Interaction of the Antitumor Compound Cryptophycin-52 with Tubulin†

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ABSTRACT: Cryptophycin-52 (LY355703) is currently undergoing clinical evaluation for cancer chemotherapy. It is a potent suppresser of microtubule dynamics in vitro, and low picomolar concentrations appear to inhibit cancer cell proliferation at mitosis by stabilizing spindle microtubules. In the present study, using [^{3}H]cryptophycin-52, we found that the compound bound to tubulin at a single high-affinity site [apparent K_a $(3.6 \pm 1) \times 10^6$ L/mol, 34 °C]. The binding of cryptophycin-52 to tubulin was rapid, not appreciably temperature-dependent, and very poorly reversible. However, we could remove [^{3}H]-cryptophycin-52 from [^{3}H]cryptophycin-52−tubulin complex by denaturing the complex with either urea treatment or boiling. These data suggest that the binding of cryptophycin-52 to tubulin is not covalent. A van't Hoff plot of the binding data indicated that the binding of cryptophycin-52 to tubulin is primarily entropy-driven with a minimum enthalpy contribution. In addition, cryptophycin-52 perturbed the far-ultraviolet circular dichroic spectrum of tubulin and it inhibited the colchicine-induced guanosine triphosphatase activity of tubulin, indicating that its binding to tubulin induces a conformational change in the tubulin. Competition experiments with vinblastine suggest that the binding site for crytophycin-52 may overlap with the vinblastine binding site.

Microtubules are highly dynamic polymers built by the self-association of $\alpha\beta$ tubulin dimers. Microtubules can undergo two unusual nonequilibrium dynamic behaviors. The ends of the microtubules can switch between states of slow growth and rapid shortening, a process called dynamic instability (*1, 2*). Also, microtubules can grow predominantly at one end and shorten at the opposite end by a process called treadmilling (*3*−*5*). It is believed that both dynamic instability and treadmilling are important for microtubule organization and function in cells, especially during mitosis, which requires extremely rapid dynamics to properly segregate the duplicated chromosomes to the daughter cells (*6*−*9*). Microtubules have become excellent targets for anticancer drug development, and several of the most valuable anticancer drugs such as vinblastine and taxol appear to inhibit cell proliferation at the metaphase/anaphase transition of mitosis by suppressing microtubule dynamics (*8*−*12*).

The depsipeptides known as the cryptophycins are among the most potent antimitotic agents thus far described. The parent compound of the series, cryptophycin-1 (originally called cryptophycin A), was isolated from a cyanobacterium (*Nostoc* sp.) and was found to block cells at mitosis in the low picomolar concentration range by an apparent action on microtubules (*13*−*15*). In vitro, cryptophycin-1 has been shown to inhibit the assembly of tubulin into microtubules (*16*−*18*) and to strongly suppress microtubule dynamic instability (*19*).

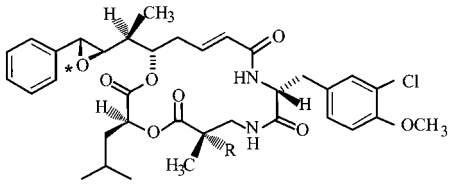

FIGURE 1: Chemical structure of cryptophycin-52. R = H, cryptophycin-1; R = CH_3, cryptophycin-52. The asterisk marks the position of the epoxide ring.

Cryptophycin-52 (LY355703) (Figure 1), a new member of the cryptophycin family that is produced by total chemical synthesis (*20*), is currently undergoing clinical evaluation for cancer chemotherapy. Cryptophycin-52 has remarkably potent antiproliferative activity against cultured human tumor cells and animal tumor models (*11, 21*). For example, the concentration of cryptophycin-52 required to inhibit HeLa cell proliferation by 50% is only 11 pM (*11*). The drug is between 40- and 400-fold more potent than paclitaxel or the vinca alkaloids in several tumor cell lines (*21*). Of particular interest is that tumor cells that are resistant to paclitaxel and the vinca alkaloids due to overexpression of multidrug resistance transport proteins are sensitive to cryptophycin-52 (*21*). At high concentrations (>10 times the IC_{50}), cryptophycin-52 blocks HeLa cell proliferation at mitosis by depolymerizing spindle microtubules. However, like many microtubule-targeted antimitotic drugs, low concentrations of cryptophycin-52 inhibit mitosis without significantly reducing the spindle microtubule mass or chromosome organization. These actions of cryptophycin-52 at low

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concentrations appear to be due to the suppression of microtubule dynamics (*11*).

We recently determined that cryptophycin-52 potently suppresses microtubule dynamic instability in vitro, at concentrations that do not appreciably reduce the microtubule polymer mass (*11*). Its major actions are to suppress the rate and extent of growing and shortening at microtubule ends and to increase the percentage of time that the microtubules spend in an attenuated state, neither growing nor shortening detectably. The powerful suppression of dynamics by cryptophycin-52 appears to result from the binding of a very few molecules of the compound to the microtubule. By analyzing the effects of cryptophycin-52 on dynamic instability in relation to its binding to microtubules, we determined that only five or six cryptophycin-52 molecules bound per microtubule, presumably at the microtubule ends, are sufficient to suppress dynamicity by 50% (*11*).

The binding of cryptophycin-52 to tubulin has not yet been characterized. Thus in the present study we used radiolabeled cryptophycin-52 and fluorescence spectroscopy to characterize the binding of the drug to tubulin. Our data indicate that cryptophycin-52 binds extremely tightly to a single high-affinity binding site in tubulin and that cryptophycin-52 binding induces a conformational change in the tubulin that may be important in the ability of the drug to increase stability at microtubule ends.

MATERIALS AND METHODS

Materials. Bovine brain microtubule protein consisting of ~70% tubulin and 30% microtubule-associated proteins was isolated without glycerol by two cycles of polymerization and depolymerization (*5*). Tubulin was purified from the microtubule protein by phosphocellulose chromatography and stored as frozen pellets at −70 °C (see 5). Cryptophycin-52 was synthesized by the Lilly Research Laboratories, Indianapolis, IN. [*methoxy*-^{3}H]Cryptophycin-52 [specific activity 82 Ci/mmol (3.0 TBq/nMol); radiochemical purity 99.2%] was synthesized through contract with Amersham International, Buckinghamshire, England. Bis-ANS was obtained from Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma (St. Louis, MO).

Binding of [^{3}H]Cryptophycin-52 to Tubulin. Tubulin (3 μM) was mixed with different unlabeled cryptophycin-52 concentrations (0−23 μM) along with trace amounts of [^{3}H]-cryptophycin-52 in PME buffer (10 mM sodium phosphate, 0.2 mM $MgCl_2$, and 1 mM EGTA, pH 6.8) at 34 °C and incubated for 5 min. Bound cryptophycin-52 was separated rapidly from free drug by spin-column centrifugation in a clinical centrifuge at high speed as previously described (*22*). Briefly, 180 μL samples of reaction solution were centrifuged through 1 mL columns filled with Bio-Gel P6 (Bio-Rad) and equilibrated with PME buffer. Bound [^{3}H]cryptophycin-52 was determined by scintillation counting. Background radioactivity measured in the absence of tubulin was <1% of the experimental values. Protein concentration was determined by the method of Bradford (*23*) with bovine serum albumin as the standard. Data are the average of four independent experiments.

Determination of GTP Exchange. To obtain tubulin with GDP in the exchangeable site, tubulin-GTP (3 mg/mL) in 100 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA buffer, pH 6.8 (PEM buffer), was first incubated with 1 mM EDTA and 6 mM GDP for 30 min at 0 °C. The tubulin was then separated from all free nucleotide by centrifuging the solution rapidly through a 1-mL Bio-Gel P6 spin column. Fresh excess GDP (100 μM) was added, the solution was incubated for an additional 5 min at 0 °C, and the solution was centrifuged a second time to remove all unbound nucleotide. The purified tubulin-GDP solution (2 μM) was then incubated at 0 °C for 30 min in the absence or presence of a range of cryptophycin-52 concentrations. [^{3}H]GTP (final concentration 100 μM) was then added and incubation continued for an additional 15 min. Finally, the amount of [^{3}H]GTP bound per tubulin dimer was determined after the tubulin-GTP complex was separated from all unbound nucleotide by centrifugation through a 1 mL Bio-Gel P6 spin column as previously described.

Measurement of Colchicine-Induced GTPase Activity. Tubulin (25 μM) was incubated with colchicine (100 μM) for 90 min at 35 °C to form tubulin−colchicine (TC) complex [approximately 0.85−0.9 mol of bound colchicine/mol of tubulin (*24, 25*)]. The TC complex was separated from unbound colchicine by centrifugation through a 1 mL Bio-Gel P6 spin column equilibrated in PEM buffer. Trace amounts of [^{32}P]GTP were added to the TC complex solution (final TC complex concentration 5 μM) along with 6 mM $MgCl_2$ and 300 μM unlabeled GTP, and the solutions were equilibrated with a range of cryptophycin-52 concentrations by incubation at 0 °C for 30 min. The temperature was then raised to 35 °C to induce GTP hydrolysis. GTP hydrolysis was quenched after 30 min by adding a suspension of activated charcoal [10% activated charcoal, 10% (v/v) acetic acid, and 2.5 mM sodium phosphate] (*26*). The quenched samples were incubated at 0 °C for 10 min and then centrifuged for 20 min at room temperature to remove GTP (Baxter Scientific Microcentrifuge, room temperature). The radioactivity remaining in the supernatant was determined by scintillation counting.

Fluorescence Spectroscopy. Fluorescence measurements were performed in a Perkin-Elmer LS 50B spectrofluorometer. Spectra were taken by multiple scans, and buffer blanks were subtracted from all measurements. The inner filter effects were corrected by measuring the change of fluorescence intensity of a tryptophan solution in the presence of cryptophycin-52 (*22, 27−28*). The excitation and emission wavelengths were 295 and 336 nm, respectively. The fraction of binding sites (α) occupied by cryptophycin-52 was determined from the relationship $\alpha = (F_0 - F)/(F_0 - F_m)$, where F_0 is the fluorescence intensity of tubulin in the absence of cryptophycin-52, F is the corrected fluorescence intensity when the tubulin and cryptophycin-52 are in equilibrium, and F_m is the calculated fluorescence intensity of the fully liganded tubulin (*27*). F_m was determined by plotting $1/(F_o - F)$ versus $1/L$ (L = total ligand concentration) and extrapolating $1/L$ to 0. The association constant, K_a, was determined from the relationship $K_a = [\alpha/(1 - \alpha)](1/Lf)$,

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PME buffer, 10 mM sodium phosphate, 0.2 mM $MgCl_2$, and 1 mM EGTA, pH 6.8; PEM buffer, 100 mM 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM $MgCl_2$, and 1 mM EGTA, pH 6.8; β-ME, β-mercaptoethanol; bis-ANS, bis-8-anilino-1-naphthalenesulfonic acid; GTP, guanosine triphosphate; GDP, guanosine diphosphate.

Table 1: Inhibition of Tubulin Polymerization by Cryptophycin-52 in the Absence and Presence of β-ME

cryptophycin-52 (μM)	inhibition of polymer mass[a] (%)	
	control	3 mM β-ME
0	0	0
1	52	53
2	71	69
4	82	84

[a] The control polymer level was 1 mg/mL.

where Lf = the free ligand concentration and $Lf = L - \alpha$-[C], where [C] is the molar concentration of ligand binding sites, with the assumption of a single binding site per tubulin dimer.

RESULTS

Kinetics and Reversibility of Cryptophycin-52 Binding to Tubulin. In our initial experiments we observed that the binding of [^{3}H]cryptophycin-52 to tubulin occurred rapidly, with maximum stoichiometry being reached within 1 min of incubation at a number of different cryptophycin-52 concentrations (data not shown). The presence of a potentially reactive epoxide ring in the cryptophycin-52 structure (asterisk, Figure 1) suggested to us that the compound might form a covalent bond with a nucleophilic side chain in the tubulin dimer. To examine the reversibility of the binding, we incubated [^{3}H]cryptophycin-52 with purified tubulin, isolated the [^{3}H]cryptophycin-52−tubulin complex by gel-filtration chromatography, and then dialyzed the complex at 4 °C for 48 h. Complex formation between cryptophycin-52 and tubulin was very poorly reversible. In a typical experiment, the initial stoichiometry was 1.14 mol of cryptophycin-52 bound/mol of tubulin and the final stoichiometry after 48 h of dialysis was 0.72 mol of cryptophycin-52 bound/mol of tubulin. Inclusion of excess unlabeled cryptophycin-52 (125 μM) during dialysis did not appreciably reduce the stoichiometry of bound [^{3}H]cryptophycin-52.

We examined whether the binding of cryptophycin-52 to tubulin might be covalent in several ways. Cysteine residues are strong candidates for reactive groups that could form covalent interactions through a reactive epoxide. Thus, we examined the effects of β-mercaptoethanol (β-ME) on the ability of cryptophycin-52 to inhibit microtubule assembly in PEM buffer. As shown in Table 1, β-ME did not reduce the ability of cryptophycin-52 to inhibit microtubule assembly. We also directly determined the stoichiometry of [^{3}H]cryptophycin-52 binding to tubulin in the absence and presence of 100 mM β-ME and found that the βME did not inhibit the binding of the compound to tubulin (data not shown). Together these results indicate that the epoxide group of cryptophycin-52 does not react with sulfhydryl groups in tubulin. We also found that 1 mM histidine, lysine, or glutamic acid had no effect on the binding of cryptophycin-52 to tubulin (data not shown), suggesting that these residues in tubulin also are not involved with formation of a covalent complex with the drug.

Denaturing of proteins with high molar concentrations of urea can be used to determine whether a ligand is covalently or noncovalently bound. Thus, purified [^{3}H]cryptophycin-52−tubulin complex, isolated on a size-exclusion Bio-Gel P6 column, was denatured by incubation with 6 M urea (30 min, 35 °C), and the stoichiometry of cryptophycin-52 binding per tubulin dimer was determined after gel filtration a second time. We found that 98.5% of the bound cryptophycin-52 was lost from the complex after incubation in 6 M urea (four independent experiments). Similar results were obtained by denaturing the [^{3}H]cryptophycin-52−tubulin complex by immersion in boiling water for 30 min. Taken together, the results indicate that the binding of cryptophycin-52 to tubulin is noncovalent.

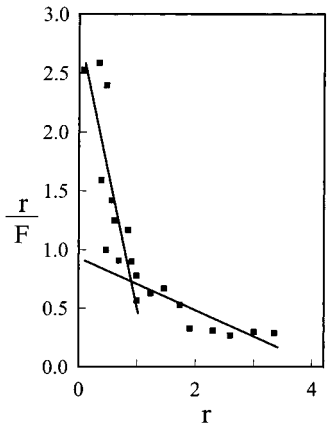

FIGURE 2: Scatchard analysis: binding of [^{3}H]cryptophycin-52 to tubulin. Tubulin (3 μM) was incubated at 0 °C for 5 min with different concentrations of [^{3}H]cryptophycin-52 (0.3−23 μM). Free [^{3}H]cryptophycin-52 was separated from tubulin-bound [^{3}H]cryptophycin-52 by use of a spin column as described under Materials and Methods. *F* represents the concentration of unbound cryptophycin-52 in solution, and *r* represents the stoichiometry of cryptophycin-52 bound per tubulin dimer. One of four replicate experiments is shown. The binding constant reported is the average of the four experiments.

Determination of the Binding Constant and the Stoichiometry of Cryptophycin-52 Binding to Tubulin. The apparent binding constant and the binding stoichiometry between cryptophycin-52 and tubulin were determined by use of [^{3}H]-cryptophycin-52. Tubulin (3 μM) was incubated with a range of [^{3}H]cryptophycin-52 concentrations between 0.3 and 23 μM at 0 °C for 5 min, and the concentration of bound [^{3}H]-cryptophycin-52 was determined after spin-column centrifugation (Materials and Methods). The data yielded a nonlinear Scatchard plot (*29*), indicating the presence of at least two classes of cryptophycin-52 binding sites (Figure 2). There was a single high-affinity site per tubulin dimer ,with an apparent association constant of $(2.2 \pm 0.3) \times 10^6$ M^{-1}, and an indeterminate number of low-affinity sites, with apparent association constant(s) in the range of $(0.12 \pm 0.01) \times 10^6$ M^{-1}. The low-affinity binding may represent nonspecific sites or may be generated by aggregation of tubulin dimers at the high concentrations of cryptophycin-52 (*16, 18*).

In a second approach, we determined the binding of cryptophycin-52 to tubulin in the high-affinity range by analyzing the effects of drug binding on the intrinsic tryptophan fluorescence of tubulin (*30, 31*). As shown in Figure 3, cryptophycin-52 inhibited tubulin fluorescence in a concentration-dependent manner. Analysis of the data yielded a dissociation constant of 0.1 ± 0.01 μM, which is consistent with the binding constant obtained by use of radiolabeled cryptophycin-52 (Figure 2).

Energetics of Cryptophycin-52 Binding to Tubulin. The association constant for the high-affinity binding site did not

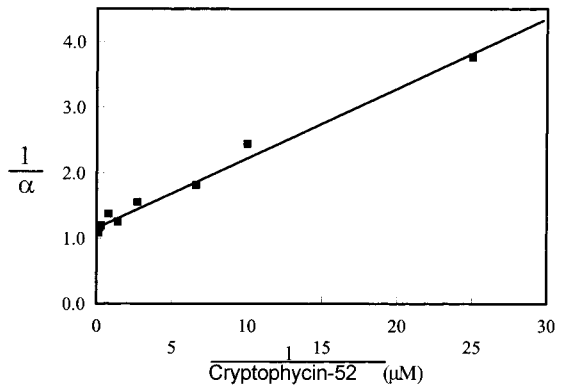

FIGURE 3: Binding of cryptophycin-52 to tubulin as determined by fluorescence spectroscopy. α = fraction of binding sites occupied by cryptophycin-52. Tubulin (2 μM) in PEM buffer was incubated in the absence or presence of cryptophycin-52 at 25 °C for 30 min and the fluorescence changes were determined as described under Materials and Methods. The data represent one of four replicate experiments.

Table 2: Binding Constant for Cryptophycin-52 at Different Temperatures[a]

temp (°C)	high-affinity K_a (M^{-1})	n
0	$(2.2 \pm 0.3) \times 10^6$	0.94
21	$(3.1 \pm 0.8) \times 10^6$	0.85
34	$(3.6 \pm 1.0) \times 10^6$	0.91

[a] On average four experiments were carried out at each temperature. Values are given ± SEM. K_a = apparent association constant; n = number of binding sites.

vary greatly as a function of temperature [only 1.5-fold between 0 and 34 °C (Table 2)]. Based on the available data with three temperatures, the calculated enthalpy and entropy values were approximately 2.3 kcal/mol and 37 eu, respectively. Thus, the binding reaction between cryptophycin-52 and tubulin is primarily entropy-driven.

Effects of Cryptophycin-52 on the Properties of Tubulin. One of the prominent characteristics of tubulin in solution is that it rapidly loses its colchicine binding activity and its ability to polymerize, a process called tubulin decay (*32*−*34*). A number of drugs that bind to tubulin such as vinblastine stabilize the tubulin and reduce the rate of decay (*32*−*36*). Bis-ANS is a useful probe for measuring tubulin decay because when it is bound to tubulin, its fluorescence increases as tubulin decays (*33*−*36*). Thus, we used bis-ANS fluorescence to examine the effects of cryptophycin-52 on tubulin decay. Tubulin (2.5 μM) was incubated in the absence or presence of 5 μM cryptophycin-52 or 5 μM vinblastine at 37 °C, and at the indicated times, bis-ANS (25 μM) was added and the bis-ANS fluorescence was determined. In the absence of cryptophycin-52 (△), tubulin decay with time was apparent from the increase of fluorescence (Figure 4). Cryptophycin-52 (5 μM) suppressed the rate of tubulin decay to approximately the same extent as 5 μM vinblastine (Figure 4). Thus, cryptophycin-52, like vinblastine, which may bind to tubulin at or near the same site (see below), stabilizes tubulin against decay (Figure 4).

Microtubule polymerization and dynamic instability require hydrolysis of GTP (*9*, *37*, *38*), which binds reversibly to the β subunit in the tubulin dimer (*39*). We wanted to determine whether cryptophycin-52 might exert its effects on polymerization and dynamics by inhibiting GTP binding

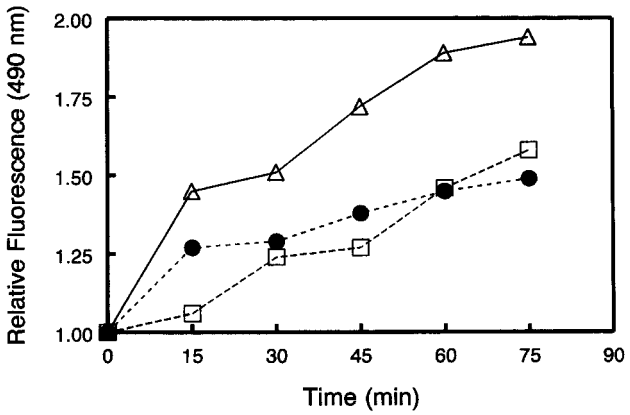

FIGURE 4: Effects of cryptophycin-52 on tubulin decay. The increase in tubulin-bis-ANS fluorescence with time at 37 °C is shown in the absence (△) and presence of 5 μM cryptophycin-52 (□) or 5 μM vinblastine (●) in PEM buffer. The excitation and emission wavelengths were 430 and 490 nm, respectively.

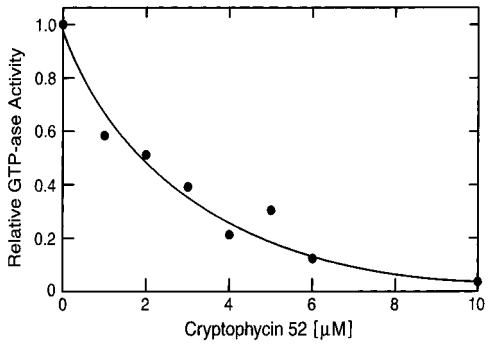

FIGURE 5: Reduction of colchicine-induced GTPase activity of tubulin by cryptophycin-52. The GTPase activity of 5 μM tubulin−colchicine complex was determined at 35 °C in the absence and presence of different concentrations of cryptophycin-52 (Materials and Methods).

or hydrolysis. We prepared tubulin with GDP present in the exchangeable nucleotide binding site and measured [^{3}H]GTP exchange into the site in the absence or presence of a range of cryptophycin-52 concentrations (Materials and Methods). Cryptophycin-52 (1−5 μM) had no effect on the rate of GTP exchange. For example, the stoichiometry of [^{3}H]GTP exchange into the nucleotide site in the absence and presence of 5 μM cryptophycin-52 under the conditions used (Materials and Methods) was 0.69 ± 0.06 and 0.63 ± 0.04 mol of GTP/mol of tubulin, respectively.

Soluble tubulin has a very low intrinsic GTPase activity, but the binding of colchicine to tubulin increases the activity, apparently due to a colchicine-induced conformational change (*26*, *28*, *40*). It is possible that the change in tubulin associated with the colchicine-induced increase of tubulin GTPase activity is similar to the increase in tubulin GTPase activity that occurs during polymerization. To determine whether cryptophycin-52 could modulate tubulin GTPase activity, we determined the effects of tubulin-bound cryptophycin-52 on colchicine-stimulated GTPase activity. We found that cryptophycin-52 inhibited colchicine-stimulated tubulin GTPase activity in a concentration-dependent manner with half-maximal inhibition occurring at a concentration of ∼2.3 μM (Figure 5). As a control, it was necessary to determine whether the inhibition of colchicine-induced GTPase activity might be due to an ability of cryptophycin-52 to inhibit the binding of colchicine to tubulin. We

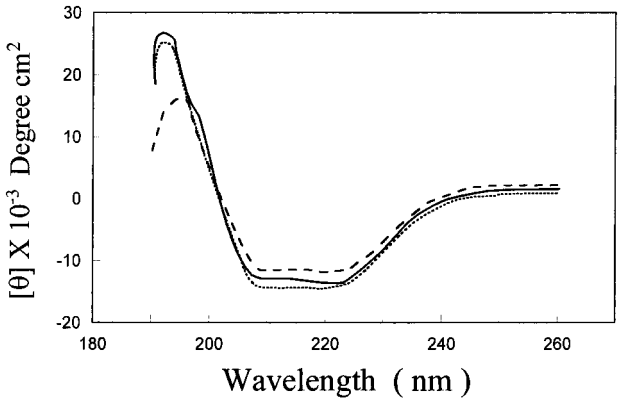

FIGURE 6: Far-UV CD spectra of 10 μM tubulin in the absence (---) and presence of 10 μM (—) and 20 μM cryptophycin-52 (— — —) in PEM buffer.

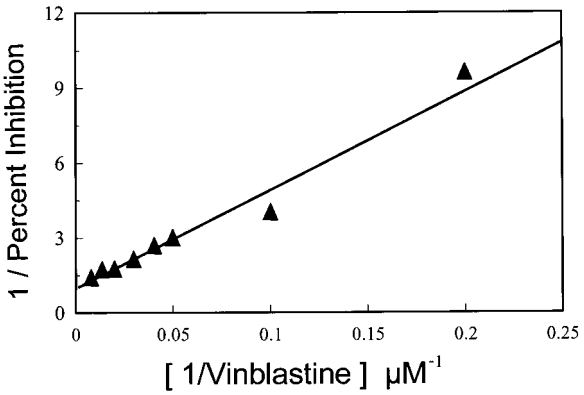

FIGURE 7: Effects of vinblastine on the binding of [^{3}H]cryptophycin-52 to tubulin. Different concentrations of vinblastine (0−200 μM) were added simultaneously with 2.5 μM [^{3}H]cryptophycin-52 to tubulin (2.5 μM) at 0 °C in PEM buffer, and the temperature was raised to 34 °C. After 30 min of incubation, the amount of [^{3}H]cryptophycin-52-bound to tubulin was determined as described under Materials and Methods.

considered this to be unlikely because cryptophycin-1 does not inhibit the binding of colchicine to tubulin. Free colchicine has minimal intrinsic fluorescence, but when it binds to tubulin, its fluorescence increases (*40*). Thus, we analyzed the binding of 15 μM colchicine to 4 μM tubulin in the presence of cryptophycin-52 by monitoring the increase in colchicine fluorescence (*30, 41*). We found that cryptophycin-52 (5−25 μM) had no effect on the fluorescence increase (data not shown), indicating that cryptophycin-52 does not bind to tubulin at the colchicine site. Since cryptophycin-52 does not affect the binding of GTP or colchicine to tubulin and does not inhibit GTP exchange, we can conclude that the binding of cryptophycin-52 to tubulin either reverses a conformational change induced by colchicine binding that causes the acceleration of GTP hydrolysis or that the binding of cryptophycin-52 to tubulin inhibits GTP hydrolysis directly.

We also determined whether the binding of cryptophycin-52 to tubulin could affect the secondary structure of tubulin by circular dichroism spectroscopy (*27, 31*). As shown in Figure 6, both 10 μM and 20 μM cryptophycin-52 altered the far-UV spectrum, indicative of a conformational change that involves a possible alteration in α-helical content. Interestingly, vinblastine did not produce any detectable change in the tubulin structure as determined by circular dichroism spectroscopy (data not shown; *27*).

Possible Interaction of Cryptophycin-52 with the Vinca Binding Domain of Tubulin. The depolymerizing antimitotic drugs are generally classified into two major groups, those that bind in the vicinity of the vinca binding site and those that bind in the vicinity of the colchicine site. A large number of structurally diverse compounds are known to bind to tubulin in the vinca domain. Previously, Bai et al. (*18*) reported that cryptophycin-1 inhibits the binding of vinblastine to tubulin in a noncompetitive manner. With the availability of [^{3}H]cryptophycin-52, we were in a position to determine whether vinblastine could inhibit the binding of cryptophycin-52 to tubulin. Thus, tubulin (2.2 μM) was incubated with 2.5 μM [^{3}H]cryptophycin-52 in the absence or presence of a range of vinblastine concentrations (5−200 μM), and binding was determined by column centrifugation (Materials and Methods). As shown by the double-reciprocal plot in Figure 7, vinblastine inhibited the binding of [^{3}H]-cryptophycin-52 to tubulin, with half-maximal inhibition, determined from the slope of the line, occurring at 50 μM vinblastine. The data are consistent with the idea that the binding site for vinblastine and cryptophycin-52 are similar or overlapping (*16*−*18*). However, many attempts to determine the nature of the inhibition of cryptophycin-52 binding by vinblastine (i.e., competitive or noncompetitive) were unsuccessful in that the type of inhibition could not be discerned.

DISCUSSION

Binding of Cryptophycin-52 to Tubulin. The cryptophycins are a family of novel antimitotic compounds with clinical potential for the treatment of cancer (*11, 13, 21*). Remarkably low (picomolar) concentrations of cryptophycin-52 inhibit the proliferation of HeLa cells at mitosis by an apparent action on spindle microtubules. We recently reported that cryptophycin-52 binds tightly to the ends of microtubules (K_d 47 nM) and that the binding of only five to six molecules of cryptophycin-52 per microtubule is sufficient to suppress microtubule growing and shortening dynamics by 50% (*11*). Because of cryptophycin-52's high potency and powerful effects on microtubule dynamic instability, it is reasonable to think that the tubulin binding site for cryptophycin-52 may be an important site involved in the regulation of microtubule polymerization dynamics in cells and, thus, an important drug-target site.

By analyzing the binding of [^{3}H]cryptophycin-52 to tubulin by Scatchard analysis, we identified a single high-affinity binding site for the compound with an apparent association constant of 3.6×10^6 M^{-1} (34 °C) (Table 2). A similar high-affinity binding constant was obtained by analyzing the ability of the compound to quench intrinsic tubulin fluorescence (Figure 3). Because the binding of cryptophycin-52 to tubulin may induce self-association of tubulin dimers, the determined affinity contstant should be regarded as an apparent affinity constant.

The binding of cryptophycin-52 to tubulin, like the binding of vinblastine to tubulin, is relatively independent of temperature (*27, 42*). van't Hoff analysis of binding data at three different temperatures indicated that the binding reaction is primarily entropic. A number of additional low-affinity binding sites were also found, but these appear to be nonspecific. We also found that cryptophycin-52 did not

inhibit the binding of colchicine to tubulin, consistent with previous results for cryptophycin-1 (*16, 17*).

Considerable evidence indicates that the cryptophycins may bind to tubulin in the vicinity of the vinca binding site (*16*−*18*). For example, Bai et al. (*18*) reported that cryptophycin-1 inhibits the binding of radiolabeled vinblastine to tubulin in a noncompetitive fashion. Here, with radiolabeled cryptophycin-52, we found that vinblastine inhibited the binding of cryptophycin-52 to tubulin with half-maximal inhibition occurring at 50 μM vinblastine (Figure 7). However, attempts to determine the nature of the inhibition were unsuccessful. The concentration of vinblastine required to inhibit cryptophycin-52 binding to tubulin was high (50 μM). This concentration is well above that required to induce tubulin aggregation (*16, 18*). Thus, it remains possible that the ability of vinblastine to inhibit the binding of cryptophycin-52 to tubulin is due to a vinblastine-induced aggregation of tubulin that obscures the cryptophycin-52 binding site. Other investigators have reported that cryptophycin-1 can inhibit the binding of vinblastine to tubulin noncompetitively (*18*). Because the cryptophycins also can induce tubulin aggregation, conversely, it is possible that the inhibition of vinblastine binding activity by the cryptophycins is also due to an aggregation-induced loss of the vinblastine binding site by cryptophycin-52.

The apparent irreversibility of the effects of cryptophycin-1 on microtubules in cells (*13*) and the presence of a potentially reactive epoxide ring in the cryptophycin-52 structure prompted us to examine the reversibility of cryptophycin-52 binding to tubulin. We prepared [^{3}H]cryptophycin-52−tubulin complex and monitored the loss of radiolabeled drug from the complex under several conditions. The binding was very poorly reversible, and only 36% of the previously bound cryptophycin-52 was lost after 48 h of dialysis in either the absence or presence of excess cryptophycin-52. Because of the instability of tubulin in solution, we could not tell whether the limited amount of loss we detected was due to slow dissociation of the cryptophycin-52 from the native complex or to tubulin denaturation. We found we could release bound [^{3}H]cryptophycin-52 from a cryptophycin-52−tubulin complex by denaturing the tubulin either with 6 M urea or by boiling. These data suggest that the binding of cryptophycin-52 to tubulin is noncovalent and are in agreement with the results obtained with cryptophycin-1 (*19*). Together with the data from Table 2 indicating that the energetics of the binding reaction are primarily entropy-driven, it appears that the high-affinity binding of cryptophycin to tubulin may occur in a hydrophobic pocket.

We also found that binding of cryptophycin-52 to tubulin induces a conformational change in the tubulin. Cryptophycin-52 induced a small but highly reproducible change in the far-UV CD spectrum of tubulin, suggesting that the binding of the drug to tubulin perturbs tubulin secondary structure. In contrast, the binding of vinblastine to tubulin had no detectable effect on the far-UV CD spectrum of tubulin (data not shown; also see ref *27*), indicating that the nature of its binding is different from that of cryptophycin-52. We cannot completely eliminate the possibility that the increase in the CD signal caused by cryptophycin-52 binding is due to a protection mechanism that prevents the time-dependent loss of secondary structure.However, this possibility does not seem very likely because vinblastine, which protects tubulin against time-dependent decay, had no effect on the far-UV CD spectrum of tubulin. Cryptophycin-52 binding also decreased the intrinsic fluorescence of tubulin, consistent with the idea that its binding alters tubulin conformation in the vicinity of a fluorophore such as a tryptophan residue. Cryptophycin-52 binding to tubulin reduces the rate of tubulin decay (Figure 4), a process involving the loss of the ability to bind colchicine and loss of the ability to polymerize into microtubules (*32*−*36*). This is consistent with the idea that the binding of cryptophycin-52 to tubulin maintains the tubulin in a native-like state.

Possible Mechanism of Action of Cryptophycin-52 at Microtubule Ends. The binding of just a few cryptophycin-52 molecules per microtubule strongly stabilizes the microtubules in vitro, indicating that at low concentrations cryptophycin-52 must act at microtubule ends (*11*). Because cryptophycin-52 has such a high affinity for tubulin, it is reasonable to think that when low concentrations of cryptophycin-52 are added to a steady-state microtubule suspension, most of the drug would be complexed with the tubulin in the soluble tubulin pool and little would remain free in solution. If so, then cryptophycin-52 must bind to microtubule ends as a cryptophycin-52−tubulin complex. Furthermore, the conformational change induced in tubulin by the binding of cryptophycin-52 must modify the tubulin so that when it is bound at the end, the normal association and dissociation of tubulin is modified (*11*). One possibility is that the presence of tubulin−cryptophycin-52 complexes at microtubule ends may induce an isodesmic self-association of tubulin dimers at the ends, as proposed for vinblastine (*44*−*47*), thus increasing the affinity between adjacent dimers and stabilizing the ends.

It is interesting that colchicine, which binds to a different site in tubulin than does cryptophycin-52, also acts at microtubule ends as a drug−tubulin complex. Further, the effects of tubulin−colchicine complex on microtubule dynamic instability are remarkably similar to those of cryptophycin-52 (*24*). Thus, a similar modulation of microtubule dynamics can be achieved by drug−tubulin complexes with drugs that bind to distinct regions of tubulin.

It is widely believed that alternation between the gain and loss of a stabilizing GTP or GDP•P_i cap at microtubule ends is responsible for the dynamic instability behavior of microtubules (*37, 38*). It is reasonable to think that cellular regulatory proteins may control gain and loss of the stabilizing cap by modulating GTP hydrolysis or GTP exchange, and it follows that drugs might act by mimicking the regulatory proteins (*9*). In the present study we found that cryptophycin-52 did not inhibit the binding or exchange of GTP with tubulin. We could not measure the effects of cryptophycin-52 on the intrinsic GTPase activity of tubulin because the GTPase activity of free tubulin is very low. However, we could measure the effects of cryptophycin-52 on colchicine-induced tubulin GTPase. We found that cryptophycin-52 strongly inhibited the GTPase activity of colchicine−tubulin complex, either directly or indirectly through an induced conformational change in the tubulin. The high GTPase activity of tubulin−colchicine complex is not due to self-association of tubulin dimers similar to that which occurs during microtubule assembly (see refs *48* and *49*). However, colchicine−tubulin complex may have a native-like conformation similar to the conformation of

tubulin at microtubule ends. It is possible that the mechanism responsible for the increased tubulin GTPase activity that occurs when colchicine binds to tubulin may be similar in certain ways to the mechanism that triggers GTP hydrolysis when tubulin adds to a microtubule end. Thus, the inhibition of colchicine−tubulin complex GTPase activity by cryptophycin-52 suggests that the drug may inhibit the GTPase activity of tubulin at the microtubule ends. Inhibition of GTPase activity at microtubule ends may play a role in the ability of cryptophycin-52 to suppress microtubule dynamics and stabilize microtubule ends, perhaps by retaining the tubulin in the capped (GTP) state.

A potentially important feature of the action of cryptophycin-52 in tumor cells is that the drug exhibits reduced susceptibility to P-glycoprotein-mediated multidrug resistance as compared with many currently used antimitotic anticancer drugs such as vinblastine and taxol. The very poorly reversible binding of cryptophycin-52 to tubulin may contribute significantly to its superior activity against resistant tumor cell lines. Upon addition to tumor cells, a large fraction of the intracellular cryptophycin-52 may remain indefinitely bound to tubulin and thus not be available for drug efflux.

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