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INTERACTION OF USTILOXIN A WITH BOVINE BRAIN TUBULIN

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Abstract—Ustiloxin A is a modified peptide derived from false smut balls on rice panicles, caused by the fungus *Ustilaginoidea virens*; structurally, it resembles phomopsin A. Ustiloxin A is cytotoxic and is an inhibitor of microtubule assembly *in vitro*. Because of its resemblance to phomopsin A, we examined its interaction with tubulin and compared the results with those obtained with phomopsin A and dolastatin 10, both of which were found previously to have very similar effects. We determined that ustiloxin A inhibited the formation of a particular intra-chain cross-link in β-tubulin, as do vinblastine, maytansine, rhizoxin, phomopsin A, dolastatin 10, halichondrin B and homohalichondrin B; this is in contrast to colchicine and podophyllotoxin which do not inhibit formation of this cross-link. Ustiloxin A also inhibited the alkylation of tubulin by iodo[^{14}C]acetamide, as do phomopsin A and dolastatin 10; vinblastine was almost as potent an inhibitor of alkylation as ustiloxin A, whereas maytansine, halichondrin B and homohalichondrin B have little or no effect. In addition, ustiloxin A inhibited exposure of hydrophobic areas on the surface of the tubulin molecule. In this respect, ustiloxin A was indistinguishable from phomopsin A but slightly more effective than dolastatin 10 and considerably more effective than vinblastine; this provides a strong contrast to maytansine, rhizoxin, and homohalichondrin B which have no effect on exposure of hydrophobic areas and to halichondrin B which enhances exposure. Lastly, ustiloxin A strongly stabilized the binding of [^{3}H]colchicine to tubulin. The combination of ustiloxin A with colchicine stabilized tubulin with a half-life of over 8 days, comparable with results obtained with phomopsin A and colchicine. A comparison of the structures of ustiloxin A, phomopsin A and dolastatin 10 raised the possibility that the strong stabilization of the tubulin structure may require a short segment of hydrophobic amino acids such as the modified valine-isoleucine sequence present in all three compounds. The rest of the structure, specifically the large ring of ustiloxin A and phomopsin A, may serve to place this sequence in an appropriate conformation to interact with tubulin.

Key words: tubulin; microtubule; ustiloxin A; colchicine; vinblastine; protein cross-linking

Microtubules, composed of the 100 kDa protein tubulin, are involved in a variety of cellular functions [1]. Because of their role in mitosis, drugs that bind to tubulin and inhibit assembly are of interest as potential anti-tumor drugs. We have developed three probes that are sensitive indicators of the nature of the interaction of an anti-mitotic drug with tubulin. In the first assay, we examine the effect of the drug on the formation of two intra-chain covalent cross-links in β-tubulin induced by EBI§ [2]. One of these cross-links, designated β^*, is between Cys^{239} and Cys^{354} [3]. The other cross-link, designated β^s, is between Cys^{12} and either Cys^{201} or Cys^{211} [4]. The second assay examines the effect of a drug on the rate of exposure of tubulin sulfhydryl groups, as measured by iodo[^{14}C]acetamide [5]. The third assay measures the exposure of hydrophobic areas on tubulin using the fluorescent probe BisANS [6].

By using these assays we can classify tubulin ligands into three broad categories. The first includes colchicine, podophyllotoxin and nocodazole, which inhibit β^* formation, enhance β^s formation, and inhibit the reaction of tubulin with iodo[^{14}C]-acetamide and the binding of tubulin to BisANS [6–8]; the drugs in this category are competitive inhibitors of colchicine binding to tubulin [9–11]. The second category includes vinblastine, GTP, maytansine, rhizoxin, phomopsin A, dolastatin 10, halichondrin B and homohalichondrin B; these drugs inhibit β^s formation while enhancing that of β^* [8, 12–15]; except for GTP, the compounds in this category are competitive or non-competitive inhibitors of vinblastine binding to tubulin [16–21]. The third category includes BisANS, at its high-affinity site, which does not affect the formation of either cross-link [22].

The second category is a complex one. Vinblastine itself causes only partial inhibition of β^s formation, whereas all the others in this group cause full inhibition [8]. Alkylation of tubulin by iodo[^{14}C]acetamide is inhibited by vinblastine, dolastatin 10 and phomopsin A; very minor inhibition is noted with homohalichondrin B and none with maytansine and halichondrin B. Vinblastine is a

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§ Abbreviations: EBI, *N*,*N*′-ethylenebis(iodoacetamide); and BisANS, bis-5,5′-[8-(*N*-phenyl)aminonaphthalene-1-sulfonic acid].

strong inhibitor of BisANS binding; dolastatin 10 is stronger and phomopsin A stronger yet [6, 20, 23]; in contrast, maytansine, rhizoxin and homohalichondrin B have no effect on BisANS binding, and halichondrin B slightly enhances it [6, 18, 21]. The complexity of these effects is underlined by the fact that maytansine and rhizoxin inhibit vinblastine binding competitively, whereas phomopsin A, dolastatin 10 and halichondrin B do so non-competitively [17–21].

The time-dependent exposure of sulfhydryl groups and hydrophobic areas is a reflection of the decay of tubulin, which is also manifested by the loss of ability to assemble and to bind to [^{3}H]colchicine [9]. Phomopsin A, vinblastine and dolastatin 10 stabilize colchicine binding [9, 13, 20, 23]. Maytansine and rhizoxin do not stabilize colchicine binding [17, 18]. Halichondrin B, although a non-competitive inhibitor of vinblastine binding, does not stabilize colchicine binding [21]. The most striking effect on [^{3}H]colchicine binding is that of phomopsin A; when tubulin is incubated with both [^{3}H]colchicine and phomopsin A for 3 days at 37°, essentially no decay of colchicine binding is observed [23], suggesting that the two drugs in combination can completely stabilize the conformation of tubulin.

Ustiloxin A is a modified peptide derived from the fungus *Ustilaginoidea virens* or false smut ball [24]; structurally, ustiloxin A resembles phomopsin A (Fig. 1).* Ustiloxin A is a cytotoxic drug that inhibits microtubule assembly with an IC_{50} of 0.5 μM [25]. Because it somewhat resembles phomopsin A, it is possible that it may have a set of interactions with tubulin similar to those of phomopsin A and dolastatin 10. We assayed the interaction of ustiloxin A with tubulin and found that it was very similar to phomopsin A and dolastatin 10. Ustiloxin A inhibited formation of the β^s cross-link, while enhancing that of β^*, placing this compound into the vinblastine category of ligands as defined above. In addition, ustiloxin A inhibited the reaction of tubulin with iodo[^{14}C]acetamide and also the binding of BisANS as effectively as phomopsin A. However, when the very similar actions of ustiloxin A, phomopsin A and dolastatin 10 are compared, it becomes difficult to see exactly where in the structure the activity lies. The large ring that ustiloxin A shares with phomopsin A, which is the only part that the two structures have in common, is not present in dolastatin 10. The one common feature shared by all three structures is the modified valine-isoleucine sequence; perhaps the nature of the interaction of these compounds with tubulin is determined by this short sequence of hydrophobic amino acids.

MATERIALS AND METHODS

Materials. Ustiloxin A was purified from false smut balls provoked by *U. virens* as detailed in Ref. 24. Previous papers have described how all other materials were obtained or prepared [8, 26]. Ustiloxin A was dissolved in dimethyl sulfoxide prior to experiments. In all experiments involving ustiloxin A, control samples lacking ustiloxin A contained an equivalent volume of dimethyl sulfoxide.

Tubulin preparation. The microtubules used in these experiments were prepared from bovine cerebra, and tubulin was purified from these microtubules as reported previously [27]. For some experiments, tubulin was purified on a double column of Sephadex G-25 and phosphocellulose in order to remove GTP and $MgCl_2$ [8]. Tubulin used in the fluorimetry and [^{3}H]colchicine binding experiments was purified in a buffer consisting of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.4, containing 0.1 mM EDTA, 1 mM EGTA, 1 mM GTP, 0.5 mM $MgCl_2$ and 1 mM β-mercaptoethanol [27]. Tubulin for the alkylation experiments was prepared in the same buffer, but lacking β-mercaptoethanol; tubulin for the cross-linking experiments was prepared in the same buffer minus β-mercaptoethanol, GTP and $MgCl_2$.

Cross-linking. Tubulin samples were incubated with EBI and then processed and analyzed as previously described [8]. To measure the yield of the β^* cross-link, tubulin was subjected to electrophoresis on 5.5% polyacrylamide gels according to the system of Laemmli [28]. To measure the yield of the β^s cross-link, the system of Banerjee *et al.* [29] was used. In these experiments, tubulin contained reduced and carboxymethylated conalbumin, which was used as an internal standard to measure the yield of cross-linked products. In addition to the intra-chain cross-links, the yield of the high-molecular-weight cross-linked aggregate was also determined. This aggregate is too large to migrate onto the gel; its yield is measured indirectly by measuring the ratio of tubulin to reduced and carboxymethylated conalbumin on the gel and comparing it with that obtained in a sample that has not been treated with EBI. The decreased ratio observed after EBI treatment is taken to reflect formation of this aggregate and used to calculate its yield [2].

Alkylation. Tubulin samples were incubated with iodo[^{14}C]acetamide and then precipitated and filtered; the radioactivity of the filters was determined as described previously [5].

Fluorimetry. Samples of tubulin that had been incubated at 37° were mixed with BisANS and placed in an SPF500C spectrofluorometer (SLM) set in the ratio mode. Excitation and emission were, respectively, 385 and 490 nm. This method has been described previously [6].

Other methods. The binding of [^{3}H]colchicine to tubulin was measured by filtration on DEAE-cellulose filters according to the procedure of Borisy [30]. Binding of [^{3}H]vinblastine to tubulin was measured using the non-equilibrium procedure of Wilson *et al.* [31]. Protein was determined, using bovine serum albumin as a standard, by a modification [32] of the procedure of Lowry *et al.* [33].

RESULTS

The fact that ustiloxin A and phomopsin A are

* In previous publications [24, 25], ustiloxin A was referred to as "ustiloxin"; recently, however, other congeners have been discovered (Iwasaki *et al.*, manuscript in preparation). Hence, the compound has been renamed "ustiloxin A" to distinguish it from its congeners.

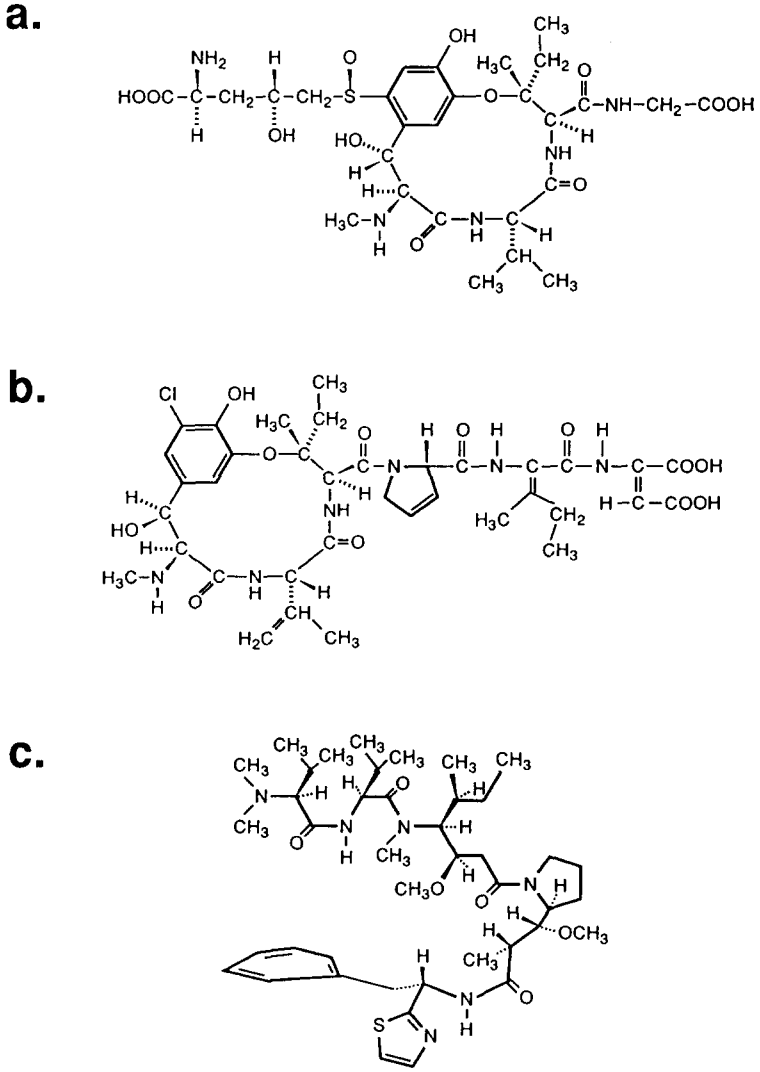

Fig. 1. Structures of ustiloxin A (a), phomopsin A (b), and dolastatin 10 (c).

Table 1. Effect of ustiloxin A on the binding of [^{3}H]vinblastine to tubulin*

Addition	[^{3}H]Vinblastine (mol bound/mol tubulin)	% of Control
None	0.082 ± 0.003	100 ± 4
Ustiloxin A, 20 μM	0.009 ± 0.002	11 ± 3

* Aliquots (500 μL) of tubulin (1.0 mg/mL) were incubated in the presence of 10 μM [^{3}H]vinblastine (113 Ci/mol). Triplicate 10-μL aliquots of each sample were placed on DE81 paper squares, and the binding of [^{3}H]vinblastine was determined by the procedure of Wilson *et al.* [31]. Values are means ± SD.

structurally similar suggests that ustiloxin A binds to tubulin in the same region as does phomopsin A, namely, the vinblastine-binding region. If this is true, then ustiloxin A is likely to inhibit the binding of [^{3}H]vinblastine to tubulin. Table 1 shows that this is indeed the case; 20 μM ustiloxin A inhibited the binding of 10 μM [^{3}H]vinblastine to tubulin by 89%.

Tubulin was reacted with EBI in the presence of ustiloxin A. In one set of samples, the reaction was done in the presence of 1 mM GTP to inhibit

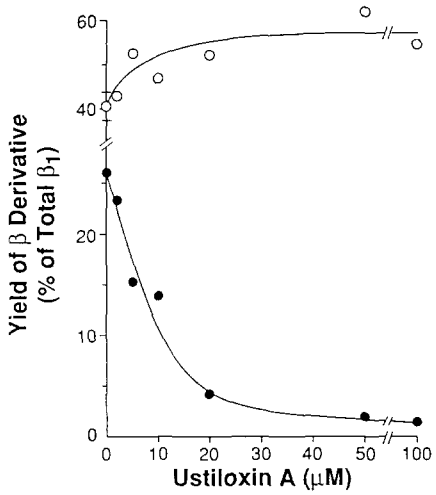

Fig. 2. Effect of ustiloxin A concentration on the EBI-induced formation of the β^s and β^* cross-links. Aliquots (250 μL) of tubulin (0.66 mg/mL), containing reduced and carboxymethylated conalbumin (0.20 mg/mL), were incubated with 0.91 mM EBI for 1 hr at 30° in the presence of the indicated concentrations of ustiloxin A. One set of samples (●) contained 50 μM podophyllotoxin to inhibit β^* formation. The other set (○) contained 1 mM GTP to inhibit β^s formation. The figure shows the yields of the β^s (●) and β^* (○) cross-links. The yields of the β^s and β^* cross-links in the absence of ustiloxin A represent the average of duplicate aliquots; the range of the values is shown.

formation of the β^s cross-link and to permit accurate measurement of the effect on formation of the β^* cross-link. In another set of samples, the reaction was done in the presence of 50 μM podophyllotoxin to inhibit formation of β^* and permit that of β^s. Figure 2 shows that ustiloxin A inhibited β^s formation and enhanced β^* formation. Half-maximal inhibition of β^s formation was obtained at about 8 μM ustiloxin A. This result suggests that ustiloxin A should be classified in the vinblastine category of tubulin ligands.

As mentioned above, ligands in the vinblastine group are highly variable in terms of their effects on the conformation of tubulin. To examine this point, the effect of ustiloxin A on the reaction of tubulin with iodo[^{14}C]acetamide was measured and compared with the effects of vinblastine and phomopsin A (Table 2). Ustiloxin A inhibited the alkylation of tubulin to a greater extent than did vinblastine, but its effect was the same as that of phomopsin A. Usually, the effect of a ligand on the reaction of tubulin with iodo[^{14}C]acetamide is similar to its effect on the formation of the high-molecular-weight cross-linked aggregate by EBI; both of these involve sulfhydryl groups. In the presence of 50 μM podophyllotoxin, formation of the high-molecular-weight cross-linked aggregate decreased from 34 ± 2% in the absence of ustiloxin A to 6% in the presence of 100 μM ustiloxin A. Similarly, in the presence of 1 mM GTP, formation of the high-molecular-weight cross-linked aggregate decreased from 36 ± 1% in the absence of ustiloxin A to 27% in the presence of 100 μM ustiloxin A.

In many, but not all, tubulin ligands, the effect on the alkylation of tubulin is paralleled by its effect on the exposure of hydrophobic areas on tubulin; both of these are manifestations of tubulin decay. Exposure of hydrophobic areas is measured by their ability to bind to the fluorescent probe BisANS. In the experiment shown in Fig. 3, we compared the effects of ustiloxin A with those of phomopsin A and rhizoxin. Clearly, ustiloxin A inhibited exposure of hydrophobic areas as much as did phomopsin A. Rhizoxin, by itself, had no effect on BisANS binding. In this respect, rhizoxin and ustiloxin A were apparently competing, because when used in combination, they had a slight inhibitory effect. In combination, at 20 μM concentrations, they inhibited BisANS binding by an average of 8 ± 4%. Similar results were obtained using 10 μM concentrations of all the drugs (data not shown).

The classical manifestation of tubulin decay is the loss of the ability to bind to colchicine. Ligands in the vinblastine category are highly variable in this respect. Figure 4 shows that ustiloxin A stabilized colchicine binding. When tubulin was incubated in the absence of [^{3}H]colchicine, the half-time of decay of the ability to bind to [^{3}H]colchicine was 3.8 hr in the absence and 59 hr in the presence of 20 μM ustiloxin A. When the sample was preincubated with [^{3}H]colchicine, the half-time of decay was 12.7 hr in the absence and 205 hr in the presence of 20 μM ustiloxin A.

DISCUSSION

The results of the experiment shown in Fig. 2 indicate clearly that ustiloxin A belongs to the vinblastine group of tubulin ligands; all the members of this group inhibit β^s formation by the cross-linker EBI and enhance formation of the β^* cross-link. Half-maximal inhibition of β^s formation occurred at 8 μM ustiloxin A (Fig. 2); this figure is comparable with those reported previously for the other members of this group of tubulin ligands: dolastatin 10 (7 μM), phomopsin A (2 μM), maytansine (3–5 μM), rhizoxin (2.5 μM), halichondrin B (3 μM), homohalichondrin B (6 μM), and vinblastine (10–20 μM) [8, 12–15]. Our results permit us to further classify ustiloxin A among the subcategories in this group. Ustiloxin A was a strong inhibitor of tubulin alkylation by iodo[^{14}C]acetamide and of BisANS binding; this distinguishes it dramatically from the ligands maytansine, rhizoxin, halichondrin B, and homohalichondrin B, which either do not affect alkylation or BisANS binding or else inhibit them only slightly or, in the case of halichondrin B, cause enhancement. The sharp contrast with rhizoxin is shown by the experiment in Fig. 3, where rhizoxin clearly countered the inhibitory effect of ustiloxin A; this could reflect either that rhizoxin and ustiloxin A are competing for binding to tubulin or that they can both bind but their effects compete. In short, ustiloxin A resembled vinblastine, dolastatin 10 and phomopsin A, all of which are strong inhibitors of

Table 2. Effect of ustiloxin A on the alkylation of tubulin by iodo[^{14}C]acetamide*

Addition	mol ^{14}C/100 kDa	% of Control
None	2.88 ± 0.11	100 ± 4
Ustiloxin A, 20 μM	1.07 ± 0.03	37 ± 4
Vinblastine, 20 μM	1.51 ± 0.03	53 ± 2
Phomopsin A, 20 μM	1.03 ± 0.06	36 ± 3

* Aliquots (250 μL) of tubulin (0.66 mg/mL) were incubated with 1.36 mM iodo[^{14}C]acetamide (0.66 Ci/mol) for 1 hr at 37°. The extent of alkylation by iodo[^{14}C]acetamide was measured as described in Materials and Methods. Values are means ± SD; N = 4 aliquots used.

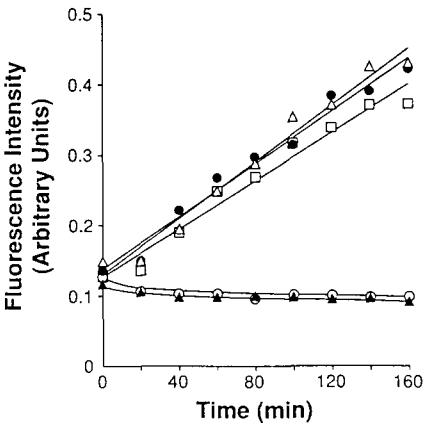

Fig. 3. Effects of ustiloxin A, phomopsin A, and rhizoxin on the binding of BisANS to tubulin. Samples of tubulin (0.2 mg/mL) were incubated at 37° in the absence (●) or presence of 20 μM concentrations of either ustiloxin A (○), phomopsin A (▲), rhizoxin (△) or ustiloxin A and rhizoxin together (□). At the indicated times, 1-mL aliquots of each sample were removed and made 10 μM in BisANS. The aliquots were placed in fluorescence cuvettes; the fluorescence was measured as described in Materials and Methods.

both alkylation by iodo[^{14}C]acetamide and binding of BisANS.

Within that smaller category of ligands, ustiloxin A most closely resembled phomopsin A and dolastatin 10. First, ustiloxin A was a stronger inhibitor of alkylation than vinblastine; the same appears to be true for both phomopsin A and dolastatin 10 [14]. Second, in the experiment in which the effect of ligands on BisANS binding was measured, ustiloxin A and phomopsin A gave a curve that, after the first 20 min, was essentially flat as already shown for phomopsin A [23]; dolastatin 10 is very similar, giving a curve in which BisANS binding appears to increase, but only at 5% of the control rate [14]. In contrast, in the presence of vinblastine, there is often a significant increase in BisANS binding, about 30–33% of the control rate [12, 14, 23]. One minor, but perhaps significant, observation is that both ustiloxin A and phomopsin A cause a decrease in BisANS binding in the first 20 min of incubation after which BisANS binding remains at a level significantly lower than that at the beginning of the incubation; this was not observed with dolastatin 10 [14, 23]. Third, ustiloxin A was a very potent stabilizer of colchicine binding. The stabilization was comparable with that caused by phomopsin A, which is significantly stronger than that of vinblastine. For example, the ability to bind to colchicine decays with a half-time of 8 hr in the presence of vinblastine and 28 hr in the presence of phomopsin A, much closer to the figure of 59 hr observed with ustiloxin A [23]. A similar pattern is seen when the effects of ligands are observed during co-incubation with colchicine: the half-times of decay in the presence of vinblastine and phomopsin A are, respectively, 23–35 hr and 15 days to 17 weeks [13]; with ustiloxin A the half-time was 8.5 days. In short, the effects of ustiloxin A are most similar to those of dolastatin 10 and phomopsin A, and ustiloxin A should be classified with these other two drugs as a very potent stabilizer of tubulin structure.

Despite the fact that the effects of ustiloxin A, dolastatin 10 and phomopsin A are so similar to each other, it is not immediately obvious exactly what structural features are necessary for this constellation of effects. All three compounds are modified peptides made of identifiable amino acids, many of which have been modified slightly. A pseudo-sequence of the three compounds would give the following: phomopsin A, YVIPVD; dolastatin 10, VVIPF; ustiloxin A, YVIG. All three have significant additions to these "sequences." In the case of dolastatin 10, a $CH_2CH(OCH_3)$ is inserted into the "backbone" between the α-carbon and the carbonyl group of the isoleucine and a $CH(OCH_3)CHCH_3$ between the α-carbon and the carbonyl group of the proline. In addition, dolastatin 10 has other substituents added to the sequence. Ustiloxin A and phomopsin A strongly resemble each other in that they both contain a large ring structure, which is not present in dolastatin 10. The rings are not identical: beginning at the phenyl ring, phomopsin A has a chloride substituent and ustiloxin A has a large side chain; the orientation of the H and OH on the C next to the phenyl ring is reversed; the valine in ustiloxin A has a double bond in phomopsin A. Ustiloxin A has a glycine substituent

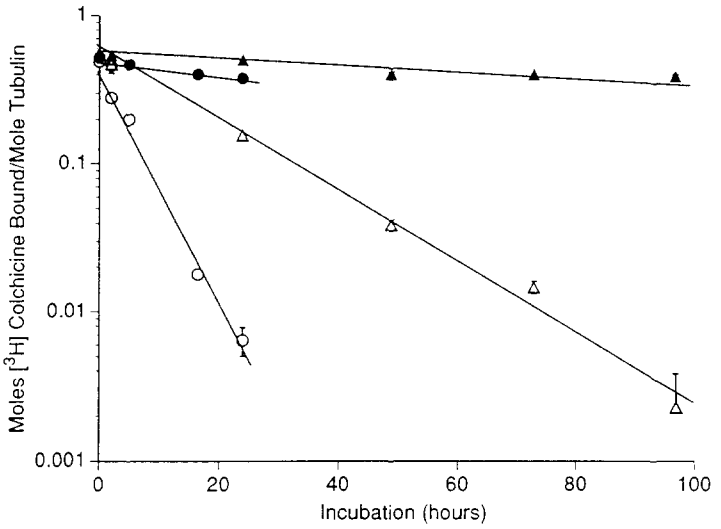

Fig. 4. Effect of ustiloxin A on the binding of [^{3}H]colchicine to tubulin. Samples of tubulin (0.3 mg/mL) were incubated at 37° in either the absence (○,●) or presence (△,▲) of 10 μM [^{3}H]colchicine (439 Ci/mol). Certain samples also contained 20 μM ustiloxin A (●,▲). For the samples that did not contain [^{3}H]colchicine, at the indicated times, triplicate aliquots (200 μL) were withdrawn and incubated for 2 hr at 37° in the presence of 9 μM [^{3}H]colchicine. The aliquots were then filtered, and the amount of [^{3}H]colchicine bound to the protein was determined as described in Materials and Methods. Of the samples that were preincubated with [^{3}H]colchicine, at the indicated times triplicate aliquots were withdrawn and filtered, and the amount of [^{3}H]colchicine bound was determined. Values are means ± SD.

where phomopsin A has a pseudo-PVD. The common element in all three drugs is the pseudo VI sequence. In fact, since tyrosine and valine are both hydrophobic, one could argue that the common element is the YVI of phomopsin A and ustiloxin A and the VVI of dolastatin 10.

In separate studies Bai *et al.* [34, 35] used the following peptide analogs of dolastatin 10: two tripeptides, pseudo-VVI and pseudo-IPF, and a tetrapeptide, pseudo-VIPF, all synthesized with the same substituents and modifications as in the intact molecule of dolastatin 10. They found that the two tripeptides do not affect vinblastine binding and are weak inhibitors of microtubule assembly. The tetrapeptide weakly inhibits vinblastine binding and is a better inhibitor of microtubule assembly. All three inhibit GTP hydrolysis. The tripeptide pseudo-IPF is the least effective at inhibiting microtubule assembly and GTP hydrolysis. All three peptides are weak inhibitors of β^s formation, with the tetrapeptide being more effective than the two tripeptides. These results suggest that the pseudo-VI portion of these peptides is the most critical for activity, and it is perhaps significant that this is a structural theme common to both ustiloxin A and phomopsin A. Perhaps the other parts of the structure are necessary to hold the pseudo VI in a conformation where it fits correctly into the tubulin molecule. It is likely that this is the effect of the large ring structure present in both ustiloxin A and phomopsin A. Even though the substituents on this ring differ between the two molecules, it is perhaps the size and general arrangement of the ring that serves this purpose. Conceivably the lack of the ring in dolastatin 10 or the change from pseudo-YVI in ustiloxin A and phomopsin A to pseudo-VVI in dolastatin 10 is responsible for the fact that dolastatin 10 is unable to induce the apparent conformational change in tubulin manifested by the decreased exposure of hydrophobic areas caused by incubation for 20 min with either ustiloxin A or phomopsin A. Although the details of this argument are speculative, it seems that the strong stabilizing effect on tubulin of these modified peptide ligands is correlated with their containing a short segment of hydrophobic amino acids. This conclusion is consistent with the earlier observation of Lacey *et al.* [19] who found that analogues of phomopsin A with either the chloride removed or the terminal pseudo-aspartate removed or altered interact with tubulin essentially as well as does phomopsin A; these analogues have the same short segment of hydrophobic amino acids as does phomopsin A.

We have reported previously that phomopsin A, of all known tubulin ligands, is the most effective at stabilizing the tubulin molecule against decay. Our findings reported here indicate that the effects of ustiloxin A are indistinguishable from those of phomopsin A and suggest that ustiloxin A could be effective in stabilizing tubulin for structural studies, in addition to being a useful probe for studies on the conformation of the tubulin molecule.

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