Mechanism of Action of the Unusually Potent Microtubule Inhibitor Cryptophycin 1[†]

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ABSTRACT: Cryptophycin 1 is a remarkably potent antiproliferative compound that shows excellent antitumor activity against mammary, colon, and pancreatic adenocarcinomas in mouse xenographs. At picomolar concentrations, cryptophycin 1 blocks cells in the G_2/M phase of the cell cycle by an apparent action on microtubules. The compound binds to tubulin, inhibits microtubule polymerization, and depolymerizes preformed microtubules *in vitro*. Its exceptionally powerful antitumor activity (many-fold greater than paclitaxel or the vinca alkaloids) raises important questions about its mechanism of action. By quantitative video microscopy, we examined the effects of cryptophycin 1 on the dynamics of individual microtubules assembled to steady state from bovine brain tubulin. At low nanomolar concentrations, in the absence of net microtubule depolymerization, cryptophycin 1 potently stabilized microtubule dynamics. It reduced the rate and extent of microtubule shortening and growing and increased the frequency of rescue. The results suggest that cryptophycin 1 exerts its antiproliferative and antimitotic activity by binding reversibly and with high affinity to the ends of microtubules, perhaps in the form of a tubulin−cryptophycin 1 complex, resulting in the most potent suppression of microtubule dynamics yet described.

Cryptophycin 1 (Figure 1) is a remarkably potent antiproliferative compound from the cyanobacterium *Nostoc* sp. that is active against human solid tumors in murine xenographs (*1*−*3*).[1] Cryptophycin 1 inhibits cell growth and, in parallel, induces mitotic accumulation at very low (picomolar) concentrations by an apparent action on microtubules (*1*−*4*). Unlike many other antimitotic drugs, the effects of cryptophycin 1 on cells are poorly reversible and the drug is a poor substrate for the multidrug resistance transport protein (*1*). Cryptophycin 1 binds tightly to tubulin (*5*); it inhibits microtubule assembly and inhibits nucleotide exchange on tubulin. It competitively inhibits dolastatin 10 binding to tubulin, and noncompetitively inhibits vinblastine binding to tubulin (*2*, *4*, *5*). In addition, the compound induces the formation of small ring-shaped oligomers of tubulin that resemble the tubulin aggregates induced by vinblastine (*2*, *5*). The antitumor activity and exceptional antiproliferative potency of cryptophycin 1 (100−1000-fold greater potency than paclitaxel and vinblastine) has raised important questions about its mechanism of action. Our previous work has indicated that the most potent antiproliferative and antimitotic actions of taxol and vinblastine are due to the suppressive effects of these drugs on the dynamics of growing and shortening events at microtubule ends (*6*−*13*). Thus, we sought to examine whether the exceptional potency of cryptophycin 1 might result from a potent effect on microtubule dynamics.

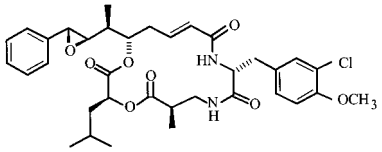

FIGURE 1: Structural formula of cryptophycin 1.

Microtubules are tube-shaped polymers composed of $\alpha\beta$ tubulin heterodimers arranged parallel to a cylindrical axis. They are involved in a variety of cell processes including intracellular transport, structure and shape determination, and mitosis (*14*, *15*). Microtubules are intrinsically dynamic polymers. Both *in vitro* and in cells, microtubule ends alternate stochastically between growing and shortening states, which is thought to be due to a gain and loss at the microtubule ends of a stabilizing GTP or GDP·P_i cap (*16*−*18*). Recent evidence indicates that the dynamics of microtubules are critically involved in microtubule function. For example, microtubule dynamics are essential for chromosome movement during mitosis, which involves dynamic interactions between the kinetochores of the chromosomes and the spindle microtubules (*19*−*22*).

Microtubules are the principal targets of antimitotic compounds. For example, vinblastine and colchicine are well-known potent inhibitors of microtubule polymerization that inhibit cell proliferation at metaphase of mitosis (*6*, *7*, *23*). Paclitaxel, which increases microtubule polymerization, similarly inhibits mitosis at metaphase (*9*). While all three drugs produce profound effects on the microtubule polymer mass at relatively high drug concentrations, they inhibit proliferation of HeLa cells at mitosis at relatively low concentrations without appreciably changing the microtubule

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[1] Abbreviations: PMME buffer, 87 mM Pipes, 36 mM Mes, 1.8 mM $MgCl_2$, 1 mM EGTA, pH 6.8; PEM buffer, 100 mM Pipes, 1 mM MgSO4, 1 mM EGTA, pH 6.9; DTT, dithiothreitol; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-morpholineethanesulfonic acid.

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polymer mass or disrupting spindle microtubule organization. In cells, paclitaxel and vinblastine potently suppress microtubule dynamics at drug concentrations that perturb or block mitosis (*6*, *7*, *9*, *13*, *24*; A.-M. Yvon, P. Wadsworth, and M. A. Jordan, unpublished data). These results have indicated that the most potent mechanism by which these antimitotic drugs act is by suppression of spindle microtubule dynamics rather than by depolymerization or excessive polymerization of spindle microtubules.

In the present study, we determined the effects of cryptophycin 1 on microtubule dynamics of individual bovine brain microtubules at steady state by video microscopy. In addition, we examined the nature of the binding of cryptophycin 1 to tubulin to determine whether the poor reversibility of its effects in cells might be due to covalent binding of cryptophycin 1 to tubulin.

EXPERIMENTAL PROCEDURES

Purification of Tubulin. Bovine brain microtubule protein was isolated without glycerol by three cycles of polymerization and depolymerization. Tubulin was purified from the microtubule protein by phosphocellulose chromatography as previously described (*10*). The tubulin solution was quickly frozen as drops in liquid nitrogen and stored at −70 °C until used. Protein concentration was determined by the method of Bradford (*25*) using bovine serum albumin as the standard.

Video Microscopy. Tubulin pellets were thawed and centrifuged at 4 °C to remove any aggregated or denatured tubulin. To polymerize microtubules, tubulin (12.5 μM) was mixed with *Strongylocentrotus purpuratus* flagellar seeds (*10*) in 86 mM Pipes, 36 mM Mes, 1.8 mM $MgCl_2$, 1 mM EGTA, pH 6.8 (PMME buffer), containing 1.5 mM GTP and incubated at 37 °C in the absence or presence of different concentrations of cryptophycin 1 for 30 min. The seed concentration was adjusted to achieve 3−6 seeds per microscope field. After 30 min of incubation, samples of microtubule suspensions (3.5 μL) were prepared for video microscopy and the dynamics of individual microtubules were recorded at 37 °C as previously described (*10*). The microtubules were observed for a maximum of 45 min after they had reached steady state. Under the experimental conditions used, microtubule growth occurred predominantly at the plus ends of the seeds as determined by the growth rates, the number of microtubules that grew, and the relative lengths of the microtubules at the opposite ends of the seeds (*10*, *26*, *27*). Microtubule length changes were analyzed as described previously (*27*). We considered the microtubule to be in a growing phase if the microtubule increased in length by >0.2 μm at a rate >0.15 μm/min and in a shortening phase if the microtubule shortened by >0.2 μm at a rate >0.3 μm/min. Length changes equal to or less than 0.2 μm over the duration of six time points were considered as attenuation (or pause) phases. Between 18 and 34, microtubules (mean of 25) were analyzed for each experimental condition.

The "catastrophe frequency" [a catastrophe is a transition from the growing or attenuated state to shortening (*26*)] was calculated by dividing the number of catastrophes by the sum of the total time spent in the growing plus attenuated states for all microtubules for a particular condition. The "rescue frequency" [a rescue is a transition from shortening to growing or attenuation, excluding new growth from a seed (*26*)] was calculated by dividing the total number of rescue events by the total time spent shortening for all microtubules for a particular condition. The catastrophe and rescue frequencies were also calculated on the basis of number of transitions per length grown or shortened (*28*, *29*). Dynamicity was calculated as the rate of total detectable tubulin dimer exchange (both gain and loss) at a microtubule end (*10*).

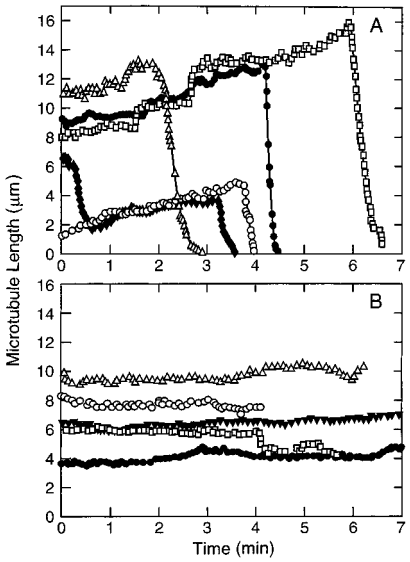

FIGURE 2: Life history traces of microtubules at steady state (A) In the absence and (B) in the presence of 100 nM cryptophycin 1. Individual microtubules alternate among phases of growth, shortening, and attenuation. For example, the microtubule indicated by open squares in panel A grew from 0 to 1.8 min, remained in an attenuated state between 1.8 and 2.3 min, grew between 2.3 and 3.4 min, remained in an attenuated state from 3.4 to 4.4 min, grew between 4.4 and 5.9 min, and shortened from 5.9 to 6.6 min. The lengths of individual microtubules were measured from real time video tape recordings as described in Experimental Procedures.

Binding of Cryptophycin 1 to Tubulin. The effect of prolonged incubation of cryptophycin 1 with tubulin on the reversibility of cryptophycin 1 binding was examined by incubating tubulin (10 μM) and cryptophycin 1 (10 μM) in PEM buffer (37 °C, 2−20 min) followed by ethanol precipitation of tubulin and any tubulin−cryptophycin 1 complex. Noncovalently bound cryptophycin 1 released into the supernatant from experimental samples and from a nontubulin control was determined by HPLC after centrifugation (Sorvall, SS34 rotor, 20 000 rpm, 1 h, 4 °C).

Materials. Cryptophycin 1 used in this study was synthesized or purified at the University of Hawaii (*1*, *3*, *30*); initially it was provided as a kind gift from Dr. Charles Smith, Fox Chase Cancer center, Philadelphia, PA.

RESULTS

Effects of Cryptophycin 1 on Microtubule Dynamics at Steady State. Several life history traces showing typical length changes at the plus ends of microtubules in the absence of cryptophycin 1 are shown in Figure 2A. As previously documented, the microtubules alternated between phases of growing and shortening and also spent a small fraction of time in an attenuated or pause state, neither growing nor shortening detectably. Addition of cryptophycin 1 suppressed microtubule dynamics. As shown in Figure 2B, 100 nM cryptophycin 1 markedly reduced the rates and extents of shortening and growing events and dramatically increased the duration of phases of attenuation or pause.

Table 1: Effects of Cryptophycin 1 on the Dynamics of Individual Microtubules

	concentration (nM)				
	0	5	20	50	100
rate (μm/min)					
growing	0.80 ± 0.11[a]	0.84 ± 0.12	0.57 ± 0.08	0.45 ± 0.05	0.42 ± 0.04
shortening	14.6 ± 1.6	12.0 ± 1.2	8.4 ± 1.4	6.1 ± 1.6	3.6 ± 1.3
% time in phase					
growing	73.8	63.9	46	51.9	48.4
shortening	11.9	12.7	12	8.9	11.1
attenuation	14.3	23.4	42	39.2	40.5
number of microtubules	34	24	29	20	18

[a] ± are SEM.

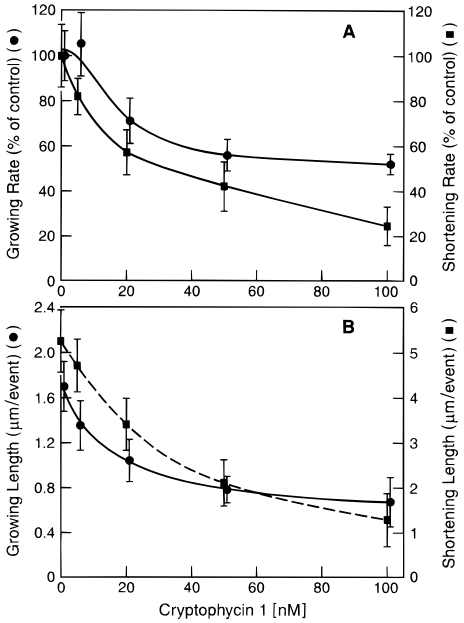

FIGURE 3: Effect of cryptophycin 1 on (A) rates and (B) lengths of microtubule growing (circles) and shortening (squares) per growing or shortening event at plus ends at steady state. The rates of individual microtubule growing and shortening events were measured from real time video tape recordings as described in Experimental Procedures. The mean growing length per growing excursion was determined by dividing the summed growing lengths for all microtubules for a particular condition by the total number of growing events measured for that condition. The mean shortening length per shortening excursion was determined similarly. Error bars = SEM.

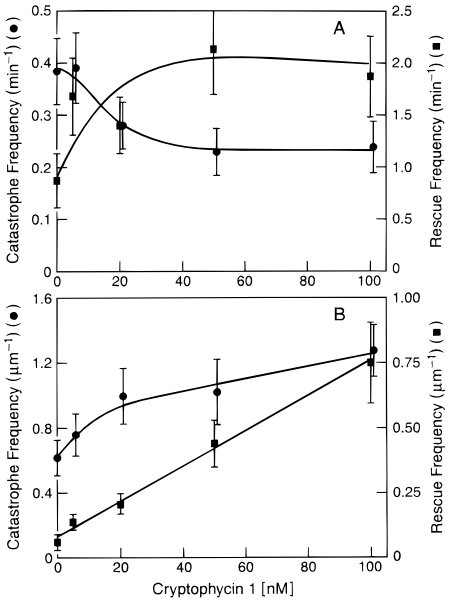

FIGURE 4: Effects of cryptophycin 1 on the frequency of rescue (squares) or catastrophe (circles). (A) Catastrophe and rescue frequencies calculated on the basis of time growing or shortening. (B) Catastrophe and rescue frequencies calculated on the basis of excursion length. See Experimental Procedures.

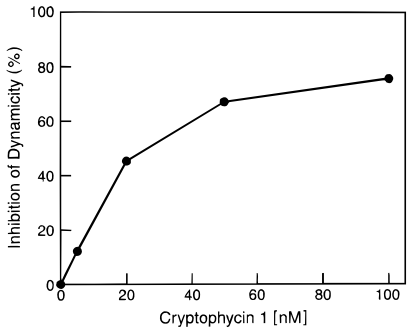

FIGURE 5: Suppression of dynamicity by cryptophycin 1. Dynamicity, a measure of overall dynamics, was calculated from the summed lengths of all detectable growing and shortening events divided by the total time including time microtubules spent in the attenuated or pause state (Experimental Procedures). Control microtubules had a dynamicity of 2.33 μm/min.

The actions of cryptophycin 1 on microtubule dynamics at steady state were determined quantitatively (Table 1, Figures 3−5). Cryptophycin 1 potently suppressed the rate of shortening in a concentration-dependent manner. Addition of only 20 nM cryptophycin 1 reduced the shortening rate by 44%, and at the highest concentration studied (100 nM), cryptophycin 1 reduced the shortening rate by 75%, from 14.6 ± 1.6 μm/min to 3.6 ± 1.3 μm/min (Figure 3A, squares). The length microtubules shortened per shortening event (Figure 3B, squares) was also potently decreased by cryptophycin 1 in a concentration-dependent manner at all concentrations ≥5 nM. For example, the mean shortening length was reduced 76% at 100 nM cryptophycin 1 from 5.2 μm per shortening event to 1.2 μm per shortening event. Thus, microtubules shortened more slowly and to lesser extents in the presence of cryptophycin 1 than in its absence.

Cryptophycin 1 suppressed the shortening rate more strongly than the growing rate (Table 1, Figure 3A). For example, the growing rate was reduced by only 48% at the highest concentration of cryptophycin 1 examined (100 nM, from 0.80 to 0.42 μm/min) while the shortening rate was reduced by 75% at this concentration. The mean length grown during a growing event was also diminished by cryptophycin 1 (Figure 3B). Interestingly, even the highest concentration of cryptophycin (100 nM) examined by video microscopy reduced the polymer mass insignificantly, by only 4 ± 9%. Thus, inhibition of net microtubule growth was balanced by equivalent inhibition of net microtubule

shortening at cryptophycin concentrations ≤100 nM. The kinetics of tubulin addition and loss at microtubule plus ends were markedly more sensitive to cryptophycin 1 than bulk phase polymerization.

The frequencies of transition among the growing, shortening, and pause states (the catastrophe and rescue frequencies, see Experimental Procedures) are considered to be important in the regulation of microtubule dynamics and function in cells (*31*−*34*). We calculated the transition frequencies on the basis of the time spent growing or shortening (Figure 4A) as well as on the basis of microtubule length grown or shortened (Figure 4B) (*28*, *29*). Cryptophycin 1 had a marked effect on rescue frequencies; it doubled the frequency of rescue measured on the basis of time (Figure 4A, squares) and vastly increased the rescue frequency per length of microtubule length lost in a shortening event (Figure 4B, squares). For example, 100 nM cryptophycin 1 increased the number of rescues per micrometer 13-fold (from 0.06 rescues per μm shortened to 0.75 rescues per μm shortened). The catastrophe frequency calculated on the basis of time was reduced by cryptophycin 1, but the compound induced more catastrophes per micrometer of growth (Figure 4, circles). The net effect was a marked damping of excursion length, which is clearly visible in the life history traces of individual microtubules (Figure 2B). Thus, the dramatic increase in the number of rescues per time and length shortened coupled with a modest reduction in the time-based catastrophe frequency resulted in extensive kinetic stabilization of microtubule dynamics.

Dynamicity is an important parameter that reflects the overall dynamics of the microtubules [see Experimental Procedures (*10*, *13*)]. As shown in Figure 5, dynamicity was strongly suppressed by cryptophycin 1. At 25 nM cryptophycin 1, dynamicity was inhibited by 50%, and 50−100 nM drug inhibited dynamicity by 70−80%.

Does Cryptophycin 1 React Covalently with Tubulin? The presence of a reactive epoxide ring in cryptophycin 1, the potent effects of the compound on microtubule dynamics *in vitro*, and the apparent irreversibility of its effects in cells (*1*) suggested the possibility that cryptophycin 1 might form a covalent bond with tubulin by reacting with a nucleophilic side chain in tubulin. This idea was tested by two experimental strategies. In the first strategy, 10 μM tubulin was incubated with an equimolar concentration of cryptophycin 1 (10 μM) (37 °C, 20 min) and the protein was then precipitated with ethanol. This procedure would deplete the supernatant of any cryptophycin 1 bound covalently to the tubulin. The cryptophycin 1 content of the supernatant as determined by HPLC (see Experimental Procedures) was not reduced by incubation with tubulin, thus suggesting that a covalent tubulin−cryptophycin 1 complex was not formed (data not shown). This experiment would not have detected the reaction of a small percentage of the cryptophycin 1; however, such a reaction would be unusual in that it would imply either a very slow reaction or that a small population of tubulin molecules binds cryptophycin 1 differently.

In the second strategy, we reasoned that if a nucleophile in tubulin were to react with the epoxide ring, a tubulin sulfhydryl group would be a strong candidate for such a reactive group. Thus, the effect of dithiothreitol (DTT) on inhibition of microtubule assembly by cryptophycin 1 was examined (Table 2). Tubulin was assembled in the presence or absence of DTT and in the presence or absence (control) of cryptophycin 1. We found that the mass of microtubule polymer assembled was the same in the presence or absence of DTT, indicating that the epoxide ring is not reactive to tubulin sulfhydryl groups at the pH value of the assembly reaction. This was confirmed by preincubating DTT with cryptophycin 1 before initiating microtubule assembly; again, we found no effect of DTT on the amount of microtubule polymer formed. Together these results suggest that the mechanism of action of cryptophycin 1 may not involve a covalent binding of the compound to the protein.

Table 2: Effect of Dithiothreitol on Assembly Inhibition by Cryptophycin 1[a]

	inhibition of assembly (%)	
cryptophycin 1 (μM)	no dithiothreitol	2 mM dithiothreitol
no preincubation		
0.5	33 ± 8	35 ± 8
1.0	55 ± 3	55 ± 7
2.0	66	68
with preincubation		
0.5	13	10
1.0	28 ± 7	28 ± 8
2.0	58 ± 9	46 ± 14
4.0	81	81

[a] Tubulin (15 μM) was incubated with cryptophycin 1 (0−4 μM) and DTT (0 or 2 mM) in PEM buffer, 8% DMSO, and 0.5 mM GTP (30 min, 37 °C); results are from seven separate experiments. The concentration of unassembled tubulin was measured after centrifugation (100000*g*, 20 min) or by centrifugation through Co-Star Spin-X filters; in controls lacking cryptophycin 8−10 μM tubulin was assembled. In some experiments, cryptophycin 1 and dithiothreitol were first incubated together for 1 h (25 °C) before addition to tubulin and initiation of polymerization.

DISCUSSION

We found that cryptophycin 1 is a remarkably potent stabilizer of microtubule dynamics. As previously reported (*2*, *4*, *5*), cryptophycin 1 inhibited microtubule polymerization, but only at concentrations ≥0.5 μM (Table 2). However, cryptophycin 1 strongly stabilized dynamics at concentrations well below 0.5 μM. At concentrations as low as 5 nM, the compound inhibited the shortening rate and length of microtubule depolymerized during a shortening event and enhanced the number of rescues per time or length shortened. Cryptophycin 1 also inhibited the growing rate and length grown, but less strongly than the shortening rate. Thus, the overall effect of cryptophycin was to strongly stabilize the microtubules by slowing microtubule dynamics and by reducing the lengths of shortening and growing excursions in the absence of any reduction in the mass of assembled microtubules.

It is clear that the powerful stabilizing effects of cryptophycin 1 on microtubule dynamics are substoichiometric with respect to soluble tubulin. The total tubulin concentration used was 12.5 μM. Determinations of the critical concentration for the bovine brain tubulin preparation used in the study indicate that approximately 10 μM tubulin was in solution and 2.5 μM tubulin was in polymer (*35*). Thus, at 25 nM cryptophycin 1 (the concentration which produced 50% inhibition of dynamicity), the ratio of the number of cryptophycin molecules to the number of tubulin dimers in solution was 1:400. Cryptophycin 1 binds tightly to soluble tubulin (*5*). With such low ratios of cryptophycin 1 to tubulin, it is reasonable to conclude that most of the cryptophycin 1

was complexed to tubulin. It is also likely that cryptophycin 1 acts in cells as a cryptophycin 1−tubulin complex.

It is clear that the powerful stabilizing effects of cryptophycin 1 on microtubule dynamics must be brought about by the binding of a very low number of cryptophycin 1 molecules per microtubule. The average length of the microtubules was 7.0 μm, and thus, the microtubule number concentration was ~210 pM. The ratio of the number of cryptophycin 1 molecules to the number of microtubules under the conditions used was relatively low, ~120:1. In order to have such a potent effect on microtubule shortening and on the transition frequencies, cryptophycin 1 or tubulin−cryptophycin 1 complexes must bind preferentially at or near the ends of the microtubules. The data indicate that a few molecules of cryptophycin 1 bound to the end of a microtubule powerfully interrupt the shortening process, transiently stabilizing the microtubule end and inducing regrowth or an attenuated (pause) state.

Microtubules are thought to be composed of an unstable tubulin−GDP core and a stable tubulin GTP (or tubulin−GDP·P_i) cap at the microtubule ends (*17*, *18*, *36*). Microtubule dynamic instability is believed to arise from differences in tubulin exchange with microtubules that have tubulin−GDP rather than tubulin−GTP at their ends (*16*, *17*, *36*, *37*). Microtubules continue to elongate as long as they maintain a cap. Loss of the cap by dissociation of tubulin−GTP or tubulin−GDP·P_i exposes the labile tubulin−GDP core and the microtubule loses subunits rapidly (catastrophe). Regain of the cap allows rescue of the depolymerizing microtubule by preventing further subunit loss and promoting regrowth. Cryptophycin 1 increased the frequency of rescue and decreased the length of microtubule lost prior to rescue; thus, it strongly facilitates the regaining of the stabilizing cap (Figure 4, squares). The compound has a mixed effect on the frequency of catastrophe, inhibiting the time-based loss of the cap but increasing the frequency of catastrophe and cap loss calculated on the basis of length grown (Figure 4, circles). These results are consistent with the relatively stable polymer mass and constant mean microtubule length that were observed at low cryptophycin 1 concentrations. Net microtubule depolymerization occurred at relatively high cryptophycin 1 concentrations because the compound produced an increased rate of cap loss coupled with a significant decrease in growth rate.

To reduce the shortening length and the shortening rate, cryptophycin 1 or a tubulin−cryptophycin 1 complex must bind and stabilize the end long enough so that addition of tubulin−GTP molecules can occur to adjacent protofilament sites, thus recapping the microtubule end transiently and slowing the overall shortening rate or promoting rescue and regrowth. Since cryptophycin appears not to copolymerize with tubulin into the microtubule (see below), cryptophycin 1 or a tubulin−cryptophycin 1 complex must also dissociate from the end in order that extensive growth and lengthening of the end can occur.

Cryptophycin 1 inhibited the rate of growth, but its effect on growth was significantly less potent than its effect on shortening. One possibility is that cryptophycin 1 or a cryptophycin 1−tubulin complex may bind transiently and with high affinity to growing microtubule ends in a manner similar to the way it binds to depolymerizing microtubule ends. However, the effect on the growing rate may be less than that on the shortening rate since growth is an inherently slower process than shortening; tubulin dimer addition events occur only 5% as fast as tubulin dimer loss events (control growing rates averaged 0.80 ± 0.11 μm/min whereas shortening rates averaged 14.6 ± 1.6 μm/min). In other words, the binding of cryptophycin 1 to a growing microtubule end may stop growth transiently by sterically hindering further tubulin addition over the bound cryptophycin 1, but the transient pause in growth is only a minor perturbation that slows the overall rate of growth and may induce pause. Once cryptophycin 1 has dissociated from the end of the microtubule, growth can resume.

It is unlikely that cryptophycin 1 or cryptophycin 1−tubulin complexes copolymerize with tubulin in microtubules in a manner similar to colchicine−tubulin complexes since copolymerization would significantly deplete the availability of cryptophycin 1 for poisoning of dynamics at microtubule ends. For example, at 25 nM cryptophycin 1, the cryptophycin 1/tubulin ratio was ~1:400. Since cryptophycin was present from the time of initiation, if copolymerization occurred, the concentration of free tubulin−cryptophycin 1 complex would be reduced, perhaps significantly, by the time of attainment of the steady state equilibrium. It is more likely that cryptophycin 1 acts, as does vinblastine, by binding to microtubule ends without undergoing copolymerization with tubulin (*38*).

Of the major drugs that suppress microtubule dynamics (vinblastine, colchicine, and paclitaxel), cryptophycin 1 most closely resembles vinblastine in its mode of action, but is more potent than vinblastine (*9*−*11*, *27*, *39*). Like vinblastine, cryptophycin 1 or tubulin−cryptophycin 1 complex appears to bind to microtubule ends with high affinity and to stabilize microtubule dynamics. Also, like vinblastine, and in contrast to colchicine, cryptophycin 1 apparently does not copolymerize with tubulin in microtubules. Similar to vinblastine and colchicine, and in contrast to paclitaxel, the binding of a few molecules of drug to the microtubule end may be responsible for potently suppressing shortening and promoting rescue. Cryptophycin 1 at a concentration of 25 nM inhibited the rate of shortening by 50% whereas comparable inhibition of shortening required 100 nM vinblastine (*27*). Cryptophycin 1 at a concentration of 100 nM increased the frequency of rescue per micrometer shortened by 13-fold whereas a comparable increase in rescue frequency required 600 nM vinblastine. Also, in contrast to vinblastine, cryptophycin 1 had a significantly stronger suppressive effect on shortening rates than on growing rates. The poor reversibility of binding of cryptophycin 1 to tubulin (*5*) contrasts with the ready reversibility of binding of vinblastine to tubulin (*23*, *40*), suggesting that cryptophycin 1 acts primarily as a complex with tubulin. Thus, cryptophycin 1 is more potent than vinblastine, and its mechanism may be similar but not identical to that of vinblastine.

Previous work indicates that cryptophycin 1 induces formation of small oligomers of tubulin, in a manner somewhat resembling that of vinblastine (*2*, *5*). Unlike vinblastine, cryptophycin 1 is a potent inhibitor of GTP binding to tubulin and the inhibition increases significantly with time, suggesting that cryptophycin may induce a slow conformational change in tubulin (*2*). Alternatively, inhibition of GTP binding may be due to the formation of oligomers or aggregates.

The irreversibility of mitotic block by cryptophycin may be an extremely important difference between cryptophycin

1 and antimitotic drugs currently in therapeutic use. If, as seems plausible from the results of Kerksiek et al. (*5*) and Bai et al. (*2*) and the results reported here, cryptophycin 1 forms a small complex with tubulin that acts at the microtubule ends and does not copolymerize with tubulin in microtubules, it may have a significant therapeutic advantage over vinblastine in the duration of intracellular retention [the binding of vinblastine to tubulin is reversible and the drug is readily pumped out of cells (*40*, M. A. Jordan, E. Tsuchiya, and L. Wilson, unpublished results)]. In addition, because cryptophycin 1 can powerfully suppress dynamics without affecting the mass of microtubule polymer, the compound may have a therapeutic advantage over paclitaxel by maintaining a normal intracellular pool of unpolymerized tubulin, thereby avoiding signaling the protein synthetic machinery of the cell to express additional tubulin (*41*) which ultimately may allow escape from paclitaxel induced mitotic block at some paclitaxel concentrations (*12*).

Implications for Cell Function. Microtubule dynamics are extremely important in mitosis, and cells display a sophisticated array of molecules and mechanisms for the regulation of their dynamics (*15*, *42*−*44*). Our results indicate that the potent anti-mitotic compound cryptophycin 1, like vinblastine, colchicine, and paclitaxel, stabilizes microtubule dynamics at low concentrations without significantly altering the microtubule polymer mass (*10*, *11*, *39*). These findings support the hypothesis that the most sensitive mechanism underlying the ability of antimitotic compounds to inhibit cell proliferation and to kill tumor cells may be the kinetic stabilization of spindle microtubule dynamics (*44*). Cryptophycin 1 is the most potent stabilizer of microtubule dynamics yet described. The results suggest that, by means of a unique high affinity binding of a cryptophycin 1−tubulin complex to the end of a microtubule, the compound potently inhibits microtubule dynamics, suppressing shortening events and enhancing rescue, thus preventing spindle maturation and the signal for the metaphase/anaphase transition (*7*, *24*, *45*). Cryptophycin 1 may mimic the actions of a potent natural regulator of microtubule dynamics, and it may offer significant therapeutic advantages over existing antimitotic drugs.

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