ABSTRACT

The antimitotic depsipeptide cryptophycin 1 (CP1) was compared to the antimitotic peptide dolastatin 10 (D10) as an antiproliferative agent and in its interactions with purified tubulin. The potent activity of CP1 as an inhibitor of cell growth was confirmed. The agent had an IC₅₀ of 20 pM against L1210 murine leukemia cells versus 0.5 nM for D10. Both drugs were comparable as inhibitors of the glutamate-induced assembly of purified tubulin, with D10 being slightly more potent. CP1, like D10, was a noncompetitive inhibitor of the binding of [³H]vinblastine to tubulin (apparent Kᵢ, 3.9 μM); and the depsipeptide was a competitive inhibitor of the binding of [³H]D10 to tubulin (apparent Kᵢ, 2.1 μM). CP1 was less potent than D10 as an inhibitor of nucleotide exchange on tubulin, but the two drugs were equivalent in stabilizing the colchicine binding activity of tubulin. CP1, like D10, caused the formation of extensive structured aggregates of tubulin when present in stoichiometric amounts relative to the protein. Whereas at lower concentrations the drugs were equivalent in causing formation of small oligomers detected by gel permeation high-performance liquid chromatography, there were notable differences in the aggregation reactions induced by the two drugs. The electron microscopic appearance of the D10-induced aggregate differed substantially from that of the CP1-induced aggregate. With D10, but not CP1, aggregate morphology was greatly altered in the presence of microtubule-associated proteins. Finally, although CP1 caused the formation of massive aggregates, as did D10, there was little turbidity change with the depsipeptide as opposed to the peptide.

INTRODUCTION

Antimitotic natural products have a long history of use as poisons and as therapeutic agents. Ever since CLC² was used to isolate tubulin (1), the target protein for this class of drug, there has been a continuing discovery of new agents remarkable for their chemical diversity and often for their potent cytotoxicity (2). Smith et al. (3) reported that the depsipeptide CP1 (previously known as “cryptophycin” and “cryptophycin A”; structure in Fig. 1, as reported by Barrow et al. in Ref. 4), a compound initially isolated as a potent antifungal agent from the terrestrial cyanobacterium Nostoc sp. (5), caused cells to accumulate in mitosis with the disappearance of intracellular microtubules. IC₅₀ as low as 20 pM were observed for cell growth, and these values for CP1 were approximately 100-1000-fold lower than those obtained with paclitaxel (Taxol), CLC, and VLB. Moreover, CP1 inhibited tubulin assembly and the binding of VLB but not CLC or a closely related agent will probably be evaluated as an experimental antineoplastic agent in clinical trials.

We have been studying interactions of antimitotic peptides (D10 and analogues; Refs. 9–14) and depsipeptides (D15 and analogues; Ref. 15) with purified tubulin, as well as the comparative antiproliferative activities of these agents (structures in Fig. 1). D10 binds in a distinct region of the tubulin α-β-heterodimer, since it noncompetitively inhibits the binding of radiolabeled Vinca alkaloids to tubulin, as do an unrelated group of sponge-derived macrocyclic polyethers termed halichondrins (16) and spongistatins (17, 18). The binding of [³H]D10 to tubulin was inhibited competitively by one of its analogues [(19αR)-isodolastatin 10] and by another peptide antimitotic agent, phomopsin A, and noncompetitively by spongistatin 1 (18). With [³H]D10, we observed tenacious, but reversible, binding to tubulin, and formation of drug-containing tubulin oligomers and coiled polymers that could be studied by HPLC, electron microscopy, or turbidity development (13, 18). D10, moreover, potently inhibited nucleotide exchange on tubulin, although it did not displace nucleotide bound in the exchangeable site. The peptide prevented time-dependent decay of CLC binding activity of tubulin (10).

In contrast, the depsipeptide D15, despite its partial structural analogy to D10 and its ability to inhibit tubulin polymerization, neither induces tubulin oligomerization nor affects the interactions of other ligands with tubulin (13, 15). Since we have not succeeded in demonstrating an interaction of [³H]D15 with tubulin,³ we have attributed this difference in properties to much weaker binding of D15 to tubulin.

Our work with the dolastatins made us particularly curious about CP1. We wondered how its properties would compare with those of the dolastatins, particularly whether its being a depsipeptide indicated a relatively weak interaction with tubulin. We found, however, that CP1 resembled D10 more than D15 in its effects on tubulin. The agent noncompetitively inhibited [³H]VLB binding to tubulin and competitively inhibited [³H]D10 binding to tubulin. Like D10, CP1 caused formation of structurally distinct tubulin aggregates, but this occurred without a change in the turbidity of the tubulin solution. CP1 induced-aggregates differed morphologically from those induced by VLB and D10 under the same reaction conditions. In addition, we confirmed the potent antimitotic activity reported by Smith et al. (3).

MATERIALS AND METHODS

Materials. CP1 was purified from the culture medium used to grow the Nostoc sp. (5). Synthetic D10 (19), [³H]D10 (13), synthetic D15 (20), (19αR)-isodolastatin 10 (21), an isomer of D10 with reversal of configuration at C-19a, and electrophoretically homogeneous bovine brain tubulin and heat-treated MAPs (22) were prepared as described previously. [⁸⁻¹⁴C]GTP,

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The abbreviations used are: CLC, colchicine; VLB, vinblastine; CP1, cryptophycin 1; D10, dolastatin 10; D15, dolastatin 15; HPLC, high-performance liquid chromatography; MAPs, microtubule-associated proteins; MES, 4-morpholineethanesulfonate.


Fig. 1. Structural formulas for CPI, DIO, and D15.

Tubulin aggregation was followed by HPLC on a 7.5 × 300-mm TSK G3000SW gel permeation column with an LKB system in line with a Ramona 5-LS flow detector. The column was equilibrated with a solution containing 0.1 M MES (pH 6.9) and 0.5 mM MgCl₂. Absorbance data were evaluated with Raytest software on an IBM-compatible computer. Tubulin aggregation was also followed turbidimetrically at 350 nm in Gilford spectrophotometers.

Negative stained specimens were examined in a Zeiss model 10CA electron microscope. About 5 μl were placed on a 200-mesh, carbon-coated, Formvar-treated copper grid, and after 5–10 s, were washed off with 5–10 drops of 0.5% uranyl acetate. Excess stain was removed by absorbance into torn filter paper.

Drug effects on the growth of L1210 murine leukemia cells were determined on cells grown under 5% CO₂ in 5 ml of RPMI 1640 supplemented with 17% fetal bovine serum, 2% glutamine, and 10 μg/ml gentamicin sulfate. Cell number was determined with a Coulter Counter following a 24-h incubation at 37°C. Mitotic index was evaluated by microscopic examination of cells fixed with methanol and stained with Giemsa.

RESULTS

CPI Is a Potent Inhibitor of Cell Growth

We have repeatedly obtained IC₅₀ₐ of 0.3–0.9 nm for DIO and 3–4 nm for D15 as inhibitors of the growth of L1210 murine leukemia
CPI Inhibits the Assembly of Purified Tubulin

Although the observation of potent antimitotic activity in a natural product strongly indicates an interaction with tubulin (2), other mechanisms of action are feasible. Moreover, both inhibitory and stimulatory effects on assembly reactions occur with agents that interact with tubulin. Furthermore, there is only a limited quantitative correlation between different drug classes relative antiproliferative activities and effects on tubulin polymerization. One of the most striking discrepancies is the low inhibitory activity of D15 on tubulin assembly, despite its potent inhibition of cell growth (15).

When we evaluated the effects of low concentrations (substoichiometric to the tubulin concentration) of CPI on tubulin assembly, we observed progressive inhibition of assembly induced by both MAPs (Fig. 2A) and glutamate (Fig. 2B). In the MAP-dependent reaction, about 50% inhibition of net assembly was observed with 2 µM CPI, using 15 µM tubulin. The glutamate-dependent assembly reaction, using 10 µM tubulin, was studied in greater detail to obtain quantitative comparisons of CPI with D10 and D15, as well as with VLB and maytansine (Table 1). Only D10 was more effective than CPI (IC50 of 1.8 µM versus 2.7 µM for CPI). D15 was a relatively weak inhibitor of the assembly reaction (IC50, 19 µM), whereas VLB and maytansine were moderately less effective than CPI. These results differ little from those of Kerksiek et al. (6) and Smith and Zhang (7).

Table 1 Inhibition of tubulin polymerization by CPI and other Vinca domain antimitotic drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition of tubulin polymerization IC50 (µM) ± SD</th>
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<tbody>
<tr>
<td>CPI</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>D10</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>D15</td>
<td>19 ± 0.3</td>
</tr>
<tr>
<td>VLB</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Maytansine</td>
<td>4.5 ± 0.2</td>
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</table>

The concentration of drug required to inhibit glutamate-induced assembly of 1.0 mg/ml (10 µM) purified tubulin was determined, as described in the text. The extent of assembly after 20 min at 37°C was determined. A minimum of three independent experiments was performed with each drug.

Table 2 Inhibition of [3H]VLB and [3H]D10 binding to tubulin by CPI and other Vinca domain drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLB</td>
<td>33</td>
</tr>
<tr>
<td>D10</td>
<td>32</td>
</tr>
<tr>
<td>(19aR)-Isodolastatin 10</td>
<td>31</td>
</tr>
<tr>
<td>Maytansine</td>
<td>34</td>
</tr>
<tr>
<td>Halichondrin B</td>
<td>38</td>
</tr>
<tr>
<td>Rhizoxin</td>
<td>2</td>
</tr>
<tr>
<td>D15</td>
<td>3</td>
</tr>
<tr>
<td>VLB</td>
<td>1</td>
</tr>
</tbody>
</table>

The 0.4-ml reaction mixtures contained 10 µM (1.0 mg/ml) tubulin, 0.1 M MES (pH 6.9), 0.5 mM MgCl2, 1% DMSO, either [3H]VLB or [3H]D10 at 10 µM, and the indicated inhibitor at 50 µM and were incubated for 30 min at room temperature. Duplicate aliquots (0.19 ml) were placed on microcolumns of Sephadex G-50 (superfine) and were processed by centrifugal gel filtration at room temperature. Averages from two independent experiments are presented in the table. Stoichiometry of binding in the control reaction mixtures without inhibitor was 0.53 mol of VLB and 0.55 mol of D10 per mol of tubulin.

Effects of CPI on Interactions of Other Ligands with Tubulin

VLB and D10. We, like Kerksiek et al. (6) and Smith and Zhang (7), found that CPI inhibited the binding of [3H]VLB to tubulin. In addition, CPI inhibited the binding of [3H]D10 to tubulin. Comparison with other drugs (Table 2), at a low inhibitor concentration (inhibitor:radioabeled ligand ratio, 1:2), showed that CPI was among the stronger inhibitors of both reactions.

Extensive kinetic analyses of inhibition of the radiolabeled drug binding reactions demonstrated a significant difference in the inhibitory effects of CPI in the two reactions. Data are presented in the Hanes format for inhibition of [3H]VLB binding (Fig. 3A) and inhibition of [3H]D10 binding (Fig. 3B). With the Hanes format, inhibitory data obtained at different inhibitor concentrations yield parallel lines for a competitive inhibitor and lines that intercept on the negative abscissa for a noncompetitive inhibitor (25). The data of Fig. 3 thus indicate that CPI is a noncompetitive inhibitor of [3H]VLB binding to tubulin and a competitive inhibitor of [3H]D10 binding to tubulin. By the terminology proposed earlier, CPI apparently binds in the "peptide site" of the "Vinca domain." Dixon analysis (25) yielded apparent Ks for CPI of 3.9 µM versus VLB and 2.1 µM versus D10 (averages of two determinations each).

GTP. All agents that bind in the Vinca domain also inhibit nucleotide exchange on tubulin, with VLB least active and maytansine, spongistatin I, D10, and phomopsin A most active (2). When added prior to radiolabeled GTP or GDP, these agents inhibit nucleotide binding to tubulin; but, as has been shown when tubulin with [8-14C]GDP bound in the exchangeable site is used, nucleotide bound in the exchangeable site is not actually displaced by any of the drugs (10, 23).

Initial studies showed that CPI also inhibited nucleotide exchange. However, CPI appeared to be relatively inactive as an inhibitor of [8-14C]GTP binding to tubulin (Fig. 4, •), with an IC50 of about 50 µM (versus about 11 µM for D10; Fig. 4, □). The activity of CPI

4400

Fig. 2. Inhibition of tubulin assembly by substoichiometric concentrations of CPI. Reaction components were described in the text. For the experiments shown in both panels, at time zero the temperature was changed from 0°C to 37°C. A. MAP-dependent assembly. Curves 1-5, 0, 1.0, 2.0, 3.0, and 4.0 µM CPI, respectively. B. glutamate-dependent assembly. Curves 1-5, 0, 1.5, 2.0, 3.0, and 4.0 µM CPI, respectively.
relative to that of D10 was dramatically enhanced by preincubating the drug with tubulin at 0°C prior to the addition of [8-14C]GTP to the reaction mixture (Fig. 4). When the drugs were added 60 min prior to the nucleotide, the IC50 for CPI was about 8 μM and that for D10 was about 5 μM (extrapolation from the data of Fig. 4). As with the other Vinca domain drugs, CPI did not displace radiolabel from tubulin-[8-14C]GDP and inhibited displacement of the bound [8-14C]GDP by nonradioislated GTP (data not presented). Smith and Zhang (7) found that CPI inhibited tubulin-dependent GTP hydrolysis, but they were unable to demonstrate an effect on nucleotide exchange. However, these workers did not evaluate CPI concentrations greater than 20 μM or a drug-tubulin preincubation, which we found were necessary to observe maximal effects on the nucleotide binding reaction.

CLC. CPI did not significantly inhibit the binding of CLC to tubulin, as was also observed by others (6, 7). Under some reaction conditions, most Vinca domain drugs enhance the binding of CLC to tubulin, and many of these drugs retard or arrest decay of the CLC binding activity of tubulin (2, 10, 24). CPI closely resembles D10 both in preventing loss of CLC binding activity in a preincubation, compared with the partially protective VLB and the nonprotective maytansine, and in strongly enhancing CLC binding without a preincubation, compared with the weaker effects of VLB and maytansine (Table 3).

**Tubulin Aggregation Induced by CPI**

In the above experiments, we never observed that reaction mixtures containing CPI became turbid. In fact, as in earlier studies with spongistatin 1–9 (18), CPI inhibited turbidity development induced by D10. Since CPI appears to bind at the same site on tubulin as D10, we examined in greater detail the effects of CPI on D10-induced aggregation.

The time course of D10-induced aggregation could be examined by turbidimetry, and Fig. 5 presents two studies with tubulin at 10 μM (1.0 mg/ml). The study shown in the main panel was performed at 0°C and that in the inset at 37°C. At 0°C, there was no turbidity change with tubulin alone (Fig. 5, curve 1) or when the protein was mixed with 10–40 μM CPI (Fig. 5, curve 2). With D10, the rate of aggregation, but not its extent, depended on drug concentration. With 10 μM D10, there was no apparent reaction for 6 min, followed by sigmoidal kinetics, with turbidity approaching a plateau after 75 min (Fig. 5, curve 3). This reaction was strongly inhibited by low concentrations of CPI (2 μM, Fig. 5, curve 6; 5 μM, Fig. 5, curve 7). With 20 μM D10 (Fig. 5, curve 4), following a 1-min lag, a rapid increase in turbidity occurred. With 40 μM D10 (Fig. 5, curve 5), turbidity rose rapidly as soon as the drug and tubulin were mixed. Raising the temperature of these reaction mixtures resulted in small decreases (10–20%) in the turbidity readings (data not shown), with no change in turbidity in the sample containing 40 μM CPI.

Because D10-induced turbidity decreased at 37°C and because we were concerned that tubulin decay might have prevented a tempera-

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**Table 3 Stabilisation of CLC binding activity of tubulin by CPI and other Vinca domain drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>pmol [3H]CLC bound per pmol tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.31</td>
</tr>
<tr>
<td>CPI</td>
<td>0.53</td>
</tr>
<tr>
<td>D10</td>
<td>0.53</td>
</tr>
<tr>
<td>VLB</td>
<td>0.46</td>
</tr>
<tr>
<td>Maytansine</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Each 0.1-ml reaction mixture contained 0.4 mg/ml tubulin, 0.1 M MES (pH 6.4), 0.1 M EDTA, 1 mM GTP, 0.5 mM MgCl2, 1 mM 2-mercaptoethanol, 1 mM EGTA, 5% DMSO, 60 μM [3H]CLC, and the indicated Vinca domain drug at 50 μM. If indicated, the reaction mixtures were preincubated for 3 h at 37°C prior to the addition of [3H]CLC in 10 μl. Following the addition of [3H]CLC, incubation was for 2 h at 37°C. The data presented in the table represent average values obtained in two independent experiments, each of which contained triplicate samples.

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**Fig. 3. Inhibition of [3H]VLB (A) and [3H]D10 (B) binding to tubulin by CPI. Reaction mixtures (0.40 ml) contained 5.0 μM (0.5 mg/ml) tubulin, 0.1 M MES (pH 6.9), 0.5 mM MgCl2, 2% DMSO, the indicated concentrations of [3H]VLB or [3H]D10, and the concentrations of CPI indicated below. Tubulin was the last component added to the reaction mixtures. After 30 min at room temperature, 0.2-ml duplicate aliquots of each reaction mixture were processed by centrifugal gel filtration at room temperature. In A, the following concentrations of CPI were used: •, none; •, 2.0 μM; ▲, 4.0 μM; and ▲, 6.0 μM. Ordinate units for S/V: μM VLB × μg tubulin/pmol VLB bound. In B, the following concentrations of CPI were used: •, none; •, 2.0 μM; ▲, 4.0 μM; and ▲, 6.0 μM. Ordinate units for S/V: μM D10 × μg tubulin/pmol D10 bound.

**Fig. 4. Effect of a 0°C preincubation of drug with tubulin on subsequent inhibition of the binding of [8-14C]GTP to tubulin: comparison of CPI (•, •, ▲, and ▲) with D10 (■, △, ◊, and ◆). Reaction mixtures (0.40 ml) contained 10 μM (1.0 mg/ml) tubulin, 0.1 M MES (pH 6.9), 0.5 mM MgCl2, 1% DMSO, and the indicated drug concentration. Following preincubation on ice (• and ▲, no preincubation; ■ and ◆, 15 min preincubation; ▲ and ◆, 30 min preincubation; △ and ▲, 60 min preincubation), 50 μM [8-14C]GTP were added to each reaction mixture. After an additional 15 min on ice, 0.2-ml duplicate aliquots of each reaction mixture were processed by centrifugal gel filtration at 4°C.

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**Fig. 5. Effect of DIO-induced aggregation by DIO on turbidity development induced by DIO. Each 0.1-ml reaction mixture contained 0.4 mg/ml tubulin, 0.1 M MES (pH 6.4), 0.1 M EDTA, 1 mM GTP, 0.5 mM MgCl2, 1 mM 2-mercaptoethanol, 1 mM EGTA, 5% DMSO, the indicated drug concentration. Following preincubation on ice (• and ◊, no preincubation; ■ and ▲, 15 min preincubation; △ and ◆, 30 min preincubation; ▲ and ▲, 60 min preincubation), 50 μM [8-14C]GTP were added to each reaction mixture. After an additional 15 min on ice, 0.2-ml duplicate aliquots of each reaction mixture were processed by centrifugal gel filtration at 4°C.**
INTERACTION OF CRYPTOPHYCIN 1 WITH TUBULIN

1.0
0.5

Fig. 5. Comparison of the effects of CPI and D10 in causing turbidity development in tubulin solutions at 0°C (main panel) and 37°C (inset). Each 0.25-ml reaction mixture contained 10 μM (1.0 mg/ml) tubulin, 0.1 M MES (pH 6.9), 0.5 mM MgCl2, 4% DMSO, and further components as indicated. Main panel: curve 1, no further addition; curve 2, 40 μM CPI (10 and 20 μM CPI also had no effect); curve 3, 10 μM D10; curve 4, 20 μM D10; curve 5, 40 μM D10; curve 6, 10 μM D10 and 2.0 μM CPI; curve 7, 10 μM D10 and 5.0 μM CPI. Inset: curve 1, no further addition; curve 2, 40 μM CPI (10 and 20 μM CPI also had no effect); curve 3, 10 μM D10; curve 4, 10 μM D10 and 2.0 μM CPI. In the experiment shown in the inset, the temperature was jumped from 0°C to 37°C within 2 min of adding D10 to the reaction mixtures.

ture-dependent aggregation reaction induced by CPI, we performed the experiment shown in the Fig. 5 inset. The temperature of the reaction mixtures was jumped to 37°C within 2 min of adding D10. Again, no change in turbidity occurred with tubulin alone (Fig. 5, curve 1) or when the protein was mixed with 10-40 μM CPI (Fig. 5, curve 2). With 10 μM D10 (Fig. 5, curve 3), following a lag of 2.5 min, there was a sigmoidal rise in turbidity, with the plateau reached after 20 min. The turbidity reading was about one-half that observed at 0°C, but when the reaction temperature was returned to 0°C, there was only a small increase in turbidity (10-20%). The 37°C reaction with 10 μM D10 was completely inhibited by 2 μM CPI (Fig. 5, curve 4).

It appeared that CPI was like maytansine, rhizoxin, and the macrocyclic polyethers and did not cause tubulin aggregation and inhibited the aggregation reaction induced by D10 (2). Even very low concentrations of D10 relative to tubulin (as low as 1:25) caused formation of small oligomers detectable by size exclusion HPLC (13). With [3H]D10, we could not document binding of drug to single tubulin molecules. The smallest radiolabeled species comigrated with a Mr 200,000 standard and was distinct from the main protein peak. We decided to determine whether CPI would inhibit formation of such small D10-induced oligomers concordant with its inhibition of the binding of [3H]D10 to tubulin. However, HPLC experiments with CPI demonstrated that such a study was not possible. CPI also caused formation of tubulin oligomers detectable by the HPLC technique. Quantitatively, the effects of D10 and CPI on oligomer formation were virtually identical, with stoichiometric amounts of either drug shifting the tubulin from the included volume to the void volume (Fig. 6). A comparable effect of stoichiometric CPI was observed by Kerksiek et al. (6).

Morphological Studies

We turned to electron microscopy for information about aggregates induced by CPI. Abundant ring-like aggregates were formed when CPI was added to tubulin, and we observed little variation in their morphology under different conditions. No specific effects could be ascribed to reaction temperature, protein or drug concentration, GTP, or MAPs. The morphology of the CPI-induced aggregate was distinct from that formed with either D10 or VLB. Because of the competitive inhibition of D10 binding (versus noncompetitive inhibition of VLB binding), we performed comparative studies with CPI and D10 (a comparative study between VLB and DK was presented previously: see Ref. 13).

Fig. 7 compares high power views of tubulin aggregates in the absence of MAPs induced by CPI (Fig. 7A) with those induced by D10 (Fig. 7B). With D10, the entire grid appeared to be covered by rings, conjoined rings, and broken rings, as observed previously (13). With CPI, there were clumps that had the appearance of multiple clustered rings, together with scattered individual rings between
clumps. The CPI rings had a diameter about one-third to one-half that of the DIO rings. VLB-induced aggregates had the appearance of ill-defined, short spirals (13).

Fig. 8 compares high power views of aggregates formed in the presence of MAPs (panel A with CPI, panel B with DIO). As before (13), the MAPs seemed to impose a greater "order" in the appearance of the DIO-induced aggregate, with most of the material having the appearance of conjoined rings. These structures probably represent tightly coiled filaments that sometimes take on the appearance of pinwheels,7 with the field shown in Fig. 8B displaying more coils and pinwheel formations than generally observed in a single field (the view was chosen for the variety of structures shown in a small area). Previously (13), we had observed that MAPs also altered the morphology of the VLB-induced aggregate, resulting in formation of the looser spiral filaments described by many workers (reviewed in Ref. 26). CPI differed from DIO and VLB in that MAPs had little effect on the morphology of the aggregate formed in terms of its electron microscopic appearance. As in the absence of MAPs (Fig. 7B), with MAPs (Fig. 8A) the CPI aggregate largely consisted of conjoined miniature rings. We have not visualized any well-defined coils or spirals in CPI-treated samples.

In view of the aggregated conjoined rings visualized by electron microscopy, it seemed strange that CPI failed to cause a change in turbidity when added to tubulin with or without MAPs. We, therefore, compared the overall low power appearance of tubulin + CPI to tubulin alone (Fig. 9). With drug, there were many electron dense clumps of varying sizes. One of the largest seen is shown in Fig. 9A. Invariably, at high power, such clumps had the fine structure of clustered small rings (Fig. 9A, inset) that results from the interaction of CPI with tubulin. When scanning grids prepared from reaction mixtures containing tubulin but no drug, we also observed many electron dense clumps (Fig. 9B). Thus far, these have been substan-

tially smaller on average than the clumps observed in the CPI samples. Invariably, at high power, the clumps from the tubulin-only controls have not shown any discrete structural morphology (Fig. 9B, inset).

DISCUSSION

The studies presented here, together with those of Kerksiek et al. (6) and Smith and Zhang (7), establish that the depsipeptide CPI interacts strongly with tubulin, inhibiting microtubule assembly. This interaction probably accounts for the antimitotic activity of the drug and for the disappearance of intracellular microtubules (3). In studies of the effects of CPI on interactions of tubulin with other ligands, we found a noncompetitive pattern of inhibition versus [3H]VLB, a competitive pattern versus [3H]D10, inhibition of nucleotide exchange that was enhanced by preincubating drug and tubulin, and strong stabilization of the [3H]CLC binding activity of tubulin. Except for enhancement of the inhibitory effect on nucleotide exchange by the drug-tubulin preincubation, these properties are identical to those of the peptides D10 and phomopsin A. To account for these properties (especially the noncompetitive pattern of inhibition of Vinca alkaloid binding), as well as structure-activity observations with DIO analogues, we proposed that DIO and phomopsin A bind to a "peptide site" on β-tubulin distinct from the "Vinca site." This model proposed that inhibition of VLB binding and nucleotide exchange occurred because occupancy of the peptide site sterically obstructed access to the Vinca site. This model proposed that inhibition of VLB binding and nucleotide exchange occurred because occupancy of the peptide site sterically obstructed access to the Vinca and nucleotide sites (10). We suggested that this complex of ligand binding sites be called the "Vinca domain."8 Our ligand data support the idea that the depsipeptide CPI also binds in the peptide site and not in the Vinca site itself.

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7 Aggregate induced by the cyclic peptide phomopsin A is identical in appearance to that induced by D10 (37).

8 There is probably a third drug binding region in the Vinca domain that forms the primary binding site for the macrocyclic polyethers, because halichondrin B noncompetitively inhibits the binding of [3H]VLB to tubulin (16) and spongistatin 1 noncompetitively inhibits the binding of both [3H]VLB and [3H]D10 to tubulin (18).
The major difference between CPI and D10 is in the structured aggregates they induce. The drugs are quantitatively similar in the formation of small tubulin oligomers. These HPLC studies were performed on columns not equilibrated with free drug, indicating that CPI, like D10 (13), binds tightly to tubulin in a slowly reversible reaction. Yet D10 causes tubulin solutions to become highly turbid, whereas no change in turbidity occurs with CPI. Electron microscopy adds to the apparent paradox. Both drugs cause formation of substantial amounts of structured aggregates with distinct morphology. The morphologies of these aggregates are different from each other, as well as from aggregates induced by VLB. The gross morphology of the CPI-induced aggregate is not greatly affected by MAPs, in contrast to substantial changes observed in the D10- and VLB-induced aggregates. These differences in drug effects probably result from subtle changes in tubulin-tubulin interactions as a function of the bound ligand.

What accounts for the limited ability of the CPI aggregate to scatter light? Two possibilities merit consideration. There appears to be some aggregate in purified tubulin preparations, as exemplified by the small void volume peak observed in the HPLC studies without drug (Fig. 6A; Ref. 6) and the unstructured, electron dense clumps observed by electron microscopy. Although such aggregates probably represent denatured tubulin, the large structured CPI-induced aggregates could derive primarily from preexisting aggregates. The HPLC studies argue against this explanation, unless soluble M, 100,000 tubulin dimers only enter relatively small oligomers upon addition of CPI to the reaction mixture. Alternatively, the absence of turbidity could result from the fine structure of the aggregate, which appears to consist of relatively small interconnecting rings, possibly with an underlying tight spiral structure.

Enough data has now been accumulated with different members of the Vinca domain class of drugs to postulate a relationship between drug stabilization of CLC binding activity and drug induction of structured aggregates. Thus far, every agent that causes aggregation (VLB, D10, phomopsin A, and CPI) prevents the time-dependent decay of the CLC binding activity of tubulin; whereas all agents that do not induce aggregation (maytansine, rhizoxin, halichondrin B, spongistatin 1, and D15) fail to stabilize the CLC binding activity of tubulin. Only agents that constrain tubulin conformation within multimeric aggregates appear to preserve the conformation of the CLC site.

This generalization does not apply to the exchangeable nucleotide site. The preincubation effect resulting in enhancement of inhibition of nucleotide exchange by CPI could result from slow aggregation, but we have no method for evaluating the time course of CPI-induced tubulin aggregation. More importantly, of the four most potent inhibitors of nucleotide exchange [maytansine (23), D10 (10), phomopsin A (10), and spongistatin 1 (17)], only the peptides cause tubulin aggregation, and VLB is the weakest inhibitor of nucleotide exchange among the Vinca domain drugs, despite its induction of an aggregation reaction (16). Thus, we continue to favor steric inhibition, with drugs differentially affecting egress and entry of nucleotide at the exchangeable site, as the most probable explanation for inhibition of nucleotide exchange by this class of drugs. However, neither allosteric effects nor aggregation effects can yet be excluded as factors causing this phenomenon. Also, there has been excellent qualitative and quantitative correlation between a drug’s inhibition of nucleotide exchange and its ability to inhibit formation of a cross-link between cysteine-12 and cysteine-201 or -211 of β-tubulin by N,N’-ethylenebis(iodoacetamide) (12, 27, 28). CPI also inhibits formation of this intra-β-tubulin cross-link (29).

Despite the quantitative similarity between D10 and CPI in most of
their interactions with tubulin, the depsipeptide is much more potent as an inhibitor of cell growth. With L1210 cells, CP1 was at least 15-fold more potent than D10 and had activity comparable to that of spongistatin 1. In our spongistatin 1 studies (17), we had compared equivalent antiproliferative doses (1× and 10× the IC50 concentrations) of spongistatin 1, halichondrin B, D10, CLC, and VLB on microtubules in PtK1 cells. We observed similar effects with all compounds, consistent with a similar mechanism of action for all agents despite a 400-fold range in IC50 values. Similarly, Smith et al. (3) observed identical effects on microtubules of A-10 cells of CP1 and VLB at equivalent antiproliferative concentrations (100× the IC50 concentrations, which differed 500-fold).

Little data is available on intracellular concentrations of antimicrotubule drugs. Singer and Himes (30) measured VLB uptake in B16 melanoma cells by an HPLC technique at 25 times and 250 times the IC50 concentration and estimated by extrapolation that at that IC50 concentration, the intracellular tubulin:drug ratio would be about 100:1. In attempting to gain insight into the greater antiproliferative activity of D10 relative to VLB, we have compared intracellular concentrations of radiolabeled drug in CA46 Burkitt lymphoma cells. At the same drug concentration, significantly more D10 than VLB enters cells. In studies at the IC50 concentration of VLB, we obtained a tubulin:drug ratio of 20:1. Our results thus far with [3H]D10 at the IC50 concentration have been uninformative because of the relatively low specific activity of the peptide. Nonetheless, we tentatively conclude that D10 does enter cells more readily than VLB, and we postulate that CP1 will be even more readily absorbed.

Assuming the low stoichiometry observed with VLB at the IC50 concentration occurs universally with antimotic agents that interact with tubulin, what accounts for their potent action in cells? At least two potential explanations merit consideration. The first is that substoichiometric drug concentrations have significant kinetic effects on the functioning of cellular microtubules, probably by interfering with the dynamic instability properties of microtubules (31). The second is that γ-tubulin is also a target of antimicrotubule agents, especially those that inhibit assembly. Interference with nucleation at the microtubule organizing center/centrosome via binding to γ-tubulin (32–34) could readily account for the disruptive effects that even IC50 concentrations of antimotic drugs cause on cellular microtubule organization (17).

Finally, although CP1 and not D10 is the central topic of this report, our comparative studies with the two peptides have led to new

Fig. 9. Comparison of the structured electron-dense aggregate observed in the presence of CP1 (A) with the nonstructured aggregate observed in the absence of drug (B). ×4500 (main panels); ×224,000 (insets). Reaction mixtures contained components as described in the legend for Fig. 7 and were incubated as described for Fig. 7. In each main panel, the arrow points to the region of the aggregate that is shown at high power.
observations on the tubulin aggregation reactions caused by the marine peptide. Most notable is the much greater turbidity development that occurs at 0°C as compared with 37°C. The temperature effect does not appear to be reversible. After a prolonged incubation at 0°C, the turbidity did not fall greatly when the reaction temperature was increased; and following a prolonged incubation at 37°C, the turbidity did not rise greatly when the reaction temperature was decreased. Despite the different turbidity readings, electron microscopy did not reveal any obvious structural difference in aggregates formed at 0°C and 37°C.

Previously, we have not observed electron micrographic differences in tubulin aggregates induced by VLB at 0°C and 37°C (13). In contrast, different, and reversible, time-resolved X-ray solution scattering patterns were observed with VLB-induced tubulin aggregates at 0°C and 37°C by Hodgkinson et al. (35) and Nogales et al. (36). The pattern differences were interpreted as indicating different pitches in the drug-induced spiral filaments. These reports indicate that it should be worthwhile studying effects of DIO and CP1 by this technique.

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REFERENCES


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