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EFFECT OF PHOMOPSIN A ON THE ALKYLATION OF TUBULIN

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Abstract—Phomopsin A, a macrocyclic heptapeptide isolated from the fungus *Phomopsis leptostromiformis*, is a potent inhibitor of microtubule assembly and of vinblastine binding to tubulin. Like vinblastine, phomopsin A stabilizes colchicine binding to tubulin. Because phomopsin A is structurally very different from either vinblastine or maytansine, it was of interest to compare its effects on tubulin sulfhydryls to those of the other two drugs. Our results indicate that the effects of phomopsin A combine those of maytansine and vinblastine. Like maytansine, phomopsin A completely inhibited formation of a covalent cross-link between cysteines 12 and 201 or 211, induced by *N,N'*-ethylenebis(iodoacetamide); like vinblastine, phomopsin A strongly inhibited alkylation of tubulin by iodo[^{14}C]acetamide. Our results are consistent with the hypothesis that phomopsin A binds to regions on tubulin overlapping those to which vinblastine and maytansine bind. We have shown previously that phomopsin A is a potent stabilizer of the tubulin molecule. We now find that when both phomopsin A and colchicine are bound to tubulin, the rate of decay of colchicine binding becomes insignificant.

Microtubules, cylindrical organelles playing crucial roles in processes such as mitosis, are composed largely of the protein tubulin, which consists of two polypeptide chains designated α and β [1, 2]. Inhibition of microtubule assembly can be mediated by a wide diversity of chemicals, collectively termed microtubule inhibitors [3]. By competitive ligand binding studies many of these inhibitors have been shown to occupy two distinct domains on the tubulin dimer, which can be loosely designated as the colchicine and the vinblastine sites [3, 4]. While much is known of the structural prerequisites for binding to these sites, their location within the protein structure is still unknown. One experimental approach which has yielded detailed information about the interactions of these drugs with tubulin is to examine their effects on the reaction of tubulin sulfhydryls with alkylating agents. Bifunctional alkylation of cysteines exclusively within the β subunit of tubulin by *N,N'*-ethylenebis(iodoacetamide) (EBI) in the presence and absence of GTP creates two distinct intra-chain cross-links; the tubulin molecules containing these cross-links can be resolved from each other electrophoretically [5, 6]. These cross-links, designated as β^* and β^s, have been identified as ligating cysteines 239 and 354 [7], and 12 and either 201 or 211 [8] for the colchicine and vinblastine sites respectively [9]. Interestingly, β^* is observed in the presence of GTP, whereas β^s is observed exclusively in GTP-deficient tubulin [6]. Inhibitors of each site have been shown not only to inhibit the cross-link corresponding to their respective binding site but also to enhance the formation of the other cross-link; thus, while vinblastine and maytansine inhibit the formation of β^s, they also enhance β^* formation [6].

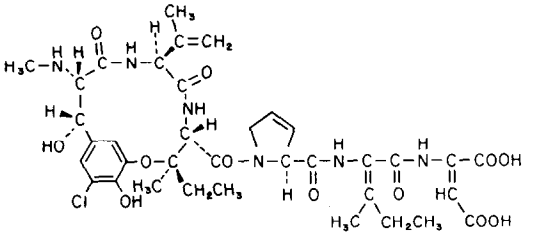

Fig. 1. Structure of phomopsin A. The above structure was derived by Mackay *et al.* [13].

Within these sites, differences between the extent of inhibition and enhancement of both β^* and β^s formation serve as an indication of structural overlap of the binding domains of the ligands. For example, vinblastine and maytansine, which are competitive inhibitors of each other's binding [10, 11], differ strikingly in their abilities to inhibit β^s formation, with the latter being considerably more effective [6]. Further differences can be seen when the effects of a ligand on monofunctional tubulin alkylation are observed; for example, vinblastine is a strong inhibitor of the alkylation of tubulin by iodo[^{14}C]acetamide, whereas maytansine has little effect on this reaction [5, 12].

Recently, phomopsin A, a 13-membered macrocycle (Fig. 1) isolated from the fungus *Phomopsis leptostromiformis*, was shown to inhibit microtubule assembly and to block [^{3}H]vinblastine binding to tubulin [14]. The structural heterogeneity of the inhibitors which appear to bind at or near the vinblastine site (Fig. 2) has prompted further studies into the nature and implications of ligand binding within the vinblastine site. The strikingly different effects of vinblastine and maytansine binding within

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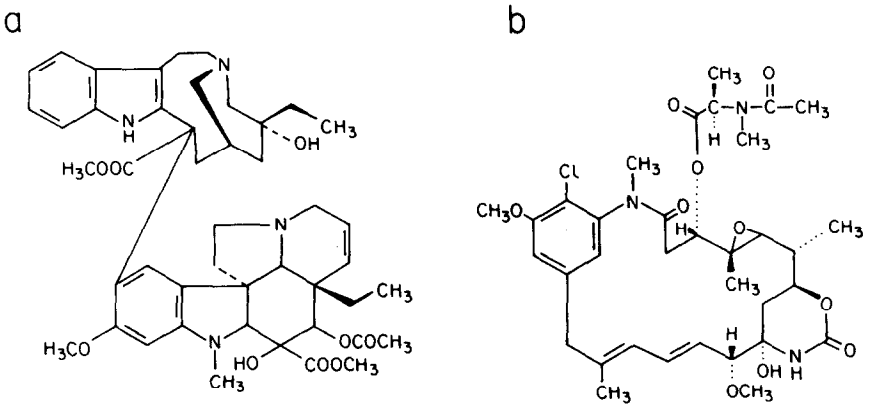

Fig. 2. Structures of vinblastine (a) and maytansine (b).

this site have been noted already [6, 12, 15, 16]. Like vinblastine, phomopsin A stabilizes binding of [^{3}H]colchicine to tubulin [14] and can induce tubulin to form spirals [17]; like maytansine, it completely blocks the increase in turbidity of tubulin preparations caused by vinblastine-induced spiral formation [18]. Furthermore, phomopsin A is superior to both vinblastine and maytansine in stabilizing decay of tubulin as measured by the time-dependent loss of ability to bind to [^{3}H]colchicine and the time-dependent increase in the binding of bis(8-anilinonaphthalene-1-sulfonate) [18], indicative of prolonged stabilization of the tertiary structure of tubulin.

In this paper we report further on the comparative nature of the interaction of phomopsin A within the vinblastine site, by examination of the effects on the mono- and bifunctional alkylation of tubulin sulfhydryl groups. We found that, like maytansine, phomopsin A completely inhibited formation of the β^s cross-link by EBI, but that, like vinblastine, phomopsin A was a strong inhibitor of the reaction of tubulin with iodo[^{14}C]acetamide. Of unique importance, we observed that phomopsin A in conjunction with colchicine appeared to inhibit completely tubulin decay. This represents a major step towards crystallization and ultimately X-ray resolution of the three-dimensional structure of tubulin.

These results have been reported previously in preliminary form elsewhere [19].

MATERIALS AND METHODS

Materials. Phomopsin A was isolated as previously described [20]. Vinblastine sulfate was a gift from the Eli Lilly Corp. (Indianapolis, IN). Other materials were prepared or purchased as described previously [21].

Tubulin preparation. Microtubules were purified from bovine cerebra and tubulin purified therefrom according to the procedure of Fellous *et al.* [22]. For alkylation and cross-linking experiments, chromatography was performed on a double column of phosphocellulose and Sephadex G-25 equilibrated in 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.4, containing 0.1 mM EDTA and 1 mM ethyleneglycolbis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, as described by Roach and Luduena [6]. For colchicine binding experiments, the tubulin was in a buffer similar to the above except that it contained, in addition, 1 mM GTP, 0.5 mM $MgCl_2$ and 1 mM β-mercaptoethanol.

Alkylation experiments. Freshly purified tubulin was reacted with iodo[^{14}C]acetamide in the presence of drugs, the incubation mixtures were passed through Millipore filters, and the radioactivity of the filters was determined [12].

Cross-linking experiments. Freshly purified tubulin was reacted with EBI, and the yields of the β^* and β^s cross-links were determined as previously described [6].

Other methods. Determination of protein concentrations was done using the modification by Schacterle and Pollack [23] of the method of Lowry *et al.* [24]. Bovine serum albumin was used as a standard. Polyacrylamide gel electrophoresis was done on 5.5% polyacrylamide gels according to the method of Laemmli* [25]. To obtain an accurate measurement of the yield of the β^s cross-link, some samples were subjected to electrophoresis on polyacrylamide gels using the modified Laemmli system of Banerjee *et al.* [26] in which the concentration of Tris in the resolving gel is 0.74 M and the pH of the resolving gel is 9.1. In this modified system, there is a greater separation between the β_1 and β^s bands, permitting a more accurate estimation of the yield of the β^s

* When reduced and carboxymethylated mammalian brain tubulin is subjected to electrophoresis on polyacrylamide gels in the system of Laemmli [25], two bands of β-tubulin are observed. The slower of these, designated β_2, is unique to brain tubulin; the faster-moving, designated β_1, is common to tubulin from all tissues yet studied [21]. When the tubulin is subjected to cross-linking with EBI under conditions where both the β^s and β^* cross-links form, as many as three new electrophoretic species may be observed. All of these species have electrophoretic mobilities higher than that of β_1. These new species (in order of increasing electrophoretic mobility) are the β^s band, containing the β^s cross-link, the β^* band, containing the β^* cross-link, and the β^{s*} band, containing both cross-links [6].

Table 1. Effect of phomopsin A on the alkylation of tubulin by iodo-[^{14}C]acetamide*

Addition	^{14}C Incorporation	
	Moles ^{14}C/100 kD	% of Control
Expt. 1		
None	5.2 ± 0.1	100 ± 2
Phomopsin A, 50 μM	3.7 ± 0.1	70 ± 2
GTP, 1 mM	4.2 ± 0.1	80 ± 3
Vinblastine, 50 μM	4.2 ± 0.1	81 ± 2
Maytansine, 50 μM	4.9 ± 0.1	94 ± 3
Expt. 2		
GTP, 1 mM	5.1 ± 0.5	100 ± 10
Phomopsin A, 100 μM + GTP, i mM	4.1 ± 0.1	80 ± 8
Vinblastine, 100 μM + GTP, 1 mM	4.2 ± 0.1	83 ± 8

* Aliquots (250 μL) of tubulin (0.66 mg/mL) were reacted with 1.36 mM iodo[^{14}C]acetamide (0.39 Ci/mol) for 1 hr at 37°. Incorporation of ^{14}C into the tubulin was measured by filtration. In Expt. 1, the sample to which no ligand was added was set as the control. In Expt. 2, all the samples contained 1 mM GTP in order to show the effects of phomopsin A and vinblastine on tubulin which has GTP bound to it to stabilize its native conformation; the sample with only GTP and no other ligand was set as the control. All samples were done in quadruplicate. Standard deviations are given.

Table 2. Effect of phomopsin A on the cross-linking of tubulin by EBI*

Addition	Yield of β^s (% of total β_1)	Yield of β^* (% of total β_1)	Yield of aggregated tubulin (% of total tubulin)
None	12, 19	19, 20	62, 65
Phomopsin A, 50 μM	0	56	21
Vinblastine, 50 μM	6, 7	33, 38	47, 52
Maytansine, 50 μM	0	36, 39	48, 50
GTP, 100 μM	4	28	54
GTP, 100 μM + phomopsin A, 50 μM	0	52	17
Podophyllotoxin, 50 μM	17, 19	0	30, 37

* Aliquots (250 μL) of tubulin (0.66 mg/mL) containing reduced and carboxymethylated con-albumin (0.4 mg/mL) were reacted for 1 hr at 30° with 0.91 mM EBI in the presence of the indicated compounds. Samples were then reduced and carboxymethylated and subjected to electrophoresis on polyacrylamide gels according to the system of Laemmli [25]. The table gives the calculated yields of the β^* and β^s cross-links and of aggregated cross-linked tubulin. Certain samples were done in duplicate.

cross-link [26]. In this system, however, the β^* and β^s bands co-migrate; hence it is necessary to use samples in which β^* formation has been inhibited by podophyllotoxin [26]. The binding of [^{3}H]colchicine to tubulin was determined by filtration of DEAE-cellulose filters according to the method of Borisy [27].

RESULTS

When we examined the effects of phomopsin A on the alkylation of tubulin by iodo [^{14}C]acetamide, we found that at a concentration of 50 μM, phomopsin A inhibited alkylation by 30% in the absence of GTP (Table 1, Expt. 1). If GTP was present to stabilize the tubulin molecule, phomopsin A (100 μM) inhibited alkylation by only 20% (Table 1, Expt. 2). In both cases, the effect of phomopsin A was comparable to that of vinblastine. Maytansine has been shown previously to have no effect on tubulin alkylation by iodo[^{14}C]acetamide in the presence of GTP [12] and to inhibit it only slightly in the absence of GTP [6]. Clearly, alkylation was inhibited more by phomopsin A than by GTP, vinblastine or

maytansine (Table 1). The inhibition of alkylation by maytansine was significantly lower than the effect of phomopsin A.

The effect of phomopsin A on the interaction of tubulin with EBI is shown in Table 2. Phomopsin A, like vinblastine and maytansine, significantly inhibited formation of the β^s cross-link by EBI and also inhibited the formation of high molecular weight cross-linked aggregated tubulin by EBI. This latter observation is consistent with previous studies that suggest that inhibition of cross-linked aggregate formation by a ligand is loosely correlated with its effect on alkylation by iodo[^{14}C]acetamide [9]. Consistent with the effects of other vinblastine site ligands, phomopsin A enhanced formation of the β^* cross-link by EBI. As can be seen in Tables 1 and 2, the effects of phomopsin A were generally greater than those of the other ligands tested. Analogous to maytansine, phomopsin A gave complete inhibition of the formation of the β^s cross-link. As can be seen in Table 2, however, vinblastine only partially inhibited β^s formation, as we previously reported [6]. The inhibition by phomopsin of β^s formation was dependent on its concentration (Fig. 3); half-maximal inhibition of β^s formation was attained at 2 μM phomopsin A. This result is comparable to those of Lacey *et al.* [14] who found that half-maximal effects of phomopsin A on inhibition of microtubule assembly, stabilization of colchicine binding to tubulin and inhibition of vinblastine binding were obtained at phomopsin A concentrations in the range of 0.25 to 0.83 μM.

We previously observed [18], using two different experimental approaches, that phomopsin A significantly stabilizes the tubulin molecule against decay, extending the half-time of decay of tubulin's ability to bind to [^{3}H]colchicine to 28 hr. Since the results of the cross-linking experiments described above suggest that phomopsin A is interacting analogous to other vinblastine-site ligands, we reasoned that its stabilizing effect on the tubulin molecule may be additive with the stabilizing effect of colchicine. Accordingly, tubulin was preincubated with [^{3}H]-colchicine in the presence or absence of phomopsin A or vinblastine. At intervals, aliquots would be removed, and the amount of bound [^{3}H]colchicine was determined. The results (Fig. 4) indicate that in the presence of *both* phomopsin A and colchicine, the decay of colchicine binding is so slow as to be essentially unmeasurable. For example, duplicate experiments, including the one shown in Fig. 4, gave half-times of decay of colchicine binding in the presence of both colchicine and phomopsin A that ranged from 15 days to 17 weeks. In contrast, the half-time of decay in the presence of colchicine alone was 5–14 hr, and, in the presence of both colchicine and vinblastine, was 23–35 hr. Clearly, the co-stabilization of tubulin by phomopsin A and colchicine yielded a synergistic interaction beyond that observed by either the individual ligands or by the combination of colchicine and vinblastine.

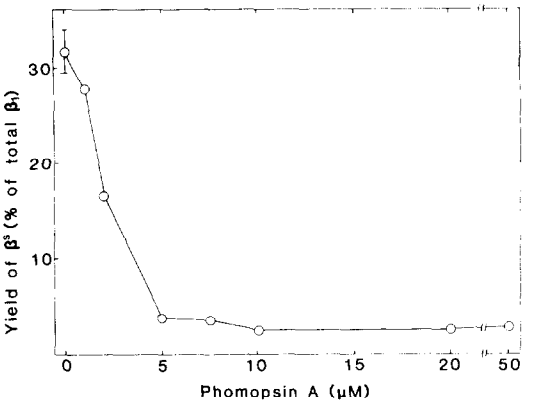

Fig. 3. Effect of phomopsin A on the formation of the β^s cross-link by EBI. Aliquots (250 μL) of tubulin (0.66 mg/mL) containing reduced and carboxymethylated conalbumin (0.4 mg/mL) were reacted for 1 hr at 30° with 0.91 mM EBI in the presence of the indicated concentrations of phomopsin A. All samples contained 50 μM podophyllotoxin to prevent formation of the β^* cross-link. Samples were reduced and carboxymethylated and subjected to polyacrylamide gel electrophoresis on the modified Laemmli system [26]. The figure shows the yield of the β^s cross-linked electrophoretic species as a percentage of the total β_1 tubulin. Control value is the mean of two measurements. *Note*: In Table 2, phomopsin A caused complete inhibition of β^s formation; in Fig. 3, however, there still appears to be a 2–3% yield of β^s even in the presence of the highest concentration of phomopsin A. The reason for the apparent discrepancy is that in the experiment shown in Fig. 3, the samples were analyzed in the modified Laemmli system, which gives a greater resolution between the β_1 and β^s bands. In this system, in the control untreated by EBI, there is a small contaminant band which migrates at the β^s position. This amounts to about 3.5% of the β_1 band. It is likely that this band may still be present in the EBI-treated samples, although we cannot be certain because its presence could be masked by the β^s band. Hence, it is possible that the true yield of β^s in the presence of high concentrations of phomopsin A is zero. In the experiment shown in Table 2, the unmodified Laemmli system was used. Here, there is no contaminant co-migrating with the β^s band. Hence, it is possible to say that the yield of the β^s band is apparently zero.

DISCUSSION

Although it is not yet known whether phomopsin A inhibition of vinblastine binding is competitive [14], its effects on the sulfhydryls of tubulin place it clearly in the same category as vinblastine and maytansine: (1) phomopsin A blocked formation of the β^s cross-link between cysteines 12 and 201 or 211; (2) as with the other two drugs, phomopsin A also enhanced formation of the β^* cross-link, between cysteines 239 and 354 (Table 2). The β^* cross-link is the one whose formation is blocked by colchicine and podophyllotoxin [9]. Unlike vinblastine, but like maytansine, phomopsin A completely blocked β^s formation. This suggests that, if vinblastine and maytansine have overlapping rather than identical binding sites [4], phomopsin A would also share that portion of the binding site where only maytansine and not vinblastine binds and which contains one of the sulfhydryls involved in the β^s cross-link. Phomopsin A, however, was very much unlike maytansine in that it strongly inhibited tubulin decay; in this respect, it was even more effective

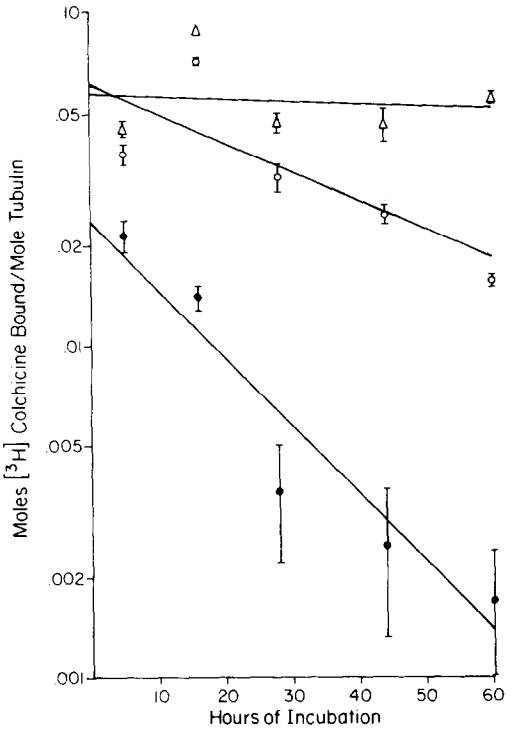

Fig. 4. Effects of phomopsin A and vinblastine on the stabilization of the tubulin–colchicine complex. Samples of tubulin (0.3 mg/mL), containing 100 μM [^{3}H]colchicine (109 Ci/mol), were incubated at 37° in the absence (●) or presence of 40 μM concentrations of either vinblastine (○) or phomopsin A (△). At the indicated times, aliquots were withdrawn and the amount of bound colchicine was determined by filtration using the method of Borisy [27]. Values are means ± SD; samples were done in triplicate.

II, III and IV and are constituted by residues 105–112, 143–148, 205–208, 297–300 and 60–69 in β-tubulin respectively. Regions III and IV are thought to comprise the guanyl binding domain while I and II are considered to comprise the phosphoryl binding domain. Regions I_A and II were reasoned to interact alternatively as Mg^{2+}-independent and -dependent options with the phosphoryl domain [28].

The fact that one of the cysteinyl residues involved in formation of the β^s cross-link is located at either 201 or 211 (bracketing the phosphoryl-binding domain II of Sternlicht *et al.*) together with the fact that β^s formation is suppressed by GTP suggest that both phomopsin A and maytansine are interacting near this phosphoryl-binding domain. This observation is supported by the direct inhibition of [^{3}H]GTP binding to the exchangeable site by maytansine but not vinblastine [29]. The analogy between the failure of vinblastine to inhibit [^{3}H]GTP binding and its relatively weak ability to inhibit β^s formation implies that a unique domain for maytansine and phomopsin A is proximal to the phosphoryl portion of the GTP binding site. Confirmation of this hypothesis by demonstration of phomopsin A inhibition of [^{3}H]GTP binding is currently being undertaken. A comparison of the effects of phomopsin A, GTP, and other ligands on the alkylation of tubulin is shown in Table 3.

The most unexpected effect of phomopsin A was its strong inhibition of tubulin decay. We have found previously that phomopsin A is more effective than vinblastine at stabilizing the tubulin molecule against decay [18]. However, when decay is examined over a 24-hr period, decay is evident even in the presence of phomopsin A [18]. It is striking that when the stabilizing effects of both phomopsin A and colchicine were combined (Fig. 4), the rate of decay at 37° became almost unmeasurably slow. This result has some interesting implications. First, it is conceivable that when phomopsin A is administered *in vivo*, its strong ability to inhibit decay may prolong the existence of a pool of phomopsin A–tubulin complexes which could repress tubulin synthesis for a long period of time and perhaps account for the very high toxicity of phomopsin A that has been observed [30, 31]. Second, if a complex of tubulin with both colchicine and phomopsin A is stable for a period of weeks at 37°, it is likely that such a complex may be stable for even longer at lower

Table 3. Effects of ligands on tubulin alkylation

Ligand	Effect on β^s formation	Effect on β^* formation	Effect on alkylation (−GTP)	Effect on alkylation (+GTP)
Vinblastine	↓	↑	↓↓	↓↓
Maytansine	↓↓	↑	↓	*
Phomopsin A	↓↓	↑	↓↓	↓↓
GTP	↓↓	↑	↓↓	*
Colchicine	↑	↓↓	ND†	↓↓
Podophyllotoxin	↑	↓↓	↓↓	↓

* No significant effect.
† ND = not determined.

than vinblastine. One may speculate, therefore, that the binding site of phomopsin A may extend into a region of the vinblastine/maytansine binding site that is occupied only by vinblastine.

In a recent model presented for the GTP exchangeable binding site, Sternlicht *et al.* [28] postulated that GTP binds to a site comprising five discontinuous sequences structured by folding of tubulin analogous to other guanine nucleotide binding proteins. Regions within the site have been designated I, I_A,

temperatures and perhaps permit crystallization of the complex. Formation of a tubulin crystal that would be amenable to structural analysis would represent a major step forward in the tubulin field.

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