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INTERACTION OF PHOMOPSIN A AND RELATED COMPOUNDS WITH PURIFIED SHEEP BRAIN TUBULIN

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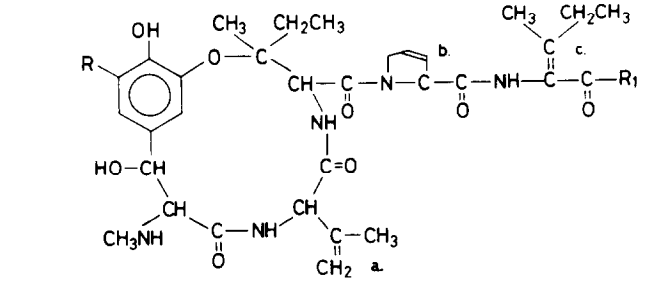
Abstract—Phomopsins comprise a family of peptide mycotoxins containing a 13-membered ring formed by an ether bridge, produced by the fungus *Phomopsis leptostromiformis*, the causal agent in lupin poisoning (lupinosis). The biochemical actions of two naturally occurring phomopsins, phomopsin A and B, and the chemical derivatives, phomopsinamine A and octahydrophomopsin A, on purified sheep brain tubulin were investigated. All analogues were potent microtubule inhibitors, blocking the polymerization of tubulin at concentrations of less than 1 μM. They inhibited [^{3}H]vinblastine binding to tubulin and, in common with vinblastine and its competitive inhibitor maytansine, enhanced the binding of [^{3}H]colchicine to tubulin. It is postulated that phomopsin A and its analogues exert their action on tubulin by interaction at or near the vinblastine binding site. Two possible mechanisms for the interaction between vinblastine or phomopsins and colchicine binding to tubulin are proposed.



	R	R_1
Phomopsin A	Cl	$-NHC(=CH-COOH)-COOH$
Phomopsin B	H	$-NHC(=CH-COOH)-COOH$
Phomopsinamine A	Cl	$-NH_2$
Octahydrophomopsin A	Cl	$-NHCH(CH_2COOH)-COOH$ (reduction at sites a., b. and c.)

Fig. 1. Structure of phomopsin A and related compounds.

Phomopsin A (Fig. 1) is a hexapeptide mycotoxin which occurs in post-harvest lupin stalks and seeds infected with the fungus *Phomopsis leptostromiformis*, the causative agent of lupinosis in domestic livestock [1]. It contains the unusual amino acids: 3,4-didehydroproline, 2,3-didehydroisoleucine, 2,3-didehydroaspartic acid, 3,4-didehydrovaline, 3-hydroxyisoleucine and *N*-methyl-3-(3′-chloro-4′,5′-dihydroxyphenyl) serine [2, 3] with a 13-membered ring formed by condensation between the 5′-phenolic group of the phenylserine and the 3-hydroxyisoleucine unit. The assignment of the cyclic structure is based on the recent results of X-ray crystallographic analysis of phomopsin A [4]. Phomopsin A induces mitotic abnormalities in the liver with associated histopathology [5], subsequently shown to be due to a colchicine-like arrest of mitosis [6]. Tönsing *et al.* [7] demonstrated that phomopsin A inhibits the polymerization of pig tubulin and induces depolymerization of existing microtubules both *in vitro* and *in vivo*.

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Although phomopsin A is extremely toxic (LD_{50}, 20–30 μg/kg in sheep [8]), its action as a microtubule inhibitor merits further investigation, given the therapeutic importance of other microtubule inhibitors in anti-tumour and anthelmintic chemotherapy [9]. In the present study, the interactions with tubulin of phomopsin A and phomopsin B, a closely related congener, which lacks the chlorine atom, and the derivatives phomopsinamine A and octahydrophomopsin A (Fig. 1) were investigated. Comparison was made with the biochemical interactions of established microtubule inhibitors: colchicine (CLC), podophyllotoxin, maytansine, taxol and vinblastine (VBL) in an effort to identify the site of action of the phomopsins on tubulin and to obtain information on structural requirements for microtubule inhibitory activity.

MATERIALS AND METHODS

Phomopsins A and B were isolated from laboratory cultures of *P. leptostromiformis* grown in lupin seed [1]. Phomopsinamine A was prepared by mild acid hydrolysis of phomopsin A [2]. Octahydrophomopsin A was obtained as the product of catalytic reduction of phomopsin A [2] and as such is an isomeric mixture in which both D- and L-isomers are present in the constituent valine, isoleucine and aspartic acid residues [2]. Maytansine and taxol were gifts from Natural Products Branch, Division of Cancer Treatment, NCI, Bethesda, MD, U.S.A. Tritiated CLC[ring A-4-^{3}H] and VBL[G-^{3}H] were purchased from the Radiochemical Centre, Amersham, England. For all inhibition studies, standard solutions of the phomopsins, maytansine, podophyllotoxin and taxol were prepared in dimethyl sulfoxide (DMSO), whereas CLC and VBL were prepared in 0.025 M morpholinoethane sulfonic acid (MES) buffer (pH 6.5). All other chemicals and biochemicals were purchased from either the Sigma Chemical Co., St. Louis, MO, U.S.A.; Boehringer Mannheim, Sydney, Australia; or H.L.S. Scientific, Sydney, Australia, and were of analytical reagent grade.

Tubulin was isolated from sheep brain as previously described [10] and stored as either tubulin or microtubules under liquid nitrogen. Protein concentration was determined using the Coomassie Blue method of Bradford [11]. Tubulin polymerization studies were performed using 0.7 ml tubulin (0.64 mg/ml) in 0.1 M MES buffer containing 1.0 mM GTP, 1.0 mM ethyleneglycolbis (aminoethylether)tetra acetate (EGTA) and 0.5 mM $MgSO_4$ (pH 6.5) with the addition of 0.007 ml of each inhibitor concentration in either buffer or DMSO. Polymerization was induced by warming to 37° and monitored spectrophotometrically at 350 nm for 15 min.

The inhibition of polymerization of tubulin was calculated from the rate of increase in absorbance over the linear portion of the trace and from the extent of polymerization at 15 min compared with the control. The concentration of inhibitor required to inhibit the rate or extent of polymerization by 50% (IC_{50} rate and IC_{50} extent respectively) was determined from the graph of percent inhibition versus concentration by interpolation at the 50% value.

For the binding experiments, microtubules were thawed and resuspended in buffer (concentration approximately 1 mg/ml) for 30 min and clarified by centrifugation at 100,000 *g* for 30 min at 4°. For stoichiometric determination of CLC binding in the presence of VBL and phomopsin A, microtubule-associated proteins were removed by phosphocellulose chromatography [12].

To determine [^{3}H]CLC binding, 5 μl of 20 μM [^{3}H]CLC (sp. act. 0.243 Ci/mol) was incubated for 1–6 hr with 95 μl tubulin solution (34–39 ug/assay) or buffer (blank) in the presence of 2 μl inhibitor in DMSO or buffer at 37°. After incubation, unbound [^{3}H]CLC was removed by addition of 0.5 ml charcoal suspension (2 mg/ml in 1% bovine serum albumin) for 10 min and centrifuged at 2000 rpm in a benchtop micro-centrifuge according to the general procedure of Sherline *et al.* [13]. Aliquots (0.4 ml) of the supernatant fraction were added to 5 ml Biofluor (New England Nuclear, Boston, MA, U.S.A.), and radioactivity was counted on a Packard Tricarb 2650 Liquid Scintillation Spectrophotometer (Downers Grove, IL, U.S.A.).

[^{3}H]VBL binding was determined by addition of 10 μl of 10 μM [^{3}H]VBL (sp. act, 4.43 Ci/mol) to 90 μl tubulin solution (38 μg/assay) or buffer (blank) in the presence of 2 μl inhibitor in DMSO or DMSO alone and incubation at 37° for 30 min. Bound and free [^{3}H]VBL were separated by applying 75 μl of the solution to a 0.5-ml DEAE-Sephacel column preequilibrated with 0.1 M MES buffer for 5 min. The column was washed with 2 ml buffer to remove unbound [^{3}H]VBL. The gel was then extruded and washed with 0.8 ml water, and the radioactivity counted in 10 ml Biofluor.

Calculation of bound [^{3}H]CLC and [^{3}H]VBL (in pmol/mg of tubulin protein) was obtained using the following expression:

$$\frac{\text{dpm bound} - \text{dpm blank}}{\text{specific activity}} \times \frac{1}{\text{protein(mg)}}$$

where the specific activity is in dpm/pmol, and normalized for the proportion of the assay counted. The percent inhibition or enhancement of [^{3}H]CLC or [^{3}H]VBL binding was calculated for each inhibitor with respect to controls. For all studies at least six inhibitor concentrations were used to characterize the interactions. Each concentration was tested in duplicate. Where inhibition occurred, the concentration causing 50% displacement was calculated. For enhancers of binding, the concentration required for half-maximum enhancement was calculated. Kinetic parameters, K_a (the association constant) and B_{max} (the maximum amount of drug bound at infinite drug concentration), were derived from double reciprocal plots of the bound and free drug concentrations (in μM).

RESULTS

Spectrophotometric traces of the inhibition of polymerization of purified sheep brain tubulin for the phomopsins, CLC and maytansine are shown in

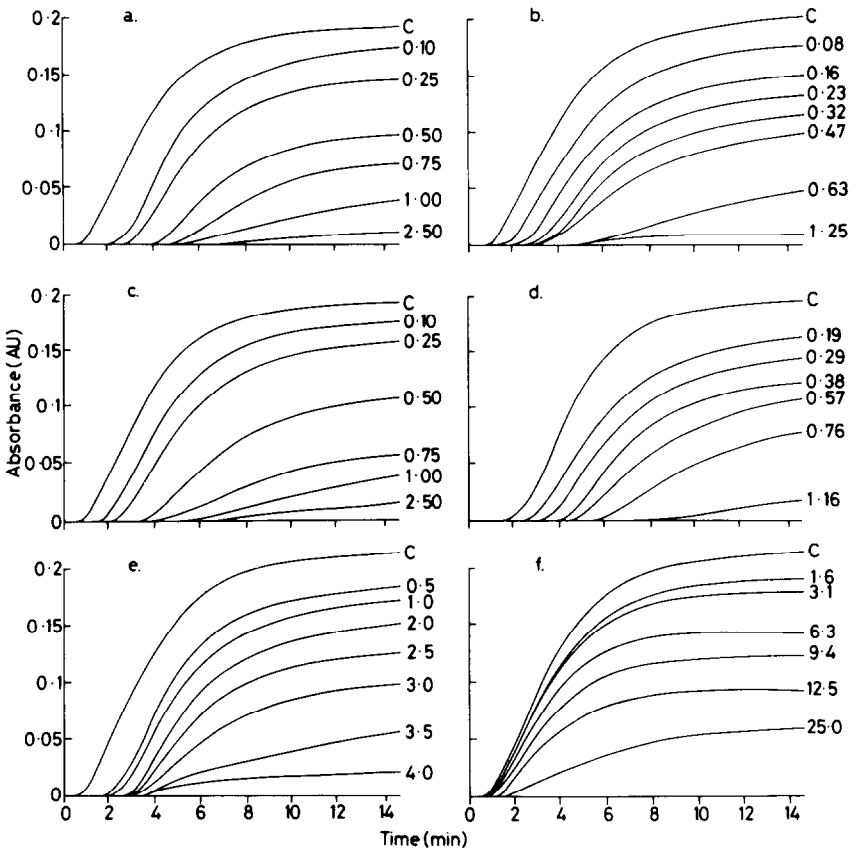

Fig. 2. Inhibition of polymerization of sheep brain tubulin by phomopsin A and related derivatives, CLC and maytansine. Key: (a) phomopsin A, (b) phomopsin B, (c) phomopsinamine A, (d) octahydrophomopsin A, (e) maytansine and (f) colchicine. Inhibitor concentrations are quoted in μM and control (uninhibited) profiles are denoted by "C".

Fig. 2. All compounds markedly inhibited both the rate and extent of polymerization by 10–85% over a 4- to 5-fold concentration range. For the phomopsins, more than 85% inhibition was achieved at 1 μM. The percent inhibition (both rate and extent) versus concentration profiles were linear over the range 10–85% inhibition [correlation coefficient (r) for all compounds was greater than 0.95]. The calculated IC_{50} rate and IC_{50} extent values are shown in Table 1.

The phomopsins were all 30-fold more potent than CLC and equipotent with VBL. Maytansine, which binds to the VBL binding site on tubulin [14], was intermediate in potency between VBL and CLC with IC_{50} values of 2.63 μM (extent) and 2.69 μM (rate). No differences between the inhibition profiles of phomopsin A, phomopsinamine A and octahydrophomopsin A were observed when compared with VBL (typically the reproducibility of inhibition profiles is $\pm 10\%$ [10]). However, the dechloro analogue, phomopsin B, was more potent with IC_{50} values of 0.40 and 0.41 μM for inhibition of rate and extent of polymerization respectively. For all compounds with the exception of octahydrophomopsin A, no differences between IC_{50} rate and IC_{50} extent were observed.

As also shown in Fig. 2, a delay in the time of onset of polymerization was observed. This delay (compared with either DMSO or buffer controls) increased from 2 min at 10% inhibition to 5 min at more than 85% inhibition for the phomopsins and was independent of structure. VBL and maytansine produced similar delays, whereas the onset of polymerisation was less than 1 min for CLC over the entire concentration range tested (10–80% inhibition).

The [^{3}H]CLC binding assay was standardized with respect to duration of incubation, ligand and protein concentration. Maximum binding at infinite time for 1 μM [^{3}H]CLC was 2144 pmol/mg as derived from a double-reciprocal plot of binding versus incubation time up to 5 hr. Binding at 5 hr represented 95% of the theoretical maximum binding at complete equilibrium (time = infinity), while binding at 1 hr represented 60% of the theoretical maximum binding. B_{max} and K_a values of 6746 pmol/mg protein and $0.22 \times 10^6\,M^{-1}$, respectively, were calculated from examination of binding saturation over the concentration range 0.18 to 50 μM for the 1-hr incubation ($r = 0.999$). In this experiment, 34 μg of twice cycled tubulin was used. Routinely this preparation was found to be approximately 90% pure, containing 10% microtubule associated proteins.* Assuming a molecular weight for tubulin dimer of 100,000 [9],

* E. Lacey, unpublished observations.

Table 1. Comparative inhibitor data for microtubule inhibitors of tubulin polymerization and [^{3}H]colchicine and [^{3}H]vinblastine binding

Compound	IC_{50} rate*	IC_{50} extent*	[^{3}H]CLC*	[^{3}H]VBL*
Phomopsin A	0.56	0.60	0.25 E†	0.83 I‡
Phomopsin B	0.40	0.41	0.17 E	0.58 I
Phomopsinamine A	0.53	0.59	0.32 E	0.56 I
Octahydrophomopsin A	0.40	0.62	0.28 E	NT§
Vinblastine	0.55	0.59	1.41 E	2.07 I
Maytansine	2.69	2.63	0.83 E	1.58 I
Colchicine	15.90	14.60	17.40 I	NT
Podophyllotoxin	NT	NT	2.90 I	NT
Taxol	NT	NT	2.34 I/E‖	I/E‖

* Concentrations quoted are $\times 10^{-6}$ M.

† E—enhancement of binding; concentration quoted is required to achieve 50% of total enhancement observed.

‡ I—inhibition of binding; concentration quoted is that required to inhibit binding by 50% of control binding.

§ Not tested.

‖ I/E—initial inhibitory action which was abolished with increasing concentration of drug.

this gave an observed molar ratio of [^{3}H]CLC to tubulin of 0.73. Interestingly, no differences in the stoichiometry were observed when determined at 5 hr or using phosphocellulose purified tubulin.

Binding of 1 μM [^{3}H]CLC over 1 hr was linear up to a protein concentration of 40 μg/assay ($r = 0.999$). At saturation (>160 μg protein/assay), more than 74% of the available label was bound to tubulin.

The solvent, 2% (v/v) DMSO, inhibited [^{3}H]CLC binding by 25%. Under standard assay conditions of 1 μM [^{3}H]CLC, 34 μg tubulin, and 2% DMSO over 1 hr, 28.3 ± 2.0 pmol [^{3}H]CLC was bound ($N = 8$). Unlabelled CLC and podophyllotoxin (a competitive inhibitor of CLC binding [15, 16]) displaced bound [^{3}H]CLC, with 50% displacement occurring at 17.4 and 2.9 μM respectively (Fig. 3). Taxol [17] also inhibited formation of the [^{3}H]CLC–tubulin complex, with 50% displacement occurring at 2.34 μM. Atypical enhancement of [^{3}H]CLC binding (from 23% to 55%) occurred when the taxol concentration was increased from 6.25 to 25 μM.

The formation of the [^{3}H]CLC–tubulin complex was enhanced in the presence of phomopsin A and its analogues, all profiles being similar to those of phomopsin A and octahydrophomopsin A presented in Fig. 3. The enhancement reached a plateau at 0.5 μM with 150–175% enhancement of binding; there were no differences between the phomopsins. Maytansine induced a maximum enhancement of [^{3}H]CLC binding of 169% at 2μM, while VBL induced maximal enhancement of 140% at 1.5 μM.

To examine this enhancement further, 2-μM solutions of phomopsin A, maytansine and VBL were incubated with [^{3}H]CLC for 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 hr (Fig. 4). The initial rate of [^{3}H]CLC binding (0 to 0.25 hr) was observed to be significantly higher in the presence of phomopsin A, VBL and maytansine (2700, 2650 and 2390 pmol/mg/hr bound respectively) compared with the appropriate buffer or DMSO control (1610 and 1350 pmol bound/hr respectively). The rate of binding was also observed

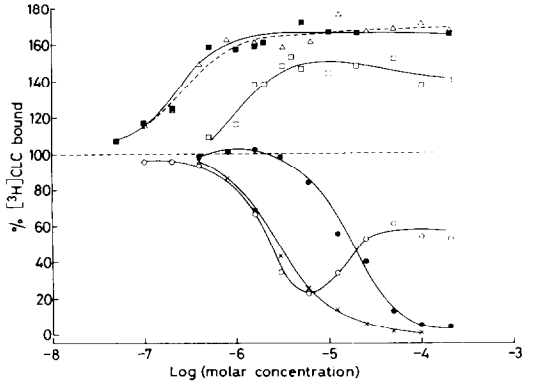

Fig. 3. Effect on [^{3}H]colchicine binding to purified sheep brain tubulin of microtubule inhibitors. Key: colchicine (●), podophyllotoxin (×), taxol (○), vinblastine (□), phomopsin A (■) and octahydrophomopsin A (△).

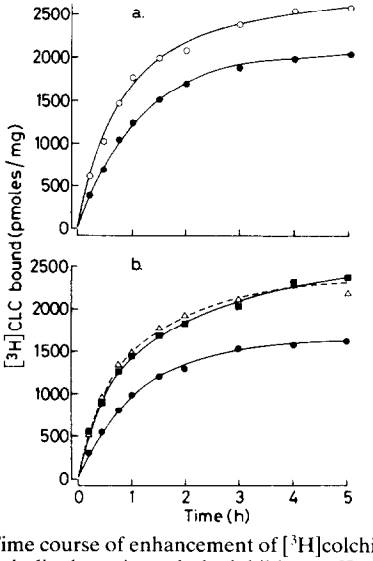

Fig. 4. Time course of enhancement of [^{3}H]colchicine binding to tubulin by microtubule inhibitors. Key: (a) vinblastine (○), and MES buffer (●); (b) phomopsin A, (■), maytansine (△) and DMSO controls (●). All inhibitors were used at 2 μM.

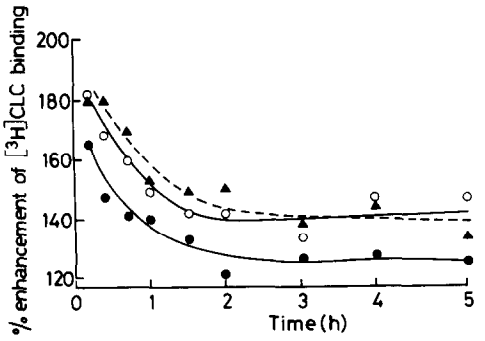

Fig. 5. Relation between enhancement of colchicine binding (%) to tubulin and the length of incubation with various microtubule inhibitors. Key: vinblastine (●), phomopsin A (○), and maytansine (▲). All inhibitors were used at 2 μM.

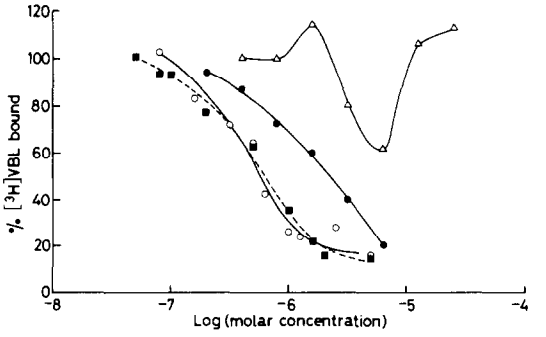

Fig. 6. Effect on [^{3}H]vinblastine binding to purified sheep brain tubulin of microtubule inhibitors: unlabelled vinblastine (●), phomopsinamine A (■), taxol (△), and phomopsin B (○).

to be dependent on the incubation time, with the percentage enhancement (with respect to the appropriate control) decreasing from 165–181% at 0.25 hr to 125–147% at 5 hr of incubation for all three compounds (Fig. 5).

The effects of phomopsin A and VBL on the stoichiometry of [^{3}H]CLC binding were assessed using phosphocellulose purified tubulin. Tubulin (32 μg, 320 pmol) was incubated for 5 hr with a range of [^{3}H]CLC concentrations from 0.195 to 50 μM in the presence or absence of 5 μM phomopsin A or VBL. From Scatchard analysis of the data, the computed x-intercepts for CLC alone or in the presence of 2% (v/v) DMSO gave CLC:tubilin stoichiometries of 0.73 and 0.61. When the effects of DMSO-induced deactivation of tubulin were accounted for, an apparent stoichiometry for the latter value of 0.73 was also obtained. In the presence of VBL and phomopsin A, this ratio was increased to 0.84 and 1.05 respectively (phomopsin A being adjusted for DMSO deactivation).

Preliminary investigation of VBL binding revealed that the complex was not stable to charcoal extraction and that DEAE paper filtration assays using the Bio-Dot filtration manifold (Biorad, Australia) were not reproducible. Reliable and reproducible separation of free and bound [^{3}H]VBL was obtained under the conditions specified; however, concentrations above 50 μM could not be tested due to incomplete elution of free label within the 2-ml wash. Larger wash quantities, for example 4 ml, eluted 20–30% bound [^{3}H]VBL. The binding of VBL was rapid and saturable with equilibrium being achieved within 10 min. Calculated B_{max} and K_a values were 4000 pmoles/mg protein and 2.4×10^4M respectively. Assuming a 2:1 molar ratio for this interaction [18], the calculated B_{max} represented only 25% of theoretical maximum binding. Thus, the low volume wash may also lead to some dissociation of the complex [19]. The low K_a value is indicative of a "low affinity" VBL binding site described for purified rat brain tubulin [18]. DMSO 2% (v/v) reduced binding by 13% compared with controls. At a tubulin concentration of 25 μg/assay, 26.01 ± 1.93 pmol [^{3}H]VBL was bound (N = 8).

The phomopsins, maytansine and unlabelled VBL inhibited [^{3}H]VBL binding (Table 1). Phomopsin B and phomopsinamine A were the most potent inhibitors of [^{3}H]VBL binding with 0.58 and 0.56 μM, respectively, causing 50% displacement (Fig. 6). Phomopsin A was only slightly less potent at 0.83 μM, whereas both VBL and maytansine were less active with 2.07 and 1.5 μM respectively. Analogous to [^{3}H]CLC binding, taxol initially inhibited [^{3}H]VBL binding, reaching a maximum inhibition of 41% at 6.25 μM with greater concentration progressively abolishing inhibition.

DISCUSSION

Phomopsin A and its related derivatives are among the most potent microtubule inhibitors thus far reported. The present study shows that the aromatic chloro-substituent, the terminal 2,3-didehydroaspartic acid, and the four exocyclic sites of unsaturation are not essential for activity nor, apparently, is a particular stereochemistry at the latter sites after reduction. However, it is not yet known whether the macrocyclic ring is essential for activity.

The inhibition of [^{3}H]VBL binding to tubulin by the phomopsins demonstrates that these compounds act in a manner analogous to maytansine, suggesting that the phomopsins bind to tubulin at or near the VBL binding site. Whether true competitive binding occurs has not been ascertained due to the limitations of the [^{3}H]VBL binding assay.

The concentration-dependent enhancement of [^{3}H]CLC binding by the phomopsins in a manner similar to that of unlabelled VLB and maytansine adds further support to this hypothesis [16, 20, 21]. Mechanistically, this enhancement has been attributed to the stabilization of the CLC binding site on tubulin [16] which would predict that enhancement of CLC binding should increase in a linear manner with first-order decay of the CLC binding site. However, the data presented suggest that this is an oversimplification since these compounds both increased the initial rate of [^{3}H]CLC binding where degradation of the binding site can be considered to be minimal and caused no significant change in enhancement with longer incubation times (2–5 hr).

This suggests that [^{3}H]CLC enhancement is due principally to non-decay phenomena. Consideration of these observations suggests that stoichiometry of the CLC–tubulin interaction has, to date, only approached the theoretical equivalence of mole for mole under equilibrium conditions using either VBL stabilized paracrystals [22] or by spectrophotometric determination of binding [23]. Indeed, reported stoichiometry for most binding techniques is typically <0.6 to 0.7 mol of colchicine per mol of tubulin [24]. This gives rise to two possible mechanisms for the "VBL type" enhancement of CLC binding:

(1) The presence of isotubulins which, in the absence of compounds binding to the VBL site, are unable to either bind CLC or form CLC–tubulin complexes sufficiently stable to be isolated by normal bound/free extraction techniques [24].
(2) The presence of multiple equilibria between CLC and tubulin, of which the initial complexes are characteristically reversible and unstable in the absence of VBL site interactions [25]. Such a hypothesis could be drawn from differences in CLC binding between the DEAE and gel filtration techniques reported by Wilson [16].

Phomopsins and maytansine caused greater enhancement of CLC binding than did VBL. This was further documented by Scatchard analysis which showed that phomopsin A increased the CLC:tubulin ratio to that of the theoretical molar equivalence.

In conclusion, the search for and study of new microtubule inhibitors are becoming of increasing importance in a number of areas of chemotherapy. These studies have led to a greater understanding of the fundamental biochemistry of tubulin. In the present study, comparison of the inhibitory activity of the phomopsins with the highly characterized microtubule inhibitors CLC, VBL, maytansine and taxol has highlighted the similarities in action of the phomopsins to those of VBL and maytansine. These compounds are structurally diverse and, despite certain similarities such as the presence of an aromatic region in conjunction with or proximal to a macrocyclic ring, the generalized structural requirements for binding at the VBL site on tubulin are still not apparent.

Although extremely toxic, phomopsins represent a new class of microtubule inhibitors.

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