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USTILOXINS, NEW ANTIMITOTIC CYCLIC PEPTIDES: INTERACTION WITH PORCINE BRAIN TUBULIN

YIN LI,* YUKIKO KOISO, HISAYOSHI KOBAYASHI, YUICHI HASHIMOTO and SHIGEO IWASAKI

Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

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Abstract—Biochemical and electron microscopic studies demonstrated that ustiloxins A–D, which are antimitotic 13-membered cyclic peptides produced by the rice plant pathogen *Ustilaginoidea virens*, strongly inhibited the polymerization of porcine brain tubulin *in vitro* and depolymerized pre-formed microtubules. The IC_{50} values of polymerization inhibited by ustiloxins A–D were determined to be 0.7, 2.8, 4.4 and 6.6 μM, respectively, under the experimental conditions used, indicating that ustiloxin A is the most potent inhibitor of tubulin polymerization currently known. Ustiloxins A–C were found to inhibit the binding of radiolabelled rhizoxin to tubulin with inhibition constants (K_i) of 0.08, 0.13 and 0.23 μM, respectively, and also inhibited the binding of radiolabelled phomopsin A as strongly as rhizoxin. These results suggest that the binding site of ustiloxins is identical with that of rhizoxin.

Key words: ustiloxin; cyclic peptide; antimitotic; tubulin; rhizoxin; phomopsin A

Ustiloxins A, B, C and D, antimitotic 13-membered cyclic peptides, were isolated from the water extract of false smuts produced on rice panicles by the pathogen *Ustilaginoidea virens* (Fig. 1) [1, 2]. Ustiloxins A and B induced abnormal swelling of rice seedling roots as does rhizoxin, a potent antimitotic antibiotic [3]. Ustiloxins A and B inhibited the mitosis of a variety of human tumour cell lines [2]. Injection of a crude mixture of ustiloxins or ustiloxin A into mice resulted in acute necrosis of isolated hepatocytes and renal tubular cells, followed by mitotic arrest and abnormal mitosis resembling that caused by colchicine [4] and phomopsin A, an anti-tubulin mycotoxin that causes lupinosis [5–7]. These facts suggested that ustiloxins interfere with microtubule function.

There are a number of antimitotic agents that bind to either the CLC† binding site or the VLB binding site [8]. We have been studying ligands that bind to the latter site, and recently reported that (i) RZX binds to tubulin in a competitive manner with respect to MAY [9] and ansamitocin P-3 [10]; (ii) RZX and MAY affect the binding of VLB to tubulin, but their binding site is not identical to the VLB site, indicating the presence of a distinct RZX/MAY site [10]; (iii) phomopsin A binds to tubulin at two (or more) sites, one of which is identical to the RZX/MAY site [11]; and (iv) dolastatin 10 also binds to the RZX/MAY site, though it, like phomopsin A, appears to have an additional binding site(s) on tubulin [12].

This paper deals with the effects of ustiloxins on microtubule assembly and on the binding of radiolabelled RZX and phomopsin A to tubulin.

* Corresponding author: Tel. 03-3812-2111 Ext. 7847; FAX 03-5684-8629.

† Abbreviations: CLC, colchicine; VLB, vinblastine; RZX, rhizoxin; MAY, maytansine; MES, (*N*-morpholino)ethanesulphone acid.

MATERIALS AND METHODS

Materials. Ustiloxins were obtained from false smuts as described by Koiso *et al.* [2]. Phomopsin A and [^{14}C]phomopsin A (14 mCi/mmol) were obtained from a shaken culture of the fungus *Phomopsis leptostromiformis*, and purified as described by Li *et al.* [11]. RZX was produced by *Rhizopus chinensis* as described by Iwasaki *et al.* [12] while [^{14}C]rhizoxin was prepared biosynthetically as described previously [13]. [^{3}H]Colchicine was purchased from Amersham (U.K.). Synthetic dolastatin 10 [14] was a kind gift from Dr T. Shioiri. Vinblastine was purchased from Sigma (St. Louis, MO, U.S.A.). Ansamitocin P-3 was donated by the Central Research Institute of Takeda Chemical Industries (Japan).

Tubulin preparation. Microtubule protein was prepared from porcine brain as described previously [15]. A typical run is as follows: fresh brains cooled on ice and washed with aqueous solution containing 100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.5, were homogenized in 0.5 mL/g of MES buffer (100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 1 mM GTP, pH 6.5) under ice cooling. After centrifugation (50,000 *g*, 30 min, 4°), the supernatant was mixed with an equal volume of glycerol buffer (MES buffer and 8 M glycerol, pH 6.5) and warmed at 37° for 40 min to polymerize tubulin. The polymerized microtubule protein was collected by centrifugation at (100,000 *g*, 45 min, 25°), resuspended in MES buffer and chilled (0°, 30 min) to depolymerize. After centrifugation of the suspension (100,000 *g*, 60 min, 4°), the supernatant was collected. The same polymerization/depolymerization process was repeated, and an equal volume of glycerol buffer was added to the supernatant solution to be stored at −70°. The microtubule protein thus obtained was used for the polymerization/depolymerization assay. For the

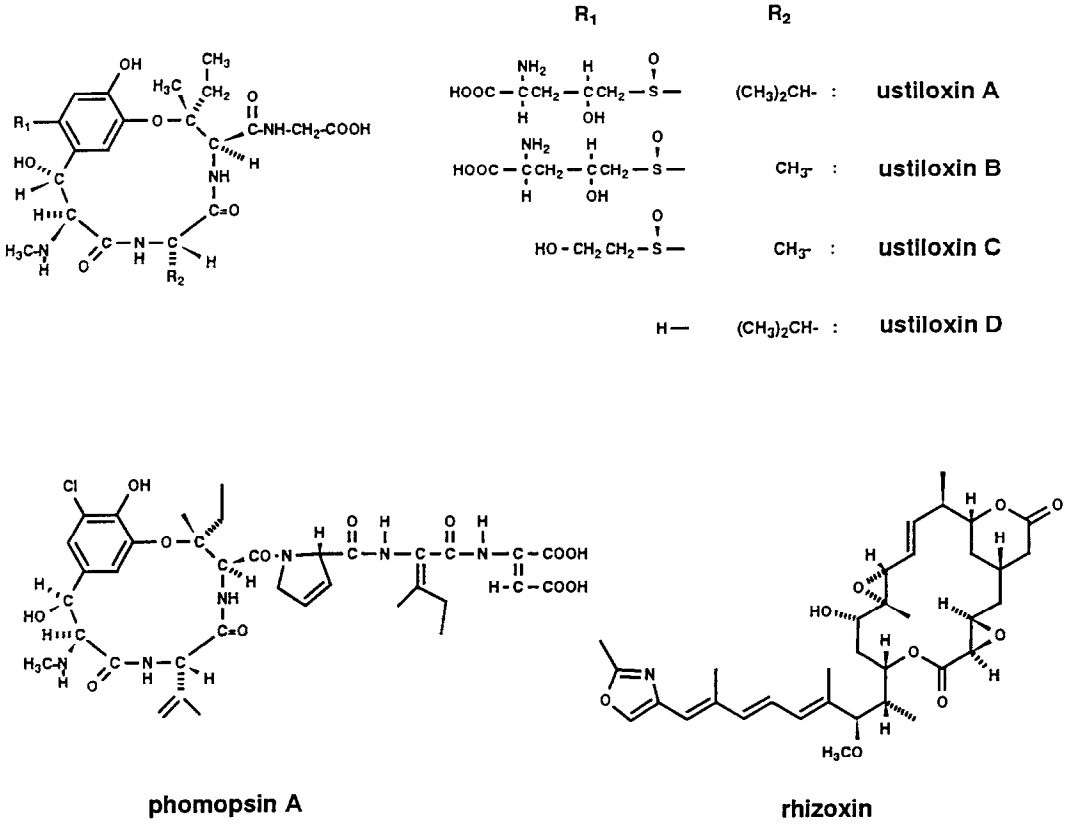

Fig. 1. Structures of ustiloxins, phomopsin A and rhizoxin.

ligand-binding assay, tubulin was purified from the microtubule protein by phosphocellulose column chromatography [16] and its purity evaluated by polyacrylamide gel electrophoresis [17]. Protein concentration was determined by the method of Lowry *et al.*, using BSA as a standard [18]. The results to be compared directly were obtained under the same experimental conditions using the same protein preparation.

Polymerization and depolymerization assay. Microtubule polymerization and depolymerization were followed by turbidity measurement at 37° in microtubule assembly buffer as described previously [10]. Experiments were repeated several times and the results were reproducible.

Electron microscopy. For electron microscopy of ustiloxin A-treated tubulin and microtubules polymerized *in vitro*, a negative staining method was used [19]. Samples (0.5 mL) containing microtubule proteins were incubated for 20 min at 37°. The mixtures were diluted in microtubule assembly buffer [20] containing 2% glutaraldehyde (microtubule proteins 0.5 mg/mL). After a few minutes, a drop of each solution was applied to a formvar film on a 150 mesh copper grid. The samples were then negatively stained with 2% uranyl acetate solution and air-dried. Specimens were examined on a JEOL 200 CX electron microscope at 100 kV.

Binding assay. The binding of [^{3}H]colchicine, [^{14}C]rhizoxin and [^{14}C]phomopsin A to tubulin was evaluated by the dextran-coated-charcoal method using 1% of charcoal (Sigma)–0.1% of dextran (Sigma)–20 mM Tris (pH 8.0)–10% DMSO [11]. Phosphocellulose-purified tubulin (final concentration; 0.5 mg/mL) and the radiolabelled ligand (final concentration; 3 μM) were incubated in microtubule assembly buffer with various concentrations of drug for 20 min at 37°. For the water insoluble compounds, DMSO was used as a co-solvent. The final concentration of DMSO did not exceed 2%, which had no influence on drug binding to tubulin.

RESULTS

Effect of ustiloxins A–D on microtubule assembly

The effects of ustiloxins A, B, C and D on polymerization of porcine brain tubulin and on depolymerization of microtubules polymerized *in vitro* are summarized in Figs 2(A) and (B), respectively.

Tubulin polymerization proceeded rapidly and was completed in less than 20 min under the conditions used. Ustiloxins A, B, C and D inhibited tubulin polymerization in a concentration-dependent manner (Fig. 2(A)) while IC_{50} values were estimated to be 0.7, 2.8, 4.4 and 6.6 μM, respectively, under the experimental conditions used. These compounds

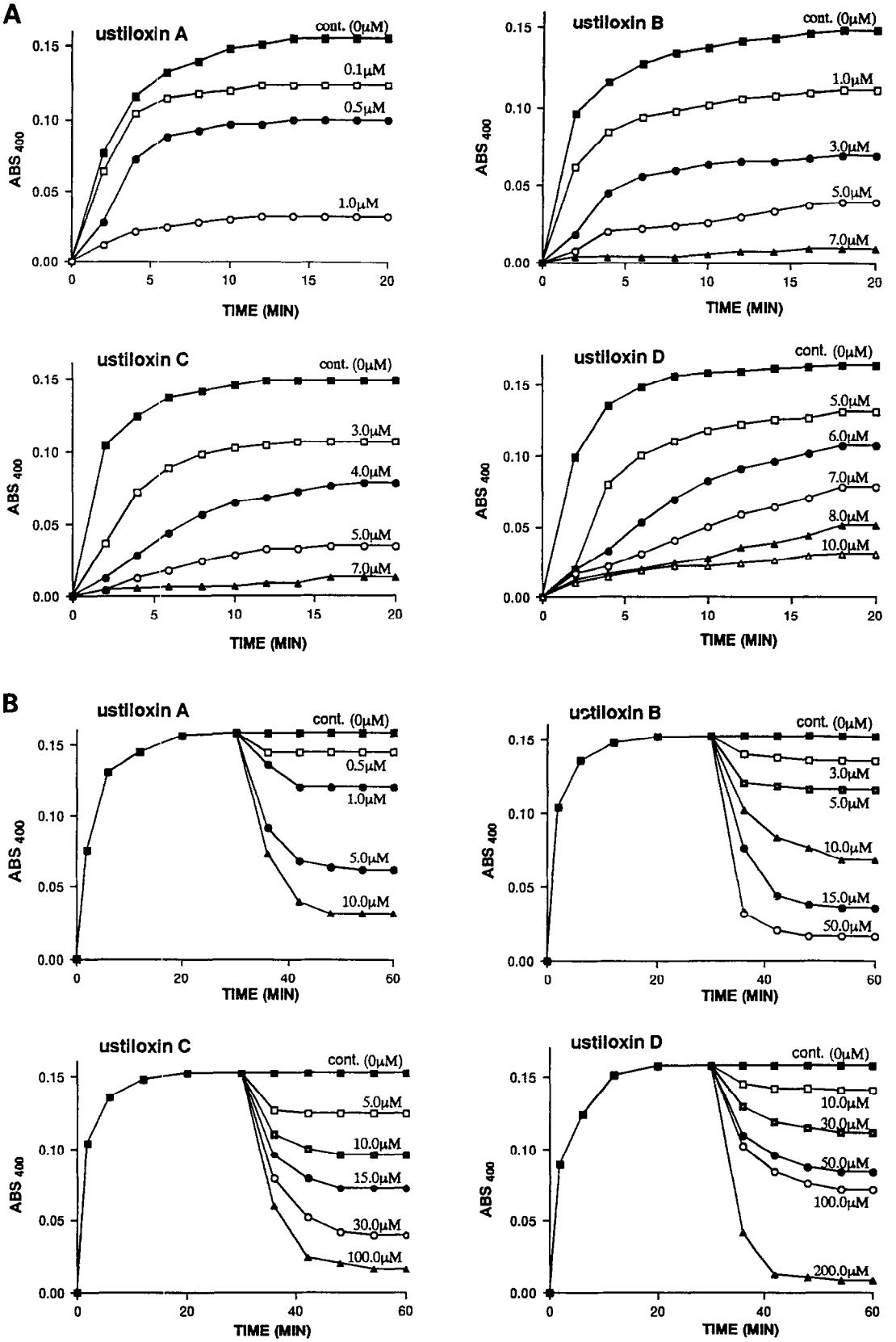

Fig. 2. Effect of ustiloxins on the *in vitro* polymerization and depolymerization of microtubule proteins. (A) Various concentrations of ustiloxins were mixed with microtubule proteins (2 mg/mL) at 0° and incubated at 37°. (B) Microtubule proteins (2 mg/mL) were incubated at 37°. Various concentrations of ustiloxins were added 30 min later.

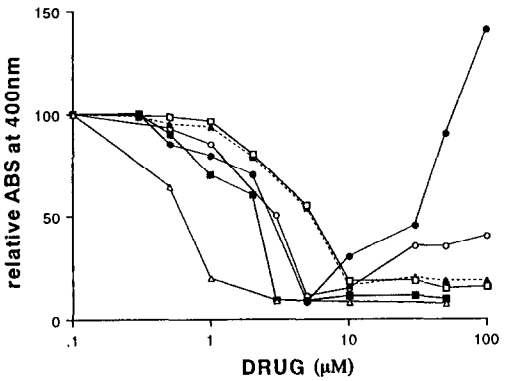

Fig. 3. Effect of various antimitotic agents on microtubule assembly. Microtubule proteins (2 mg/mL) were mixed with various concentrations of drugs at 0° and incubated at 37°. Relative absorbance after 20 min was plotted versus drug concentration. The drugs are as follows: (△) ustiloxins A, (■) phomopsin A, (□) ansamitocin P-3, (●) vinblastine, (○) dolastatin 10, and (▲) rhizoxin.

also induced the depolymerization of preformed microtubules. The time courses of polymerization and depolymerization are shown in terms of turbidity changes in Fig. 2(B). The drugs were added after 30 min incubation of the protein at 37°. Addition of 1.0 and 10 μM ustiloxin A induced approximately 25% and 80% depolymerization, respectively. Similar effects were seen with ustiloxins B (25% at 5 μM and 90% at 50 μM), C (30% at 10 μM and 90% at 100 μM) and D (30% at 30 μM and 95% at 200 μM).

Ustiloxin A is thus the most potent inhibitor of polymerization of porcine brain tubulin currently known. The other three compounds also showed inhibitory activities comparable to or greater than those of rhizoxin, ansamitocin p-3, phomopsin A, dolastatin 10 and vinblastine, whose IC_{50} values were given as 5.0, 5.0, 2.4, 3.0 and 2.0 μM, respectively, from a set of experiments. Unlike vinblastine and dolastatin 10 [12], ustiloxins induced no turbidity increase with higher concentrations (> 10 μM) (Fig. 3).

The effect of ustiloxin A on microtubule assembly was further examined by electron microscopic analysis (Figs 4(A) and 4(B)). The control sample without drug treatment showed singlet microtubules of normal cylindrical structure (Fig. 4(A)), while a sample treated with 10 μM ustiloxin A for 10 min showed dissociated microtubule proteins (Fig. 4(B)). This confirmed the result of turbidity measurement. No evidence of irregular aggregation was observed by treatment with higher concentrations of ustiloxin A.

Analysis of the ustiloxin-binding site on porcine brain tubulin

In order to characterize the binding site of ustiloxins on tubulin, we conducted binding competition experiments using other antimitotic agents as reference compounds. Ustiloxins A, B and C inhibited the binding of radiolabelled rhizoxin to tubulin with IC_{50} values of 1.0, 1.5 and 2.6 μM, respectively (Fig. 5). Their inhibition constants (K_i) were estimated to be 0.8×10^{-7} M, 1.3×10^{-7} M and 2.3×10^{-7} M, respectively, using the dissociation constant of rhizoxin (that of rhizoxin was 1.7×10^{-7} M [10] and the concentrations of these drugs giving 50% inhibition of rhizoxin binding. Inhibition of the binding of radiolabelled phomopsin A to tubulin by ustiloxins A, B and C was similarly examined. The inhibition curves are compared with that for rhizoxin in Fig. 6. The inhibition of phomopsin A binding to tubulin by each ustiloxins

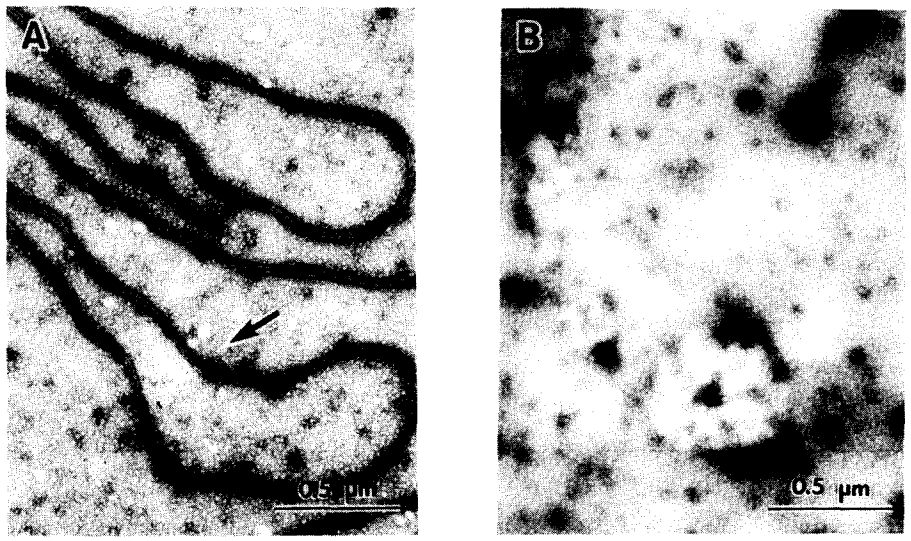

Fig. 4. Electron micrographs of microtubule proteins incubated at 37° for 30 min in (A) the absence, and (B) the presence of 10 μM ustiloxin A.

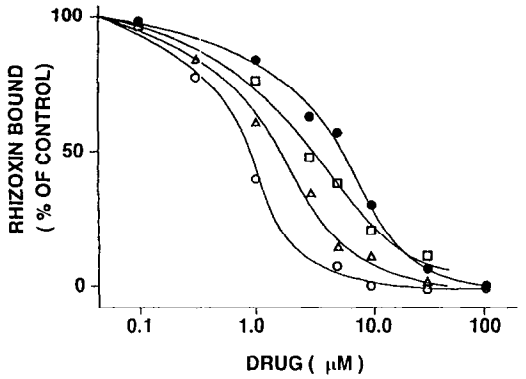

Fig. 5. Effect of ustiloxins A–C on binding of [^{14}C]rhizoxin to tubulin. (●) Rhizoxin, (□) ustiloxin C, (△) ustiloxin B, and (○) ustiloxin A.

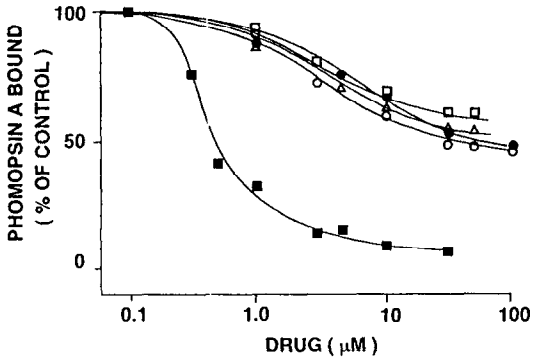

Fig. 6. Effect of various antimitotic drugs on the binding of [^{14}C]phomopsin A to tubulin. (□) Ustiloxin C, (△) ustiloxin B, (●) rhizoxin, (○) ustiloxin A, and (■) phomopsin A.

(A, B and C) was as strong as that by rhizoxin, and was, at maximum, approximately 50%, which is the same as in the case of rhizoxin. We previously reported, based on tubulin–ligand binding competition assay and the Scatchard analysis for binding of rhizoxin and phomopsin A, that (1) rhizoxin binds to tubulin at one site which is designated as the RZX/MAY site and (2) phomopsin A binds at two major sites, one of which is the RZX/MAY site [10, 11]. Present data shown in Figs 5 and 6 suggest that ustiloxins A–C bind to tubulin at the RZX/MAY site but not at the second site, where phomopsin A binds. This could not be established for ustiloxin D because of the limited amount available.

Ustiloxins showed no inhibition of [^{3}H]colchicine binding to tubulin (data not shown), but, as reported [21], ustiloxin A strongly stabilized the binding of [^{3}H]colchicine to tubulin.

DISCUSSION

Ustiloxins are unique cyclic tetrapeptides containing a 13-membered ring formed from three amino acid units through two peptide bonds and an ether linkage. The cyclic structure is closely related to that of phomopsin A (Fig. 1). Biological properties of ustiloxins also resemble those of phomopsin A; all strongly inhibit the polymerization of brain tubulin, competitively bind to tubulin with respect to rhizoxin (Fig. 5 [11]) and cause similar mycotoxicosis [4], but show no antifungal activity [2]. In addition, ustiloxin A, like phomopsin A, strongly stabilizes the binding of colchicine to tubulin [21]. These facts suggest that they act in a very similar fashion biologically. The present study, indeed, proved that both share the same binding site on tubulin (RZX/MAY site) though phomopsin A also binds to another site [11].

Ustiloxins A, B and C strongly inhibited the binding of both radiolabelled rhizoxin and radiolabelled phomopsin A to tubulin. It should be noted that ustiloxins inhibited rhizoxin-binding almost completely at the concentration of *c.* 50 μM (Fig. 5), whereas their inhibitions of phomopsin A-binding attained, at most, a maximum of *c.* 50% (Fig. 6). The inhibition patterns of phomopsin A-binding by ustiloxins are quite similar to those obtained with rhizoxin [11]. These facts indicate that ustiloxin-binding to tubulin competes with phomopsin A only at the RZX/MAY site and does not inhibit phomopsin A-binding at its second binding site (Fig. 6), though the use of radiolabelled ustiloxins is required to determine whether ustiloxins have an additional binding site on tubulin.

A common structural element present in both ustiloxin A and phomopsin A is the 13-membered ring, which may be the fundamental structural element necessary for their interaction with tubulin at the RZX/MAY site. Ustiloxin D, consisting only of the 13-membered ring system and a glycine unit, showed significant anti-tubulin activity (IC_{50}: 6.6 μM), and this compound could be a useful 'lead' in both investigating the interaction between tubulin and the ustiloxin–phomopsin class of compounds and analysing the functional requirements for RZX/MAY site ligands.

On the other hand, it would be intriguing to clarify the function of the unique tripeptidic phomopsin A side chain that may be responsible for phomopsin A-binding to tubulin at its second binding site.

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