data symbol, so only one line is shown.

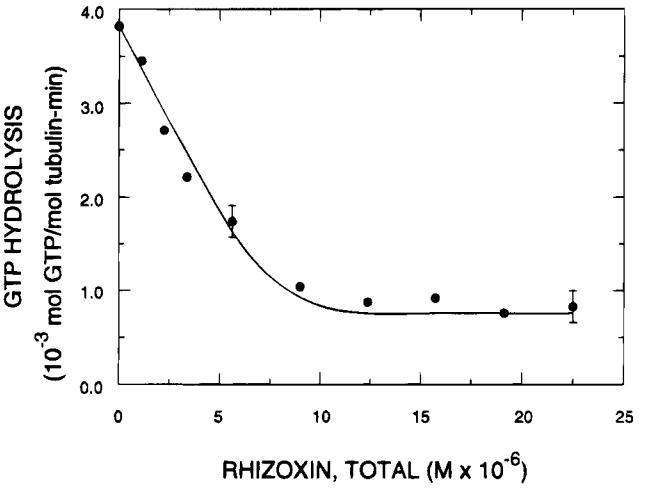

FIGURE 8: Concentration-dependent inhibition of colchicine-induced GTP hydrolysis by rhizoxin. Tubulin was incubated with excess (0.1 mM) colchicine for 20 min at 30 °C to saturate tubulin with colchicine. The complex was aliquoted, with addition of rhizoxin to yield final concentrations as indicated. Final tubulin concentration was 9 μM. Incubation continued at room temperature for 5 min. Labeled GTP was added and the mix incubated for 30 min at room temperature. GTP hydrolysis was assayed as described in Methods. GTP hydrolysis, expressed as moles of GTP hydrolyzed per mole of tubulin per minute, is shown at various concentrations of added (total) rhizoxin. In the absence of colchicine and rhizoxin, tubulin showed activity of 1.3 × 10^{-3} mol of GTP/(mol of tubulin·min). Representative errors (mean ± SD, n = 3) are shown. The line is the best single site fit to the data, with K_d = 0.2 μM.

of tubulin–colchicine complex is assayed at a fixed incubation time, but in the presence of varying concentrations of added vinca site ligands, here rhizoxin. Similar experiments were done with maytansine and vincristine. The inhibition of tubulin–colchicine GTPase activity is clearly concentration-dependent. Fitting of the rhizoxin data to a single site model yields an estimate of K_d = 0.2 μM, shown by the solid line in Figure 8. This is in agreement with published data indicating K_d = 0.2 μM (Takahashi et al., 1987). Similar experiments with the other agents yielded K_d estimates of 0.2 μM for maytansine and 2.5 μM for

vincristine, in reasonable agreement with the fluorescence titrations and with published data (data not shown).

DISCUSSION

There are two principal binding sites on tubulin for MT-destabilizing drugs: the colchicine site and the vinca site. The agents binding to these two site differ in chemical character (colchicine site agents tend to be smaller and have a less complex structure) as well as in binding site. While the binding sites are distinct, it could be supposed that the structural changes in tubulin induced by binding and resulting in poisoning of MT assembly might be similar for the two sets of ligands. However, it has been clear for some time that this is not the case. The data presented here and the results of others show that the structural changes due to colchicine and vinca ligands are different and exhibit both independent and antagonistic characteristics.

Colchicine site ligands induce changes in tubulin that alter several properties of the protein. Binding of colchicine results in significant quenching of tubulin tryptophan fluorescence, largely due to nonradiative energy transfer. Binding of some, but not all, colchicine site ligands results in stimultation of GTPase activity, unassociated with oligomerization of tubulin. Structural changes are induced by colchicine binding that result in the unfolding of the carboxyl end of an amphipathic helix in β-tubulin near residue 390 (Sackett & Varma, 1993). This is far (in linear sequence distance) from the residues implicated as binding site components by photolabeling (Uppuluri et al., 1993), though these could be close in the three-dimensional folded structure. It has been argued that the colchicine ligand-induced structural changes (e.g., enhancement of GTPase activity) are long distance effects (Perez-Ramirez et al., 1994).

Vinca site ligands, like colchicine site ligands, inhibit MT assembly but may be subdivided by their ability to induce non-MT polymers of tubulin. Vinblastine and vincristine do induce formation of non-MT polymers while maytansine and rhizoxin do not. Nonetheless, it seems clear that these agents all bind at the same site (or at least overlapping sites) on tubulin. Previous reports demonstrate that all of these agents share the property of inhibiting intrinsic tubulin GTPase activity. Thus it could be expected that the vinca site ligands would share some properties due to binding site occupancy but differ on others, for example, properties resulting from polymer formation, induced by vinca alkaloids but not by maytansine or rhizoxin.

A property that is shared by all four vinca site ligands examined here is the inhibition of tubulin tryptophan fluorescence (Figure 1). Unlike tryptophan quenching due to colchicine binding, this quenching is not due to nonradiative energy transfer, since there is little spectral overlap between tubulin emission and the absorbance of these drugs (Figure 3). Therefore, the loss of tubulin emission must be due to conformational changes in tubulin that result in movement of quenching groups in the protein to positions near tryptophans that result in quenching of tryptophan emission. These results confirm previous observations of quenching of tubulin tryptophan fluorescence by vinblastine or vincristine binding which reported single wavelength emission intensity changes (Lee et al., 1975; Prakash & Timasheff, 1983). The present results demonstrate that the quenching changes the shape of the emission spectrum as well as the intensity.

The quenching of fluorescence due to vinca site occupancy is selective, causing preferential loss of higher wavelength emission. Such loss suggests that the vinca site-induced conformational change preferentially affects more solvent-exposed tryptophans. This is demonstrated by difference spectra (Figure 2) which indicate the loss of fluorescence emission from near-surface tryptophans. More than one tryptophan is affected, and the intensity lost is similar to that of 3 equiv of free tryptophan. The structural changes induced by binding appear to result in a direct quenching of these tryptophans rather than a removal from solvent exposure (e.g., burial in the protein interior), since there is no increase in emission at lower wavelengths. Selective quenching is observed with all of the vinca site ligands, indicating that it is not a result of the oligomerization induced by vinca alkaloids but rather is a result of vinca binding site occupancy. Vinca site ligands also quench the tryptophan fluorescence of tubulin−colchicine complexes, whose tryptophan emission is already significantly quenched relative to the unliganded protein by the presence of colchicine. Furthermore, difference spectra from tubulin−colchichine with and without maytansine are quite similar in shape, area, and wavelength maximum to those in Figure 2 for tubulin with and without maytansine. This suggests that the vinca site agents quench the same tryptophans to about the same extent in both tubulin and tubulin−colchicine, despite the fact that tryptophan emission is already quenched about two-thirds by colchicine alone (Figure 1). This suggests that the tryptophans quenched by vinca site agents are not quenched by colchicine and vice versa. Thus, vinca site occupancy causes structural changes in tubulin that result in quenching of tryptophan emission, regardless of the occupancy state of the colchicine site. These results indicate that vinca site binding leads to structural changes in tubulin different from, and at least somewhat independent of, those induced by colchicine.

Proteolytic digestion can yield information about the regions of a protein structure that are exposed and vulnerable to enzymatic attack. Changes in patterns of digestion reflect conformational changes in the structure of the protein substrate, in the absence of specific effects on the enzyme(s). For example, novel proteolytic cleavages result from the colchicine-induced unfolding of a helix in β-tubulin near residue 390, producing the β-col fragment (Sackett & Varma, 1993). This property is shared by the other colchicine site ligands (Figure 4) and thus differs from proteolytically detectable structural changes induced by some vinca agents but not by others.

Vinca site ligands clearly induce different structural changes in tubulin than do colchicine site ligands: (1) they do not induce or enhance the formation of β-col by chymotrypsin or trypsin (Figures 4 and 5), i.e., they do not induce unfolding around β390; (2) they inhibit the low level of β-col (trypsin) found in the absence of colchicine site ligands (Figure 5); (3) they antagonize the unfolding around β-390 induced by colchicine, requiring about one mole of vincristine or rhizoxin per mole of tubulin to effect reversal of unfolding (Figure 6).

The suppression of cleavage producing β-col by all of the vinca site agents is particularly interesting in light of the photoaffinity labeling with rhizoxin reported by Sawada et al. (1993). They found that an azidodansyl derivative of rhizoxin labeled a peptide from β-tubulin containing residues 363−379. This overlaps with the sequence region in the

amphipathic helix affected by colchicine site ligands (Figure 4). This helix is predicted to contain roughly residues 373–392 and has been implicated in contacts necessary for normal polymer formation (Sackett & Varma, 1993). This implication is strengthened by the results of Sawada et al. (1993) and the results presented here. The results of Sawada et al (1993) indicate that the binding site for rhizoxin, and presumably the other vinca site agents, is very near, or even directly on, this helix. The results in Figures 4, 5, and 6 here demonstrate that all four vinca site ligands as well as all five colchicine site ligands examined affect this helix, although in opposite and antagonistic ways as judged by proteolysis yielding β-col. Clearly these agents could also be inducing other changes in this region, even to this helix, that do not alter proteolytic cleavage. Further experiments may clarify this point.

In addition to affecting this helix and suppressing production of β-col, vinca site ligands enhance the chymotryptic cleavage of β-tubulin producing βN and βC to a variable extent, as does colchicine (Figure 5). The order of enhancement in Mes assembly buffer is vinblastine = vincristine ≫ maytansine > rhizoxin ≈ colchicine. The distinction between the vinca alkaloids and maytansine and rhizoxin is more clear in the effect on patterns of proteolysis by trypsin. As already noted, all four agents share the property of inhibiting the minor tryptic cleavage of β-tubulin. They differ in the effects on the tryptic cleavage of α that produces αN and αC. Vinblastine and vincristine strongly inhibit this cleavage, while maytansine, rhizoxin, and colchicine have little effect.

The effects on proteolysis may be grouped into those common to all four agents and those shown only by the vinca alkaloids. All agents cause structural changes around β-390 that are the opposite of, and antagonistic to, those produced by colchicine. All agents also produce an enhancement of chymotryptic cleavage producing βN and βC. The vinca alkaloids differ in producing large enhancement of the chymotryptic cleavage and in inhibiting tryptic cleavage of α, which the other agents do not affect.

Structural changes induced by vinca site agents may also be probed by effects at the colchicine site, which involves sequences from the amino terminal and middle regions of β-tubulin (Uppuluri et al., 1993). The specificity of photolabeling of β-tubulin with radioactive colchicine has been shown to be sensitive to small perturbations. Many of these result in a decrease in β- specificity, given by the β/α ratio of label (Wolff et al., 1991, 1992). Vincristine or maytansine causes an increase in the β/α ratio (Table 1). This increase could be due to local changes in β, resulting in increased efficiency of β-labeling, changes in α, resulting in decreased efficiency of α-labeling, to an increase in the distance from the colchicine site on β to nearby α residues, or to some combination of these. We favor changes in β, since maytansine clearly increases the labeling of β, and other evidence, such as the proteolysis results, indicates maytansine effects only on β. The decreased labeling of α may be due to increased separation between α residues and the colchicine site, induced by changes in β.

Binding of vinca site agents also inhibits tubulin GTP hydrolysis (Hamel, 1990). At low temperatures these agents also inhibit GTP exchange, but this effect is not seen at temperatures used for GTPase assay (Bai et al., 1990; David-Pfeuty et al., 1979). Therefore interference with GTP exchange is not the explanation for the inhibition of GTP hydrolysis shown previously and here in Figures 7 and 8. Vinca site agents must alter the structure of β-tubulin in such a way that GTP binding is possible but hydrolysis is not. Colchicine binding, on the other hand, enhances GTP hydrolysis. In the presence of both colchicine and vinca site agents, the vinca site inhibition is dominant (Figures 7 and 8). The experiments of Figures 7 and 8 also show that the ability of vinblastine to inhibit tubulin–colchicine GTPase previously reported (David-Pfeuty et al., 1979) is shared by maytansine and rhizoxin and therefore cannot require oligomer formation, but rather vinca site occupancy *per se*. The dominance of the vinca site effects on GTP hydrolysis are similar to those on proteolysis: while colchicine alone leads to local unfolding around β-390 and stimulates GTPase activity, vinca site agents tighten folding around β-390 and inhibit GTPase activity in the presence or absence of colchicine. It might be tempting to speculate that flexibility around β-390 is coupled to GTP hydrolysis. This is made less likely by the fact that some colchicine site ligands, such as podophyllotoxin and steganacin, induce unfolding near β-390 and production of the β-col band (Figure 4) but do not stimulate GTPase activity (Hamel, 1990; Sackett, unpublished results).

The effects that are produced in common by all of the agents studied here, and that can be localized, clearly occur on β-tubulin. Those unique to the vinca alkaloids (Figure 5) occur on both α and β. A similar distinction has been between the effects of maytansine/rhizoxin and the vinca alakoids on sulfhydryl cross-linking and alkylation: all four alter the production of specific cross-links in β, but alkylation of α sulfhydryls is altered only by vinblastine, not maytansine (Sullivan et al., 1990; Luduena & Roach, 1991). The results here are consistent with a β-tubulin location for the shared binding site, as has been suggested before on the basis of different data [see Hamel (1992) and Luduena and Roach (1991)], and as indicated by photolabeling with rhizoxin (Sawada et al., 1993). This would explain why common structural effects occur on β.

Changes in α observed upon vinca alkaloids binding are then seen to be a consequence of oligomerization, not an indication of the location of the shared binding site. The oligomerization caused by vinca alkaloids may then explain why photolabeling with vinblastine labels α as well as β-tubulin (Safa et al., 1987; Wolff et al., 1991). Vinca alkaloid labeling of β-tubulin results from interaction with the shared binding site near the β 373–392 helix. Dimer–dimer contacts in the oligomer must bring part of α-tubulin near to the shared binding site on β-tubulin, allowing vinblastine labeling of α as well as β. Thus, photolabeling results are parallel to changes shown here by proteolysis: rhizoxin (and here also maytansine) affect β-tubulin, while vinblastine and vincristine affect both α- and β-tubulin.

Conclusion. Vinca site agents in this study may be grouped as oligomer-inducing (vinblastine and vincristine) and non-oligomer-inducing (maytansine and rhizoxin). All produce a number of detectable changes in tubulin conformation. Changes produced by both classes of agents include quenching of surface tryptophan fluorescence, inhibiting proteolytic cleavage near β-390 and increasing it after β-281, increasing the specificity of colchicine labeling of β-tubulin, and inhibiting GTP hydrolysis. These effects are plausibly localized to β-tubulin, although the location of the quenched tryptophans is unknown. The oligomer inducing agents induce large increases in proteolytic cleavage after β-281

and inhibit cleavage after α-339. These effects are plausibly the result of the oligomerization unique to these agents. The vinca site agent effects and colchicine effects are either additive, as in the case of trytophan fluorescence, or antagonistic, as in β-390 cleavage and GTPase activity. When antagonistic, the effects of vinca site agents are dominant over colchicine effects. These results are consistent with a shared binding site for the vinca site agents on β-tubulin, close to the carboxyl terminal helix that is disrupted by colchicine, with effects on α-tubulin linked to the oligomerization induced by the vinca alkaloids.

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