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INTERACTION OF PHOMOPSIN A WITH PORCINE BRAIN TUBULIN

INHIBITION OF TUBULIN POLYMERIZATION AND BINDING AT A RHIZOXIN BINDING SITE

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Abstract—Phomopsin A is an antimitotic cyclic peptide containing a 13-member ring including an ether linkage. It was isolated from the fungus *Phomopsis leptostromiformis* as the causal agent of lupinosis. Phomopsin A strongly inhibited microtubule assembly (IC_{50}: 2.4 μM). Our study using radiolabeled phomopsin A, prepared biosynthetically by feeding L-[U-^{14}C]isoleucine to the culture of *P. leptostromiformis*, indicated that at least two binding sites of phomopsin A exist on tubulin on the basis of a Scatchard analysis; i.e. the dissociation constants of a high affinity site (K_{d_1}) and a low affinity site (K_{d_2}) at 37° were determined to be 1×10^{-8} and 3×10^{-7} M, respectively. Phomopsin A inhibited the binding of radiolabeled rhizoxin to tubulin with an inhibition constant (K_i) of 0.8×10^{-8} M. This showed that the high affinity site of phomopsin A is identical to the rhizoxin binding site. The binding of the radiolabeled phomopsin A was also inhibited by rhizoxin and ansamitocin P-3, with an inhibition constant of 10^{-7} M, and to a lesser extent by vinblastine. Phomopsin A had no inhibitory effect on colchicine binding to tubulin.

Phomopsin A was isolated as a mycotoxin produced by the fungus *Phomopsin leptostromiformis* which is responsible for lupin poisoning in animals (lupinosis) [1]. It is a hexapeptide composed of modified amino acids with a 13-membered ring including an ether linkage (Fig. 1) [2, 3]. The structural assignment of this compound was based on an X-ray crystallographic analysis [4]. It has been reported that phomopsin A potently inhibited the polymerization of pig and sheep brain tubulin, and that its binding to tubulin did not compete with colchicine binding, but it inhibited the binding in a non-competitive manner of radiolabeled vinblastine and vincristine to tubulin [5–8].

There are a number of antimitotic agents which bind to the colchicine site and some information on the structure–activity relationships and the binding sites of these compounds has been accumulated. However, only a few compounds are known to bind to the site affecting the vinblastine-binding. Also, the relationships among these compounds are little known. We reported recently that rhizoxin binds to tubulin in a manner completely competitive to maytansine and ansamitocin P-3, a maytansine analogue, and that the binding sites may overlap, but they are not identical to the vinblastine site [9, 10]. We have now analysed directly the phomopsin A binding to porcine brain tubulin using radiolabeled compounds.

In this paper we describe: (i) phomopsin A production in shaken cultures and preparation of [^{14}C]phomopsin A, (ii) the determination of the dissociation constant (K_d) of phomopsin A, and (iii) the binding competition of phomopsin A with typical antimitotic agents such as colchicine, vinblastine, rhizoxin and ansamitocin P-3 (structural formulas are shown in Fig. 2).

MATERIALS AND METHODS

Materials. Phomopsin A was obtained from the fungus *P. leptostromiformis* ATCC-115 in shaken cultures. The medium of vegetative cultures and stores for the strain have been described by Lanigan *et al.* [11]. The medium used to produce phomopsin A consisted of glucose 2%, yeast extract 1%, KCl 0.05% and 100 mL of potato soup (extracted from 20 g of potato). Cultures were grown in 100-mL volumes of medium in 500-mL Sakaguchi-flasks for 7–10 days at 27° on a rotary shaker at 120 rpm. Phomopsin A was isolated from the fermentation broth by solvent extraction. The fermentation broth (1000 mL) was filtered and the filtrate was fractionated by Amberlite XAD-2 column chromatography eluted with water, water:methanol (1:1 v/v), and methanol, successively. The methanol eluate was evaporated to dryness in a vacuum at 40° to give the residue (157 mg) which was then separated by successive Sephadex LH-20 column chromatography eluted with methanol and silica gel column chromatography eluted with chloroform:methanol:water (9:10:1 by vol.), and methanol. The active fraction (20 mg) obtained from the methanol eluates was crystallized from acetonitrile:0.05% trifluoroacetic acid (1:1 v/v) to give colorless needles. The residual phomopsin A contained in the original eluate was purified by HPLC (Asahipac ODP-50, CH_3CN:0.05%

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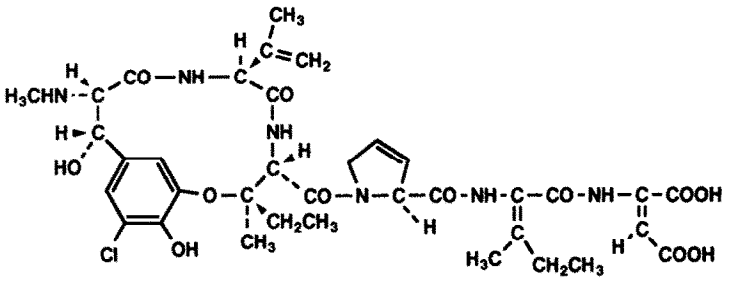
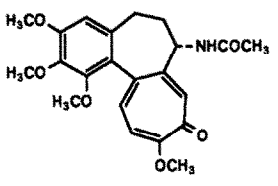
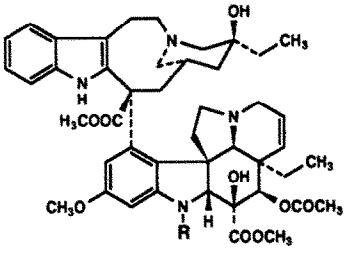


PHOMOPSIN A

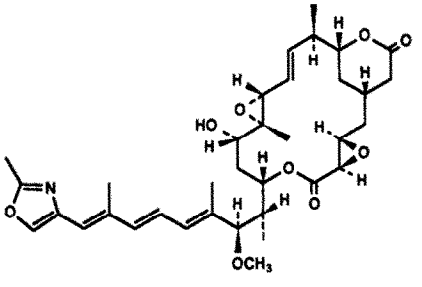
Fig. 1. Structure of phomopsin A.



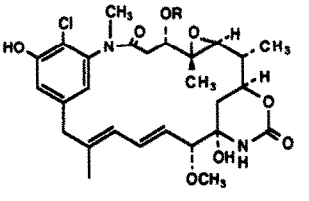
COLCHICINE



VINBLASTINE: R=CH_3
VINCRISTINE: R=CHO



RHIZOXIN



MAYTANSINE : R=$CH(CH_3)N(CH_3)COCH_3$
ANSAMITOCIN P-3 : R=$CH(CH_3)_2$

Fig. 2. Structural formulas of colchicine, *Vinca* alkaloids, maytansinoids and rhizoxin.

trifluoroacetic acid = 18:82). A total of 10 mg of phomopsin A was obtained. [^{14}C]Phomopsin A was prepared biosynthetically by feeding L-[U-^{14}C]-isoleucine (320 mCi/mmol) to the culture of *P. leptostromiformis* ATCC-115. The cultures were grown in 20 mL of the medium described above, in 100-mL cotton-plugged bottles. L-[U-^{14}C]Isoleucine (10 μCi/mL) was added after 4 days of cultivation and incubation was continued for a further 7–10 days. [^{14}C]Phomopsin A was purified by successive chromatography (polystyrene beads: Amberlite XAD-2, eluted with methanol), silica gel column chromatography (butanol:acetic acid:water = 3:1:1) and HPLC (Asahipac ODP-50, CH_3CN:0.05% TFA = 18:82). Colchicine and vinblastine were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]Colchicine (33.4 Ci/mmol) and [^{3}H]vinblastine (10.7 Ci/mmol) were purchased from Amersham (U.K.). Rhizoxin was isolated from the culture broth of *R. chinensis* Saito Rh-2. [^{14}C]Rhizoxin (69.3 mCi/mmol) was obtained biosynthetically and purified by silica gel column chromatography and HPLC as described previously [12]. Ansamitocin P-3 was donated by the Central Research Institute of Takeda Chemical Industry.

Tubulin preparation. Microtubule protein was prepared from porcine brain as described previously [13]. Tubulin was purified from microtubule protein by phosphocellulose chromatography [14]. The purity of each protein was assayed by polyacrylamide gel electrophoresis [15]. Protein concentrations were determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard. All the

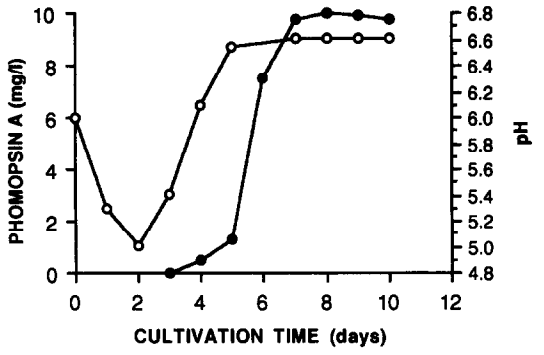


Fig. 3. Time-course of phomopsin A production in 100-mL volumes of medium in 500-mL Sakaguchi-flasks at 27° on a rotary shaker. (●) Phomopsin A; (○) pH.

experiments recorded in this paper were carried out in microtubule assemble buffer containing 100 mM 4-morphine-ethanesulfonic acid (Mes*), 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 1 mM GTP (pH 6.5).

Binding assay. The binding of [^{14}C]phomopsin A, [^{14}C]rhizoxin, [^{3}H]vinblastine and [^{3}H]colchicine to tubulin was evaluated by the D.C.C. method (using charcoal, Sigma, 1 g; dextran, Sigma, 0.1 g; 100 mL of 0.6 M NaCl–20 mM Tris pH 8.0–10% DMSO) [17, 18]. Porcine brain tubulin (0.15–0.5 mg/mL) and the radiolabeled compounds were incubated in microtubule assembly buffer with various concentrations of drug for 20 min at 37°. To the samples (0.5 mL) was added 0.2 mL of a cold D.C.C. suspension and they were kept at 4° for exactly 20 min. The mixtures were centrifuged at 10,000 *g* for 7 min at 4° and 0.6 mL of each supernatant was taken to measure the radioactivity in 4 mL of Atomlight (NEN). Concentrations of unbound drug were estimated from the total and bound drug concentrations.

Other methods. Microtubulin polymerizations were followed by turbidity measurements at 37° in microtubule assembly buffer as described previously [6, 8, 19].

RESULTS

Phomopsin A production in shaken cultures and preparation of [^{14}C]phomopsin A

The time-course of phomopsin A production in 100-mL volumes of medium at 27° on a rotary shaker and the change of medium pH are shown in Fig. 3. Phomopsin A production proceeded steadily during the first 5 days and reached a maximum in 7–9 days. A yield of phomopsin A was obtained of *ca.* 10 mg/L (purity: >90%). The medium pH decreased during the first 2 days and then rose steadily until the 5th day of cultivation. [^{14}C]phomopsin A was prepared biosynthetically by feeding L-[U-^{14}C]isoleucin to the shaken cultures. Approximately 150 μg of radiolabeled phomopsin A (14 mCi/mmol) was obtained from 60 mL of medium.

Effect of phomopsin A on polymerized microtubule proteins

The inhibitory effects of phomopsin A on the polymerization reaction of porcine microtubule protein are shown in Fig. 4A. Polymerization of tubulin proceeded rapidly and was completed in less than 10 min as shown in the turbidity curve (Fig. 4A). Phomopsin A inhibited tubulin polymerization in a concentration-dependent manner. At 5 μM of phomopsin A, there was almost complete (100%) inhibition. The IC_{50} value was estimated to be 2.4 μM under the experimental conditions used indicating phomopsin A as being twice as potent as rhizoxin [9]. Phomopsin A also depolymerized the polymerized tubulin. The time-courses of the depolymerization by the addition of various concentrations of phomopsin A are shown in Fig. 4B. The drug was added after a 30-min incubation of the microtubule protein at 37°. Adding 2.0 and 10.0 μM of the drug induced approximately 25 and 70% depolymerization, respectively, after 10 min incubation. Both the inhibition of microtubule polymerization by phomopsin A and its microtubule depolymerization effect were more potent than those of rhizoxin, maytansine or colchicine.

Analysis of phomopsin A-binding to tubulin

The time-course of phomopsin A binding to tubulin at 37° is shown in Fig. 5. It reached equilibrium in less than 3 min and the binding persisted for longer than 24 hr. The number of binding sites of phomopsin A on tubulin and the dissociation constants (K_d) were determined by Scatchard analysis using [^{14}C]phomopsin A (Fig. 6). As shown in the Figure, Scatchard plot could be represented by two straight lines by the least squares method. Good linearities were observed with r values of 0.840 and 0.880, indicating that there are at least two distinct binding sites of phomopsin A on tubulin. The dissociation constants of the high affinity site (K_{d_1}) and the low affinity site (K_{d_2}) were determined to be 1×10^{-8} and 3×10^{-7} M, respectively, under the experimental conditions used. The high affinity to tubulin of phomopsin A corresponds with its potent activity in both tubulin polymerization inhibition and microtubule depolymerization.

Interaction of phomopsin A and the typical antimitotic agents in binding to porcine brain tubulin

Phomopsin A competitively inhibits [^{14}C]rhizoxin binding to tubulin with a K_i value of 0.8×10^{-8} M at 37° (Fig. 7). This K_i value is close to the K_{d_1} value, indicating that the high affinity site is identical to the rhizoxin site. On the other hand, colchicine showed no effect on [^{14}C]colchicine binding to tubulin, indicating that phomopsin A does not bind to the colchicine site (Fig. 8).

Next we studied the inhibition of [^{14}C]phomopsin A binding to tubulin by vinblastine, rhozoxin and ansamitocin P-3 (Fig. 9). All three compounds inhibited [^{14}C]phomopsin A binding to tubulin.

* Abbreviations: Mes, 4-morpholine-ethanesulfonic acid; D.C.C., dextran-coated charcoal; DMSO, dimethyl sulfoxide.

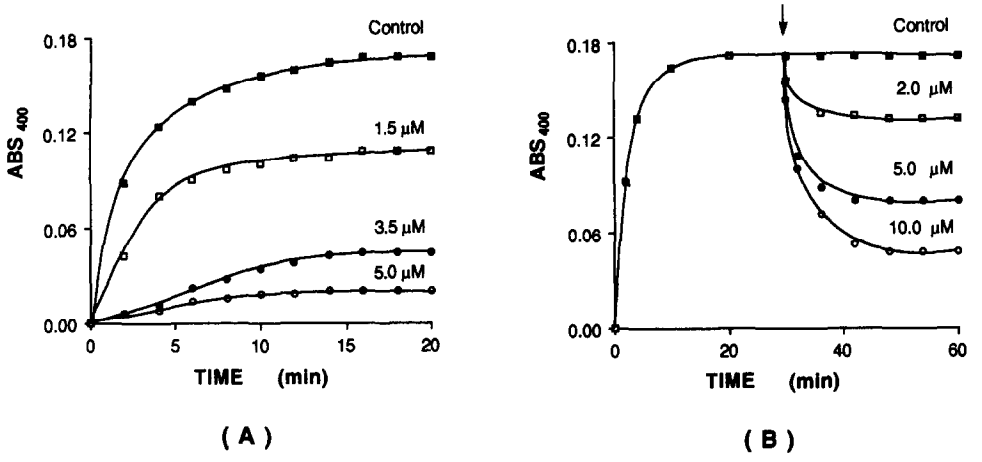


Fig. 4. Effect of phomopsin A on the *in vitro* polymerization/depolymerization of microtubule proteins. (A) Various concentrations of phomopsin A were mixed with microtubule protein (2 mg/mL) at 0° and incubated at 37°. The absorbance at 400 nm (ABS_{400}) was measured. (B) Microtubule proteins (2 mg/mL) were incubated at 37°. Various concentrations of phomopsin A were added 30 min later.

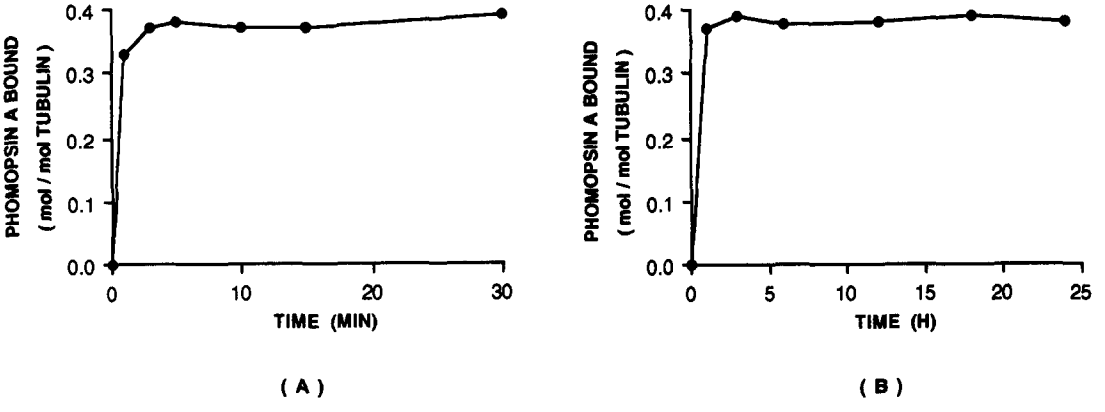


Fig. 5. Time-course of [^{14}C]phomopsin A binding to tubulin. (A) Binding of [^{14}C]phomopsin A to tubulin saturates within 3 min. Pure tubulin (0.5 mg/mL) was incubated at 37° in 100 mM Mes (pH 6.9) with [^{14}C]phomopsin A (3×10^{-6} M). To the samples (0.5 mL) were added 0.2 mL of a cold D.C.C. suspension and they were kept at 4° exactly for 20 min. The mixtures were centrifuged at 10,000 *g* for 7 min at 4°. The supernatant (0.6 mL) was used to measure the radioactivity in 4 mL of Atomlight (NEN). (B) Binding of [^{14}C]phomopsin A to tubulin persists for at least 24 hr.

Rhizoxin and ansamitocin P-3 inhibited similarly. The K_i values of rhizoxin and ansamitocin P-3 could not be determined accurately but were estimated to be in the order of 10^{-7} M from their IC_{50} values (Fig. 9; 6×10^{-6} and 6.6×10^{-6} M, respectively). These values were calculated based on our conclusion that only one of two phomopsin A binding sites can be occupied competitively by these drugs. Vinblastine showed much weaker inhibition of phomopsin A binding compared with rhizoxin and ansamitocin P-3*, in spite of the same order of binding affinity to tubulin as the other two compounds. On the other hand, vinblastine binding was inhibited strongly by phomopsin A (data not shown).

* Y. Kato; unpublished data: K_d 3.7×10^{-7} M.

DISCUSSION

Phomopsin A is a unique peptide containing a 13-membered ring and a side arm composed of six modified amino acids (Fig. 1) [2, 3]. Inhibition of microtubule assembly by this compound and its competition in the binding to tubulin with other antimitotic agents have been studied [5, 6, 7, 20, 21]. The reported data and our result (Fig. 4) indicate that phomopsin A is the most potent inhibitor of microtubule assembly among the known antimitotic agents. It has also been shown that the phomopsin A binding site on sheep and bovine brain tubulin may overlap with those of vinblastine, vincristine, maytansine and dolastatin 10 [20]. However, no direct binding assay for this compound has

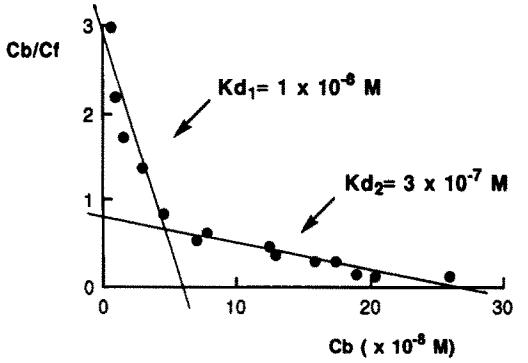


Fig. 6. Scatchard analysis of the binding of [^{14}C]phomopsin A to tubulin. Pure tubulin (0.15 mg/mL) was incubated at 37° in 100 mM (pH 6.9) for 20 min with various concentrations of [^{14}C]phomopsin A (0.75–300 × 10^{-8} M). To the samples (0.5 mL) were added 0.2 mL of a cold D.C.C. suspension and they were kept at 4° for exactly 20 min. The mixtures were centrifuged at 10,000 *g* for 7 min at 4°. The supernatant (0.6 mL) was used to measure the radioactivity in 4 mL of Atomlight (NEN). Cb: bound phomopsin A concentration; Cf: unbound phomopsin A concentration.

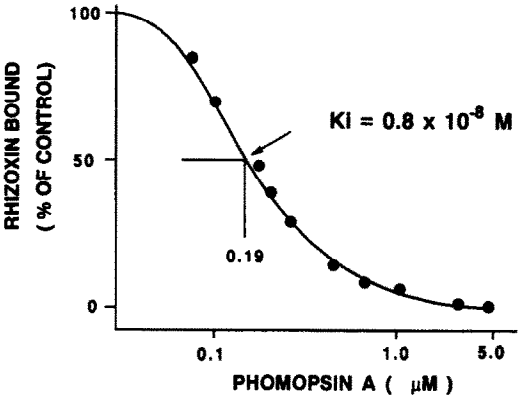


Fig. 7. Effect of phomopsin A on the binding of [^{14}C]-rhizoxin to tubulin. Each 0.5 mL of reaction mixture contained 0.15 mg/mL tubulin, 100 mM Mes (pH 6.9), 3 μM [^{14}C]rhizoxin and 5% (v/v) DMSO. The K_i value was estimated by the dissociation constant (K_d) of [^{14}C]rhizoxin binding to tubulin and the 50% inhibition of phomopsin A on the binding of [^{14}C]rhizoxin to tubulin.

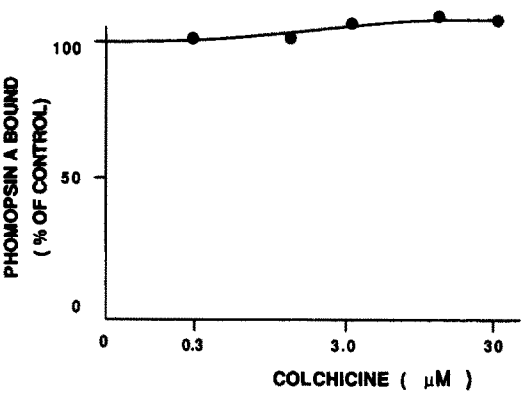


Fig. 8. Effect of colchicine on the binding of [^{14}C]phomopsin A to tubulin. Each 0.5-mL of reaction mixture contained 0.5 mg/mL tubulin, 100 mM Mes (pH 6.9), and 3 μM [^{14}C]-phomopsin A and 5% (v/v) DMSO.

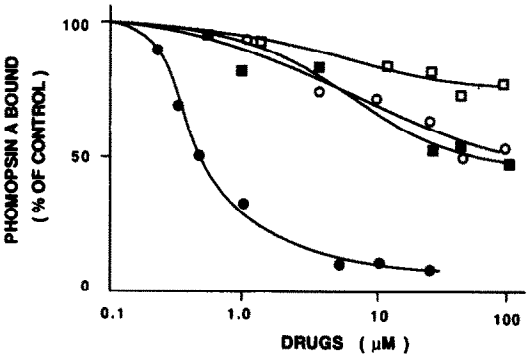


Fig. 9. Comparison of the effects of phomopsin A, rhizoxin, ansamitocin P-3 and vinblastine on the binding of [^{14}C]-phomopsin A to tubulin. Each 0.5 mL of reaction mixture contained tubulin (0.5 mg/mL), 100 mM Mes (pH 6.9), 3 μM [^{14}C]phomopsin A, 5% (v/v) DMSO and, if present, the indicated concentration of inhibiting drug: (●) phomopsin A; (■) rhizoxin; (○) ansamitocin P-3; and (□) vinblastine.

been reported, presumably because radiolabeled phomopsin A was not available. Therefore, we attempted first biosynthetic preparation of [^{14}C]-phomopsin A, which was made possible by establishing the shaken culture method. The reported culture method took more than 6 weeks [11] but it took only 1 week of the shaken culture method to obtain phomopsin A at a yield of 10 mg/L.

Scatchard analysis using radiolabeled specimen and the porcine brain tubulin gave biphasic plots (Fig. 6). Simple analysis of the plots should indicate that the number of the phomopsin A binding sites is at least two: a high affinity site and a low affinity site with dissociation constants of 1 × 10^{-8} (K_{d_1}) and 3 × 10^{-7} M (K_{d_2}), respectively. Such multiphasic plots due to tubulin aggregation have been reported as being the effects of *Vinca* alkaloids with higher protein concentration than used in our experiment [22]. We also observed an increase in turbidity of microtubule protein (2 mg/mL) induced by vinblastine at a concentration of 1 × 10^{-5} M or more [13]. However, no such phenomenon occurred with phomopsin A at a concentration of 1 × 10^{-4} M. We concluded, therefore, that the biphasic plots shown in Fig. 6 were the result not of aggregation but of the presence of two or more binding sites. Previous reports have shown that phomopsin A has no effect on the binding of radiolabeled colchicine to tubulin [6, 20]. We confirmed this result (data not shown) and observed also no effect of colchicine on binding of the radiolabeled phomopsin A (Fig. 8), indicating that neither of the two binding sites of phomopsin A overlaps the colchicine binding site.

As shown in Fig. 8, phomopsin A inhibited strongly [^{14}C]rhizoxin binding to tubulin and the observed inhibition constant (K_i = 0.8 × 10^{-8} M) was almost the same as the dissociation constant of

the high affinity binding of phomopsin A (K_{d_1}). We, therefore, concluded that the high affinity site is identical to the rhizoxin site. This conclusion was also supported by the inhibition of phomopsin A binding by rhizoxin and ansamitocin P-3 (Fig. 9). The K_i values estimated from the IC_{50} values were in the order of 10^{-7} M, which agrees with the observed K_d values of these compounds. The fact that rhizoxin and ansamitocin P-3 behaved in the same manner in inhibiting phomopsin A binding is in good agreement also with our previous conclusion, suggesting the presence of a common rhizoxin–maytansine site [8]. Both compounds inhibited the binding of phomopsin A to tubulin up to a maximum of only 50% supporting our conclusion that there are two phomopsin A binding sites, one being different from the rhizoxin–maytansine site.

On the other hand, no conclusive result was obtained concerning the relationship between the phomopsin A and vinblastine binding sites since the D.C.C. method seemed not to be suited for the analysis of [^{3}H]vinblastine, and did not give reproducible data. Nevertheless, inhibition of phomopsin A binding by vinblastine (Fig. 9) and the inhibition of vinblastine binding by phomopsin A (data not shown) were estimated to be much weaker than their respective binding affinities. The results suggest that their respective binding affects each other's binding but that they do not share the same site.

In conclusion, phomopsin A binds to tubulin at least at two sites. One of the sites is identical to the rhizoxin–maytansine site and neither site overlaps the colchicine site. The binding sites of phomopsin A may overlap partially but are not identical to the vinblastine site. It would be intriguing to study the common structural elements responsible for the binding of rhizoxin, maytansine and phomopsin A to tubulin in order to determine whether the Asn^{100} in β-tubulin is playing a role in the binding of phomopsin A to tubulin, as it is in that of rhizoxin and maytasinoids [23].

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