Interaction of cryptophycin 1 with tubulin and microtubules

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Abstract **The cryptophycins are newly discovered antimitotic agents isolated from the cyanobacterium *Nostoc*. Previous studies using cultured cells demonstrated that microtubules are the target of these compounds. We have studied the interaction of cryptophycin 1 with tubulin and microtubules in vitro. Cryptophycin 1 is an effective inhibitor of tubulin polymerization, causes tubulin to aggregate, and depolymerizes microtubules to linear polymers somewhat similar to the spiral-like structures produced by the *Vinca* alkaloids. Cryptophycin 1 also inhibits vinblastine binding to tubulin but not colchicine binding. Thus, it appears that the cryptophycins may bind to the *Vinca* site in tubulin or to a site that overlaps with the *Vinca* site.**

Key words: Cryptophycin; Vinblastine; Tubulin; Antimitotic drug

1. Introduction

The cryptophycins are natural products isolated from cyanobacteria and have been found to be highly cytotoxic [1], having strong antifungal [2–5] and antitumor [6] activities. The structure of cryptophycin 1, the major cryptophycin extracted from *Nostoc* sp. GSV224 [6] is shown in Fig. 1. This compound has antitumor activity against six tumors in vivo [6] and is much more effective against multidrug resistant strains of cell lines than vinblastine (VLB), colchicine, and paclitaxel [1].

Antimitotic drugs which act on tubulin and microtubules can be classified according to their site of interaction; the colchicine, *Vinca* alkaloid, or paclitaxel site. Drugs in the colchicine and *Vinca* classes inhibit tubulin polymerization and destabilize microtubules. Those in the paclitaxel class promote tubulin assembly and hyperstabilize microtubules. Cryptophycin 1 causes the depletion of microtubules in A-10 vascular smooth muscle cells [1], suggesting that its target molecule is tubulin and that it binds to either the colchicine or *Vinca* sites, although data on its interaction with tubulin have not been published. This report describes the effect of cryptophycin 1 on tubulin assembly and microtubules in vitro and indicates that this new antimitotic agent may bind to the *Vinca* site on tubulin.

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Abbreviations: PEM buffer, 0.1 M piperazinediethanesulfonate; 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 mM $MgSO_4$, pH 6.9; DMSO, dimethylsulfoxide; VLB, vinblastine.

2. Materials and methods

2.1. Materials

Microtubule protein was isolated from bovine brain by two cycles of assembly and disassembly [7]. Tubulin was purified from microtubule protein by phosphocellulose chromatography [8]. [^{3}H]VLB sulfate was purchased from Moravek Biochemicals. VLB was obtained from Sigma Chemical Co. Cryptophycin 1 was a gift from Merck, Sharp & Dohme Research Laboratories.

2.2. Polymerization assays

Two assays were used to measure the effectiveness of cryptophycin 1. Tubulin at 1.5 mg/ml (15 μM) was incubated at 37°C in PEM buffer containing 0.5 mM GTP, 8% DMSO and varying concentrations of cryptophycin 1. The polymerization reaction was followed by measuring the increase in apparent absorbance at 350 nm as a function of time or by sedimenting polymer after a 15 min incubation and measuring the concentration of unpolymerized protein in the supernatant.

2.3. Vinblastine and colchicine binding

Binding of [^{3}H]VLB to tubulin was measured by column centrifugation as described previously [9]. Solutions of 5 μM tubulin and 5 μM [^{3}H]VLB (3.5×10^4 DPM/nmol), with or without cryptophycin 1, were incubated in PEM buffer on ice for 15 min and 200 μl were centrifuged through a 1 ml column of Sephadex G-25 prepared in PEM buffer. The eluate was analyzed for protein by the Bradford method [10] and for radioactivity by scintillation counting.

Colchicine binding was measured using a fluorescence assay. Colchicine itself has little fluorescence when irradiated with 350 nm radiation but upon binding to tubulin the antimitotic drug has strong fluorescence, with an emission maximum at 432 nm [11]. Tubulin (3 μM) and colchicine (6 μM) were incubated for 1 h at 37°C in the absence and presence of 30 μM cryptophycin 1. Podophyllotoxin, a drug known to bind to the colchicine site, was used as a positive control. Relative fluorescence was measured in a Perkin Elmer MPF-44B spectrofluorometer at 432 nm using 350 nm as the excitation wavelength. Measurements were done against a blank of tubulin alone.

2.4. Electron microscopy

Microtubule protein was polymerized to steady state and 40 μM VLB or 40 μM cryptophycin 1 was added. After varying time periods samples were removed and diluted 5-fold into 1% glutaraldehyde in PEM buffer. After several hours in glutaraldehyde samples were added to formvar- and carbon-coated grids, stained with a 2% uranyl acetate solution, and viewed in a JEOL JEM-1200 EXII electron microscope.

3. Results and discussion

3.1. Interaction of cryptophycin 1 with tubulin and microtubules

Cryptophycin 1 is an effective inhibitor of tubulin self-assembly (Fig. 2). At a tubulin concentration of 15 μM, the concentration which caused 50% inhibition was 1 μM. This ID_{50} value is similar to that determined for VLB and colchicine (data not shown) in this assay. To determine whether the interaction of cryptophycin 1 with tubulin is freely reversible, tubulin was incubated with cryptophycin 1 for 15 min and passed through

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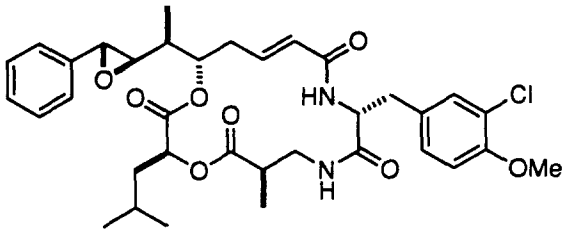

Fig. 1. Structure of cryptophycin 1.

Sephadex G-25 by column centrifugation after which the protein was tested for its ability to assemble. It was found that tubulin assembly was still inhibited, indicating that the rate of dissociation of the tubulin-cryptophycin 1 complex is slow.

Size exclusion-HPLC was used to determine whether cryptophycin 1 causes aggregation of tubulin. Chromatography of tubulin in PEM buffer containing cryptophycin 1 leads to the formation of a species with a molecular mass greater than that of the tubulin dimer (Fig. 3). The elution pattern for tubulin showed a major peak for the dimer, with an elution time of 16 min, and a smaller aggregate peak eluting at the void volume. After incubation with cryptophycin 1 for 20 min at room temperature, the profile showed the major peak eluting with the void volume, with a small shoulder at the elution time for the tubulin dimer. This result is very similar to that observed with *Vinca* alkaloids [9].

When *Vinca* alkaloids are added to microtubules formed from microtubule protein (tubulin containing microtubule-associated proteins), individual protofilaments splay from the ends of the microtubules and form spiral structures [12,13]. A similar phenomenon occurs with cryptophycin 1. Cryptophycin 1 (40 μM) was added to microtubules at steady state and incubated at 37°C. Electron microscopic examination showed the formation of aggregates, some attached to the ends of microtubules (Fig. 4A). The aggregates show some indication of a loose

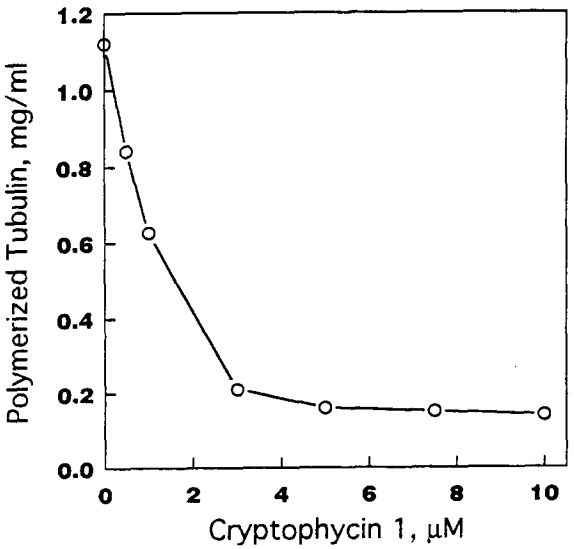

Fig. 2. Inhibition of tubulin assembly by cryptophycin 1. The amount of tubulin polymerized in 15 min is plotted against cryptophycin 1 concentration. Details are given in section 2.

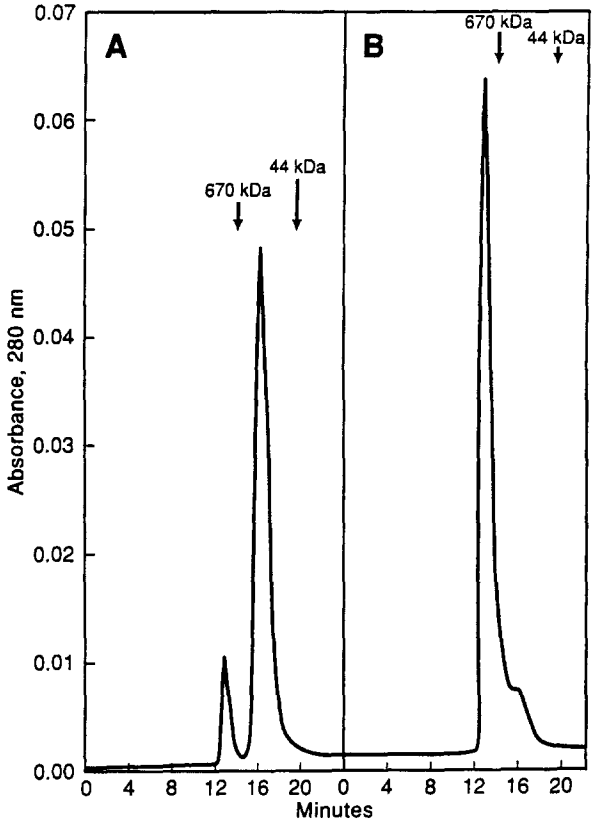

Fig. 3. Size-exclusion HPLC of the tubulin-cryptophycin 1 complex. After incubation of 5 μM tubulin with or without 5 μM cryptophycin 1 in PEM buffer for 20 min at room temperature, the sample was chromatographed on a 600 × 7.8 mm Phenomenex Biospec-sec S-2000 column using a flow rate of 0.8 ml/min. Molecular weight markers were used to calibrate the column; 670 kDa = thyroglobulin, 44 kDa = ovalbumin. (A) Tubulin alone. (B) Tubulin plus cryptophycin 1.

helical substructure that resembles the spirals produced by the *Vinca* alkaloids, but appear to be more irregular than the latter (Fig. 4B). Further detailed structural analysis is required before it is possible to say whether these structures are identical to the spiraled protofilaments formed after *Vinca* drug treatment.

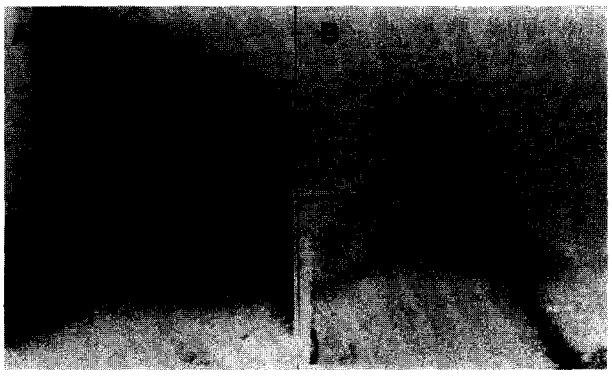

Fig. 4. Effect of cryptophycin 1 and VLB on microtubules. Microtubule protein (2.5 mg/ml) was polymerized in PEM buffer containing 0.5 mM GTP. Cryptophycin 1 (40 μM) or VLB (40 μM) were added and incubation was continued at 37°C for 20 min. Samples were then removed for negative staining. (A) Cryptophycin 1. (B) VLB.

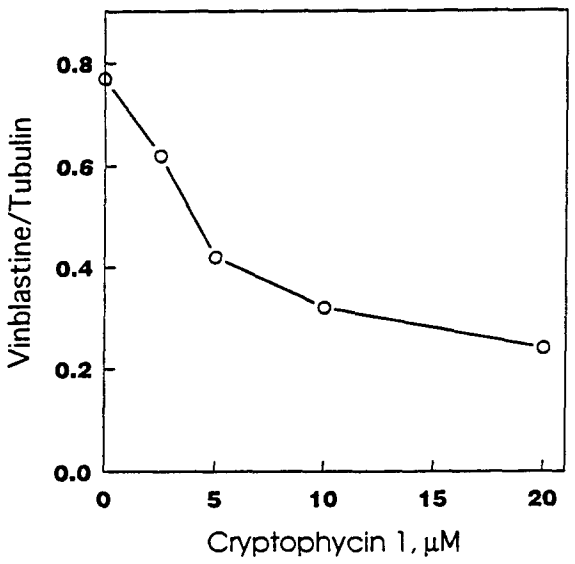

Fig. 5. Inhibition of VLB binding by cryptophycin 1. [^{3}H]VLB was incubated with tubulin in the presence of varying concentrations of cryptophycin 1 before centrifugation through a column of Sephadex G-25. Details are given in section 2.

3.2. *Effect on VLB and colchicine binding*

VLB binding was measured by separating free and bound drug by column centrifugation [9]. Fig. 5 shows the inhibition of [^{3}H]VLB binding by cryptophycin 1. Clearly, cryptophycin 1 is an effective inhibitor of VLB binding, with 6 μM causing a 50% reduction in binding at a total VLB concentration of 5 μM. Whether this inhibition is due to an interaction at the *Vinca* site or is due to the aggregating properties of cryptophycin 1 is not clear at this time.

Colchicine binding was measured by taking advantage of the fluorescence which develops when the drug binds to tubulin. Upon incubation of 3 μM tubulin with 6 μM colchicine a time-dependent increase in relative fluorescence occurred at 432 nm. The presence of a 5-fold molar excess of cryptophycin 1 over colchicine had no effect on the development of the fluorescence. On the other hand, a 2.5-fold molar excess of podophyllotoxin over colchicine completely prevented the increase in fluorescence.

4. Conclusions

Cryptophycin 1, a newly described natural product from the cyanobacterium *Nostoc*, shows properties similar to the *Vinca* alkaloids. It inhibits tubulin polymerization substoichiometrically, disassembles microtubules containing associated proteins to linear structures somewhat similar to the spiraled protofilaments produced by the *Vinca* drugs, and causes tubulin to aggregate. Moreover, it inhibits VLB binding to tubulin but not binding of colchicine. Thus, cryptophycin 1 belongs to an ever growing list of compounds that apparently interact with the *Vinca* domain in tubulin, a list which includes maytansine, dolastatin 10, phomopsin A, halichondrin B (for a review, see [14]), and spongistatins [15]. Although cryptophycin 1 does appear to bind to the *Vinca* site, it bears no structural similarity to the *Vinca* alkaloids and it is possible that the cryptophycins bind to a site on tubulin which overlaps the *Vinca* alkaloid site.

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