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DOLASTATIN 10, A POWERFUL CYTOSTATIC PEPTIDE DERIVED FROM A MARINE ANIMAL

INHIBITION OF TUBULIN POLYMERIZATION MEDIATED THROUGH THE VINCA ALKALOID BINDING DOMAIN

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Abstract—Dolastatin 10, a cytostatic peptide containing several unique amino acid subunits, was isolated from the marine shell-less mollusk *Dolabella auricularia* (Pettit GR, Kamano Y, Herald CL, Tuinman AA, Boettner FE, Kizu H, Schmidt JM, Baczynskyj L, Tomer KB and Bontems RJ, *J Am Chem Soc* **109**: 6883–6885, 1987). Since our preliminary studies demonstrated that dolastatin 10 inhibited tubulin polymerization and the binding of radiolabeled vinblastine to tubulin, an initial characterization of the properties of dolastatin 10 included a comparison to other antimitotic drugs interfering with vinca alkaloid binding to tubulin (vinblastine, maytansine, rhizoxin, and phomopsin A). Dolastatin 10 inhibited the growth of L1210 murine leukemia cells in culture, with a concordant rise in the mitotic index, and its IC_{50} value for cell growth was 0.5 nM. Comparable values for the other drugs were 0.5 nM for maytansine, 1 nM for rhizoxin, 20 nM for vinblastine, and 7 μM for phomopsin A. IC_{50} values were also obtained for the polymerization of purified tubulin in glutamate: 1.2 μM for dolastatin 10, 1.4 μM for phomopsin A, 1.5 μM for vinblastine, 3.5 μM for maytansine, and 6.8 μM for rhizoxin. Dolastatin 10 and vinblastine were comparable in their effects on microtubule assembly dependent on microtubule-associated proteins. Preliminary studies indicated that dolastatin 10, like vinblastine, causes formation of a cold-stable tubulin aggregate at higher drug concentrations. We confirmed that rhizoxin, phomopsin A, and maytansine also inhibit the binding of radiolabeled vinblastine and vincristine to tubulin. Dolastatin 10 and phomopsin A were the strongest inhibitors of these reactions, and rhizoxin the weakest. Dolastatin 10, phomopsin A, maytansine, vinblastine, and rhizoxin all inhibited tubulin-dependent GTP hydrolysis. The greatest inhibition of hydrolysis was observed with dolastatin 10 and phomopsin A, and the least inhibition with rhizoxin.

A major specific target of a substantial number of cytotoxic agents is the cellular microtubule system, which forms the framework of the mitotic spindle. All drugs which cause cells to accumulate in metaphase arrest bind to microtubule components, particularly to tubulin, their most important protein (for a recent review, see Ref. 1). Since compounds with this mechanism of action have potential as antineoplastic drugs, our laboratories are particularly interested in novel agents which interfere with microtubule structure and function.

Pettit *et al.* [2–5] have reported the isolation and initial structure determination of five unusual cytotoxic peptides derived from the sea hare *Dolabella auricularia* (a shell-less mollusk). These were all examined for effects on microtubule assembly to determine whether they might represent new antimitotic compounds. The only one of the five which inhibited the polymerization reaction was dolastatin 10 (structure in Fig. 1), and our initial studies with the natural product demonstrated a high level of activity at least equivalent to that of the potent antimitotic drugs vinblastine and colchicine. Structurally, dolastatin 10 is a linear peptide, with four amino acids (three unique, called dolavaline, dolaisoleuine, and dolaproine; both α-amino acids in dolastatin 10 are in the L-configuration) linked to a complex primary amine (dolaphenine), presumably derived from phenylalanine and also not described previously. Extremely limited quantities of the natural product initially prevented a detailed study of the interactions of dolastatin 10 with tubulin, but the total synthesis of dolastatin 10 and determination of its absolute structure have now been accomplished [6].

Consequently, we have been able to begin a detailed examination of the interactions of the peptide with tubulin. As will be demonstrated here, dolastatin 10 strongly inhibited the binding of radiolabeled vinca alkaloids to tubulin. We therefore decided to include in our description of the properties of dolastatin 10 direct comparative studies with vinblastine and with three other compounds (maytansine, phomopsin A, and rhizoxin) which also interfere with the binding of radiolabeled vinca alkaloids to tubulin (structures in Fig. 2).

While maytansine has been relatively well-studied

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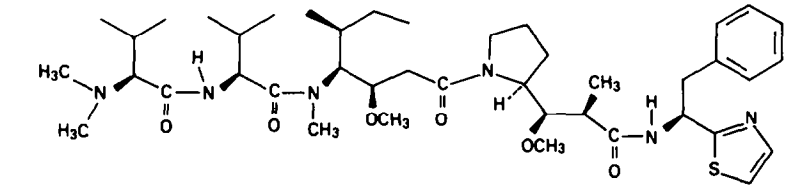
DOLASTATIN 10



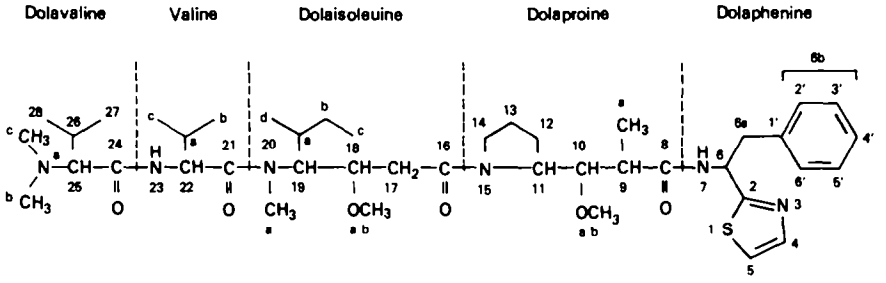


Fig. 1. Structural formula of dolastatin 10. The lower representation emphasizes the five subunit components of the peptide.

[1], including its competitive inhibition of the binding of radiolabeled vincristine to tubulin [7], rhizoxin [8, 9] and phomopsin A [10, 11] have been described only recently as antimitotic compounds interfering with microtubule assembly and the binding of radiolabeled vinblastine to tubulin. Both are natural products of fungal origin, but they are structurally quite different. Rhizoxin has a macrocyclic structure superficially similar to that of maytansine, while phomopsin A is a cyclic hexapeptide. We hope that such comparative studies will provide insights into the properties and perhaps structure of the vinca binding domain* of tubulin.

MATERIALS AND METHODS

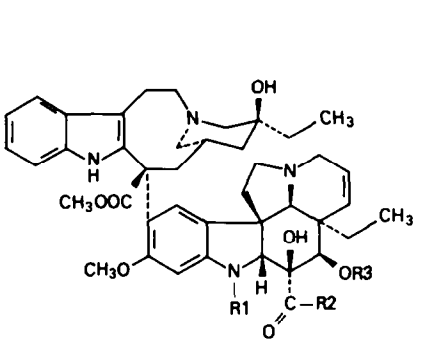
Materials. Maytansine and rhizoxin were obtained from the Natural Products Branch, National Cancer Institute. Phomopsin A was a gift from Dr. C. C. J. Culvenor. [^{3}H]Vincristine was a gift from Dr. D. G. Johns [7]. Vinblastine was obtained from Sigma. Synthetic dolastatin 10 was prepared as described elsewhere [6], and it was used in all studies presented here. The synthetic drug could be compared to the natural product only in the tubulin polymerization assay, and the two compounds behaved identically at all concentrations examined. Nonradiolabeled drugs were dissolved in dimethyl sulfoxide. Control reaction mixtures and drug-containing reaction mixtures all contained identical amounts of the solvent. GTP and [8-^{14}C]GTP were obtained from Sigma and Moravek Biochemicals, respectively, and repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25.

Electrophoretically homogeneous bovine brain tubulin and heat-treated microtubule-associated proteins (MAPs†) were prepared as described previously [12]. Purification of both protein preparations included a final gel filtration step to remove unbound nucleotide.

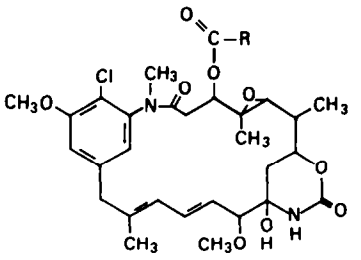
Methods. Microtubule assembly from purified tubulin + heat-treated MAPs and the glutamate-dependent polymerization of purified tubulin were followed turbidimetrically [13] at 350 nm in Gilford model 250 and 2400S recording spectrophotometers equipped with Gilford Thermoset electronic temperature controllers, as described previously [12]. Briefly, absorbance baselines were established with reaction mixtures at 0°, and microtubule assembly or tubulin polymerization was initiated by a temperature jump to 37° which took approximately 70 sec. The method used to determine IC_{50} values for inhibitory drug effects on the extent of tubulin polymerization in 1.0 M monosodium glutamate (pH 6.6 with HCl) was as described previously [14], except that values were determined graphically in at least three independent experiments. Reaction mixtures were preincubated at 37° for 15 min without GTP, and chilled on ice; GTP was added, and the

* We are using the term "domain" rather than "site" because we are not yet certain whether dolastatin 10 is a competitive or noncompetitive inhibitor of vinblastine binding. In particular, the increasing number of structurally distinct agents inhibiting vinca alkaloid binding [7, 9, 11] indicates that careful comparative studies are required. These are now in progress in our laboratory.

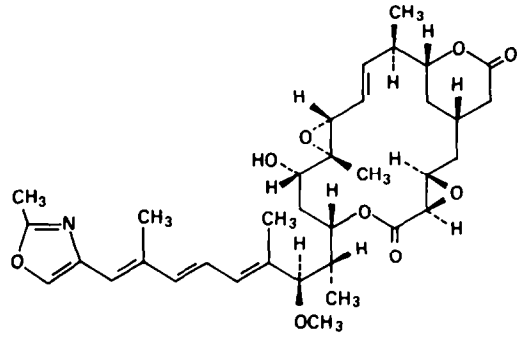
† Abbreviations: MAPs, microtubule-associated proteins; and Mes, 4-morpholineethanesulfonate.



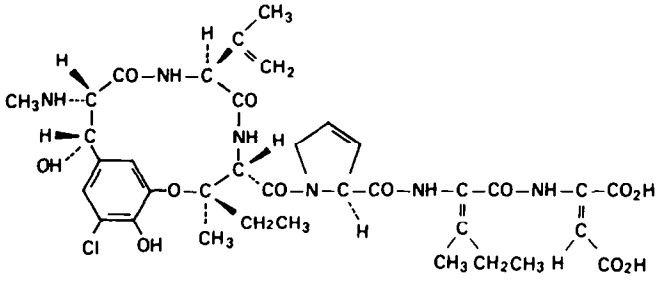
VINBLASTINE: R1 = CH_3; R2 = OCH_3; R3 = $COCH_3$
VINCRISTINE: R1 = CHO; R2 = OCH_3; R3 = $COCH_3$
VINDESINE: R1 = CH_3; R2 = NH_2; R3 = H



MAYTANSINE: R = $CH(CH_3)N(CH_3)COCH_3$
ANSAMITOCIN P-3: R = $CH(CH_3)_2$
ANSAMITOCIN P-4: R = $CH_2CH(CH_3)_2$



RHIZOXIN



PHOMOPSIN A

Fig. 2. Structural formulas of vinca alkaloids, maytansinoids, rhizoxin, and phomopsin A.

polymerization reaction was followed turbidimetrically for 20 min at 37°.

The binding of [^{3}H]vincristine to tubulin was followed by centrifugal gel filtration [15] as described in detail previously for the binding of radiolabeled vinblastine. In brief, triplicate aliquots of reaction mixtures were applied to 1-mL microcolumns of Sephadex G-50 (superfine) in tuberculin syringes, and the columns were centrifuged in a desk top centrifuge in a swinging bucket rotor. Protein and radioactivity in the filtrates were determined, permitting calculation of a molar ratio of drug to tubulin for each sample, with average values of the triplicates presented. Control studies have demonstrated that no radioactivity passes through the column in the absence of protein.

The hydrolysis of [8-^{14}C]GTP to [8-^{14}C]GDP was followed by thin-layer chromatography on polyethyleneimine-cellulose and autoradiography, with subsequent quantitation of radiolabel in the GDP and GTP spots. Details have been described previously [12].

Studies on the growth of L1210 murine leukemia cells, including determination of the proportion of cells arrested in metaphase, were performed as described previously [16]. The mitotic index of control cultures has repeatedly been 4–5%. Cells from a 5-mL culture were harvested by centrifugation, swollen for 5 min in half-strength phosphate-buffered saline, spread on a slide, fixed, and stained with Giemsa; 200–400 cells were examined under the microscope at each drug concentration.

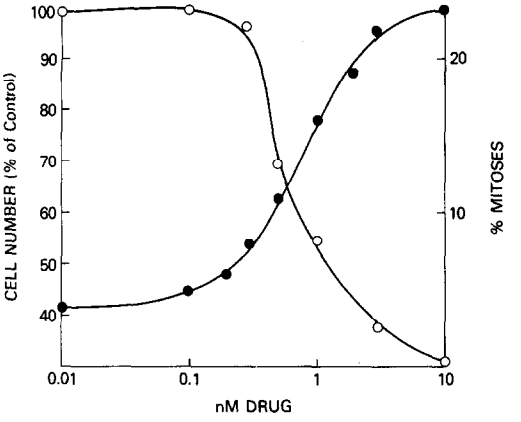


Fig. 3. Effect of dolastatin 10 on the growth of L1210 murine leukemia cells in culture and on the accumulation of cells arrested in mitosis. Cells were cultured in medium containing the indicated concentration of dolastatin 10 and either counted (open symbols) or harvested, fixed, and stained for determination of mitotic figures (closed symbols) after 12 hr. Each 5-mL culture was inoculated with 10^5 cells.

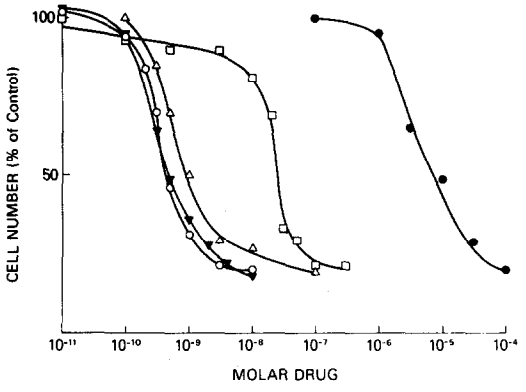


Fig. 4. Comparison of the effects of vinblastine, maytansine, phomopsin A, rhizoxin, and dolastatin 10 on the growth of L1210 murine leukemia cells in culture. Cells were cultured in medium containing the indicated concentration of drug and counted after 12 hr. Each 5-mL culture was inoculated with 10^5 cells. Symbols: (□) vinblastine; (▼) maytansine; (●) phomopsin A; (△) rhizoxin; and (○) dolastatin 10.

RESULTS

Effects of dolastatin 10 *on L*1210 *murine leukemia cells in culture.* Since initial experiments with natural dolastatin 10 established that the compound interacted with tubulin, when the synthetic peptide [6] became available we first wished to demonstrate that its mechanism of cytotoxicity was as an antimitotic agent. Figure 3 demonstrates that this is the case with L1210 murine leukemia cells grown in suspension culture. In this experiment cell growth was inhibited by 50% at a dolastatin 10 concentration of about 1 nM, with a concordant rise in the number of cells arrested in metaphase. Although the maximal mitotic index in this experiment was only 24%, substantially lower than we have observed with a number of other antimitotic compounds,* a subsequent experiment with 10 nM dolastatin 10 yielded a culture with 75% of the cells in mitotic arrest. The reason for this discrepancy is uncertain, but we have not repeated the detailed study of Fig. 3 due to the scarcity of the peptide. The antimitotic effect of dolastatin 10 at nanomolar concentrations, however, is unambiguous.

Dolastatin 10 was also compared as a cytotoxic agent to vinblastine and to three other drugs (maytansine, rhizoxin and phomopsin A) binding in the vinca domain of tubulin (Fig. 4). Dolastatin 10 and maytansine were the most potent (IC_{50} values of 0.5 nM), with rhizoxin only slightly less active (IC_{50} value of 1 nM). Vinblastine was considerably less cytotoxic with the L1210 cells, its IC_{50} value being about 20 nM. Somewhat surprisingly, the peptide phomopsin A was over ten thousand-fold less cytotoxic than dolastatin 10, with an IC_{50} value of 7 μM.

Inhibition of tubulin polymerization by dolastatin 10*: Comparison to other vinca domain drugs.* The observation that phomopsin A inhibited the binding of vinblastine to tubulin [11] suggested that dolastatin 10 (since it, too, is an unusual peptide) would also inhibit vinca alkaloid binding. This was confirmed (see below), and, therefore, we performed a detailed comparison of the effects of dolastatin 10 and vinblastine on tubulin polymerization (Fig. 5). Panel A presents a study in 0.1 M 4-morpholineethanesulfonate (Mes) of microtubule assembly dependent on MAPs. At drug concentrations up to 4 μM there was progressive inhibition of the assembly reaction with both drugs, with little difference between them, particularly if the cold-reversible component of the reaction is considered. At 10 and especially 20 μM vinblastine (curves 9 and 10), there was turbidity development even in the cold, followed by an extensive reaction at 37° which was not cold-reversible. This corresponds to the formation of extensive spiral aggregates, presumably composed of both tubulin and microtubule-associated proteins (see Ref. 15 for an electron micrograph presenting their morphology and for literature references). In a single experiment, 40 μM (curve 5) dolastatin 10 induced some turbidity development at 0°, followed by a more extensive reaction at 37° which was not cold-reversible, similar to the reaction observed with 10 μM vinblastine. We have not yet determined whether this turbidity represents the formation of discrete structures, analogous either to those induced by vinca alkaloids or

* For example, in a study [16] in which combretastatin was compared to colchicine and 2-methoxy-(2′,3′,4′-trimethoxyphenyl)tropone, the mitotic indices observed with the three drugs went as high as 50–70% at the highest drug concentrations examined. However, the result presented in Fig. 3 may only represent an experimental variation, in view of the mitotic index of 75% observed in a subsequent experiment. In addition, it should be noted that when the experiment in Fig. 3 was performed, simultaneous cultures were examined with approximately ten times the IC_{50} concentrations of vinblastine, maytansine, rhizoxin, and phomopsin A. In all cases, about 20–30% mitotic figures were observed. Further comparative studies with the five compounds have not been performed.

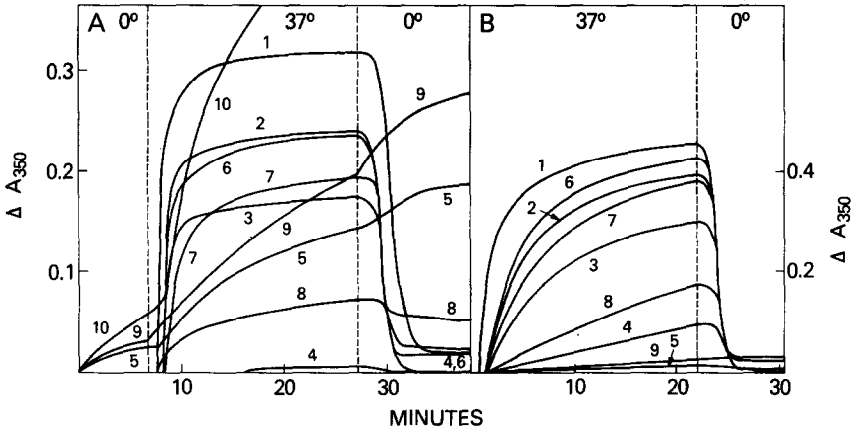


Fig. 5. Comparison of the effects of dolastatin 10 and vinblastine on tubulin polymerization. (A) Microtubule assembly dependent on microtubule-associated proteins. Each 0.25-mL reaction mixture contained 1.5 mg/mL tubulin, 0.75 mg/mL heat-treated microtubule-associated proteins, 0.1 M Mes (pH 6.4 with NaOH), 0.5 mM $MgCl_2$, 0.5 mM GTP, 4% (v/v) dimethyl sulfoxide, and drug as follows: curve 1, none; curve 2, 1.0 μM dolastatin 10; curve 3, 2.0 μM dolastatin 10; curve 4, 4.0 μM dolastatin 10; curve 5, 40 μM dolastatin 10; curve 6, 1.0 μM vinblastine; curve 7, 2.0 μM vinblastine; curve 8, 4.0 μM vinblastine; curve 9, 10 μM vinblastine; and curve 10, 20 μM vinblastine. Drugs were added to reaction mixtures held at 0° by the temperature controller, and the reactions were followed briefly in the cold. At the time indicated by the first vertical dashed line, the temperature controller was set at 37°, and it was reset at 0° at the time indicated by the second dashed line. (B) Tubulin polymerization dependent on glutamate. Each 0.25-mL reaction mixture contained 1.0 mg/mL tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 0.4 mM GTP, 4% (v/v) dimethyl sulfoxide, and drug as follows: curve 1, none; curve 2, 0.5 μM dolastatin 10; curve 3, 1.0 μM dolastatin 10; curve 4, 2.0 μM dolastatin 10; curve 5, 3.0 μM dolastatin 10; curve 6, 0.5 μM vinblastine; curve 7, 1.0 μM vinblastine; curve 8, 2.0 μM vinblastine; and curve 9, 3 μM vinblastine. All components except GTP were preincubated in a 0.24-mL volume at 37° for 15 min. Reaction mixtures were chilled on ice and GTP was added. Baselines were set in the spectrophotometer at 0°. At zero time the temperature controller was set at 37°, and it was again set at 0° at the time indicated by the dashed vertical line.

to the cold-stable oligomeric structures induced by phomopsin A [17].

To confirm that dolastatin 10 interacted with tubulin rather than microtubule-associated proteins, its effect on the glutamate-induced polymerization of purified tubulin [12] was examined and compared to that of vinblastine (Fig. 5, panel B). There was little difference in the effects of the two drugs, although dolastatin 10 reproducibly appeared to be slightly more inhibitory than vinblastine. Inhibition of the polymerization of 10 μM tubulin was extensive with 2 μM drug and complete with 3 μM drug with both compounds.*

In addition to these studies with dolastatin 10 and vinblastine, we evaluated the inhibitory effects of maytansine, phomopsin A, and rhizoxin on glutamate-induced tubulin polymerization. Table 1 summarizes the IC_{50} values obtained for the five drugs. Dolastatin 10 appeared to be the most inhibitory (IC_{50}, 1.2 μM), with phomopsin A (IC_{50}, 1.4 μM) and vinblastine (IC_{50}, 1.5 μM) almost as potent (the small differences between the three drugs may only represent experimental variation since they were not statistically significant). Maytansine (IC_{50}, 3.5 μM) was less than half as active as these three drugs, and rhizoxin (IC_{50}, 6.8 μM) was the least inhibitory of all.

Table 1. Relative inhibitory effects of vinca domain drugs on the polymerization of purified tubulin*

Drug	IC_{50} (μM)
Dolastatin 10	1.2 ± 0.08
Phomopsin A	1.4 ± 0.2
Vinblastine	1.5 ± 0.1
Maytansine	3.5 ± 0.1
Rhizoxin	6.8 ± 0.5

* The 0.24-mL reaction mixtures contained 0.25 mg tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 4% (v/v) dimethyl sulfoxide, and various drug concentrations. After a 15-min incubation at 37°, samples were chilled on ice, and GTP was added in 10 μL (final concentration, 0.4 mM). The samples were transferred to cuvettes and polymerization was followed spectrophotometrically at 37°, as described previously [14]. IC_{50}: concentration of drug which inhibited extent of assembly by 50%. Values are means ± SD, N = at least three independent determinations.

* A drug–tubulin preincubation was performed in these experiments, primarily because this has become a standard condition in our laboratory [14]. We have not observed that the preincubation greatly alters the inhibitory effects of either dolastatin 10 or vinblastine in 1 M glutamate. We should also note that high concentrations of vinblastine cause formation of extensive spiral aggregates of tubulin in glutamate [18], as well as with microtubule-associated proteins [15], but limited supplies of dolastatin 10 have not yet permitted us to examine the effects on tubulin in glutamate of higher concentrations of the peptide.

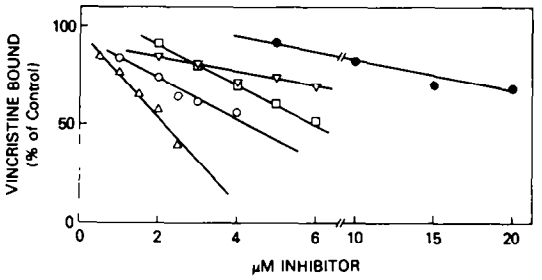


Fig. 6. Comparison of the effects of dolastatin 10, phomopsin A, maytansine, rhizoxin, and vinblastine on the binding of [^{3}H]vincristine to tubulin. Each 0.5-mL reaction mixture contained 0.5 mg/mL tubulin, 0.1 M Mes (pH 7.0), 0.5 mM $MgCl_2$, 3 μM [^{3}H]vincristine, 2% (v/v) dimethyl sulfoxide, and, if present, the indicated concentration of inhibiting drug symbolized as follows: (△) dolastatin 10; (○) phomopsin A; (□) maytansine; (▽) vinblastine; and (●) rhizoxin. After 1 hr at room temperature, 0.15 mL of each reaction mixture was placed in triplicate on 1-mL syringe-columns and processed at room temperature as described in the text; protein and radioactivity in the filtrates were determined and average values obtained. The control value was 0.54 pmol of vincristine bound per μg of tubulin. Note that the 5 μM points are placed so that the rhizoxin curve could also be drawn without interruption.

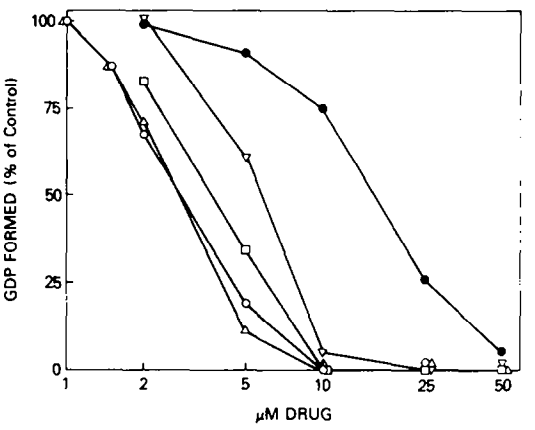


Fig. 7. Comparison of the effects of dolastatin 10, phomopsin A, maytansine, rhizoxin, and vinblastine on tubulin-dependent hydrolysis of GTP. Each 40-μL reaction mixture contained 1.0 mg/mL tubulin, 100 μM [8-^{14}C]GTP, 1.0 M monosodium glutamate (pH 6.6 with HCl), 5% (v/v) dimethyl sulfoxide and, if present, the indicated concentration of inhibiting drug symbolized as follows: (△) dolastatin 10; (○) phomopsin A; (□) maytansine; (▽) vinblastine; and (●) rhizoxin. After 5 min at 37° a 10-μL aliquot was transferred from each reaction mixture into 25% (v/v) acetic acid, and the amount of [8-^{14}C]GDP formed was determined as described in the text. In the control reaction mixture (without drug), 6.6 nmol/mL of [8-^{14}C]GDP was formed.

Inhibition of binding of radiolabeled vincristine to tubulin by dolastatin 10, phomopsin A, rhizoxin, and maytansine. Maytansine [7], rhizoxin [9], and phomopsin A [11] are all known to inhibit the binding of radiolabeled vinca alkaloids to tubulin and to have no inhibitory effect on the binding of radiolabeled colchicine to the protein. We have confirmed these results and found that dolastatin 10 behaves in an analogous fashion. Preliminary studies have been performed with both radiolabeled vinblastine and radiolabeled vincristine. Relative inhibitory effects of all compounds on vinca alkaloid binding were essentially identical with both radiolabeled ligands, and the experiments with [^{3}H]vincristine are presented in Fig. 6.

The five compounds (dolastatin 10, phomopsin A, rhizoxin, vinblastine, and maytansine) examined for inhibition of vincristine binding displayed a wide range of effects as a function of inhibitor concentration. These data permit a preliminary assignment of relative activities of these agents in terms of IC_{50} values. Dolastatin 10 was apparently the most potent inhibitor with an IC_{50} value of approximately 2 μM, followed by phomopsin A (4 μM), maytansine (6 μM), vinblastine (16 μM), and rhizoxin (33 μM). An initial study was also performed on the binding of radiolabeled colchicine to tubulin. None of these agents, including dolastatin 10, had any inhibitory effect on colchicine binding, while podophyllotoxin strongly inhibited colchicine binding as expected (data not presented).

Inhibition of tubulin-dependent GTP hydrolysis by vinca domain drugs. Both vinblastine [19] and maytansine [20] inhibit tubulin-dependent GTP hydrolysis, and it therefore was of interest to determine whether a similar inhibitory effect would be observed with the other vinca domain drugs. Figure 7 demonstrates that this is the case in a reaction system in which hydrolysis, like polymerization, was induced by 1 M glutamate. All five compounds profoundly inhibited GTP hydrolysis, if present in a high enough concentration. In this experiment 50% inhibition was observed with approximately 3 μM dolastatin 10 or phomopsin A, 4 μM maytansine, 6 μM vinblastine, or 16 μM rhizoxin. In addition to the inhibition observed after a 5-min incubation, inhibition of GTP hydrolysis was observed with all drugs after a 60-min incubation. Moreover, when MAPs instead of glutamate were used to induce tubulin-dependent GTP hydrolysis, the reaction was also inhibited by all compounds (data not presented).

DISCUSSION

The studies presented above document that dolastatin 10 is a highly cytotoxic agent acting as an antimitotic compound as a consequence of a specific interaction with tubulin. It inhibits the polymerization of purified tubulin in glutamate as well as the reaction dependent on microtubule-associated proteins. Dolastatin 10 appears to act in the vinca binding domain of tubulin, and its properties were therefore compared to those of rhizoxin, maytansine, and phomopsin A, as well as to those of vinblastine. Table 2 summarizes the relative activities of this group of drugs, normalized against the values obtained for vinblastine, in the reaction systems analyzed here: inhibition of L1210 murine leukemia cell growth, tubulin polymerization, binding of radiolabeled vincristine to tubulin, and tubulin-dependent

Table 2. Relative activities of vinca domain drugs*

Drug	L1210 cell growth	Inhibition of Tubulin polymerization relative to vinblastine	Vincristine binding	GTP hydrolysis
Dolastatin 10	40	1	8	2
Phomopsin A	0.003	1	4	2
Maytansine	40	0.4	3	2
Rhizoxin	20	0.2	0.5	0.4

* For each inhibitory effect of dolastatin 10, phomopsin A, maytansine, and rhizoxin, these drugs are compared to vinblastine, with the IC_{50} value of vinblastine serving as the numerator and that of the alternate drug as the denominator. Thus, positive values represent greater activity than vinblastine, and lower values lesser activity. The L1210 cell growth data were obtained from Fig. 3, the tubulin polymerization data from Table 1, the vincristine binding data from Fig. 6, and the GTP hydrolysis data from Fig. 7.

GTP hydrolysis. While the properties of the five drugs are not coordinate in these studies, dolastatin 10 was among the most active compounds in each series of experiments.

When dolastatin 10 was noted to inhibit the binding of radiolabeled vinca alkaloids to tubulin, we decided to compare it to other drugs reported to inhibit the binding of vincristine or vinblastine to tubulin (maytansine [7], phomopsin A [11], and rhizoxin [9]). A detailed kinetic analysis of the inhibitory effects of phomopsin A on vinblastine or vincristine binding has not yet been published. Takahashi *et al.* [9] examined the effects of ansamitocin P-3 (a close analog of maytansine, structure in Fig. 2) and vinblastine on the binding of radiolabeled rhizoxin to tubulin and of rhizoxin and ansamitocin P-3 on the binding of radiolabeled vinblastine. They concluded that rhizoxin and ansamitocin P-3 bound at the same site on tubulin, but that this differed from the vinblastine site. This conclusion differed from the earlier conclusion of Mandelbaum-Shavit *et al.* [7] with radiolabeled maytansine and radiolabeled vincristine that the two drugs share a common binding site. While the studies presented here do not permit conclusions about whether one or more binding sites are present for these compounds on tubulin, they do demonstrate that both dolastatin 10 and phomopsin A are highly effective inhibitors of the binding of radiolabeled vincristine to tubulin, whereas rhizoxin is the weakest in this group. We are presently attempting to analyze this group of compounds to determine whether one or more drug binding sites are involved in their interactions with tubulin.

It should be noted that our results differ significantly from those of Takahashi *et al.* [9] in that we found maytansine substantially more effective than rhizoxin as an inhibitor of [^{3}H]vincristine binding, whereas they found ansamitocin P-3 and rhizoxin nearly identical in their inhibitory effects on [^{3}H]vinblastine binding. Our results are, however, in good agreement with those of Lacey *et al.* [11] who also found phomopsin A more active than maytansine which, in turn, was more active than nonradiolabeled vinblastine as an inhibitor of [^{3}H]vinblastine (as opposed to [^{3}H]vincristine) binding.

We examined dolastatin 10, as well as the four other drugs, for potential inhibition of colchicine binding to tubulin, but they were all without effect. Unlike Lacey *et al.* [11], we did not observe any enhancement of colchicine binding by any of these agents. This is probably because we routinely examine colchicine binding under a reaction condition that results in the nearly complete stabilization of the colchicine binding activity of tubulin [21]. Enhancement of tubulin stability by its ligands has been described by many workers, with increased binding of one ligand observed in the presence of one or more other ligands [1].

The narrowest range of activities among the five drugs was observed in the tubulin polymerization and GTP hydrolysis assays. This is not surprising, since interference with mitosis is a major manifestation of the cytotoxicity of all of them. Again, dolastatin 10 was among the most potent compounds as an inhibitor of both polymerization and GTP hydrolysis, while rhizoxin was the least active. Although different reaction conditions were used in their studies of polymerization as compared to ours, Takahashi *et al.* [9] found rhizoxin to be a less potent inhibitor of microtubule assembly than was vinblastine; and Lacey *et al.* [11] found phomopsin A and vinblastine to have comparable inhibitory effects on the polymerization reaction, with maytansine less inhibitory. Our polymerization results are similar to those reported by both these groups.

It is worth noting specifically that all five drugs profoundly inhibit tubulin-dependent GTP hydrolysis if present in a high enough concentration. This, as well as their common inhibition of vincristine binding, is consistent with their binding in a single domain on the tubulin molecule, particularly since most agents which bind at the colchicine site stimulate rather than inhibit GTP hydrolysis [1, 14, 16, 19, 20].

The widest range of activities was observed in the comparative cytotoxicity study. Dolastatin 10, maytansine, and rhizoxin were the most effective agents, with phomopsin A over 10,000-fold less effective in inhibiting the growth of the L1210 murine leukemia cells. The poor activity of phomopsin A is perhaps surprising in view of its toxicity for livestock; but in other published studies in which its effects on

cells in culture were specifically examined, phomopsin A was only used in micromolar concentrations [17, 22]. The toxicity of the drug in animals may result from its metabolism, while its relatively weak effect on the L1210 murine leukemia cells may indicate poor uptake, possibly resulting from effects of the two carboxylic acid groups in its side chain.

Structurally, the five compounds studied here are quite diverse. The macrocyclic structures of maytansine and rhizoxin may represent their major common feature, especially in view of the apparent competitive inhibition of rhizoxin binding by the maytansinoid ansamitocin P-3 [9]. Phomopsin A, too, has a macrocyclic ring, although it is smaller than that of maytansine and rhizoxin.

There is no obvious common structural feature with the vinca alkaloids to rationalize the inhibition of their binding to tubulin by the other agents, which is quite strong for maytansine, phomopsin A, and dolastatin 10. Further, the discrepancy in the literature regarding competitive inhibition of vincristine/vinblastine binding by maytansine and rhizoxin [7, 9] raises the question whether a single site or multiple sites are involved.

While dolastatin 10 and phomopsin A are both peptides, and hence it is tempting to speculate that they share a common site on tubulin, at first glance they appear to be structurally quite different. The most prominent structural feature of phomopsin A—the macrocyclic ring containing four amino acid residues and its ether linkage—is completely absent in the linear dolastatin 10. Moreover, phomopsin A is composed solely of α-amino acids (six), while only two of the five residues of dolastatin 10 are α-amino acids.

Nonetheless, a number of structural analogies do exist betwen the two peptides, especially if one compares dolastatin 10 to octahydrophomopsin A, which has the four nonaryl carbon–carbon double bonds reduced and which is equipotent with phomopsin A in its interactions with tubulin [11]. Both peptides contain phenyl (possibly derived from phenylalanine) and tetrahydropyrrole (possibly derived from proline) rings, and several hydrophobic residues which appear to be derived from isoleucine and valine. The active conformations of the two peptides may thus be similar, but the specific structural features that make both compounds such effective inhibitors of tubulin polymerization are uncertain at this time. One peptide or the other is required in a radiolabeled form to establish unambiguously whether they bind at a common site on tubulin. In addition, it will be of great interest to compare the crystal structures of the two molecules, but only that of phomopsin A is currently known [10].

One more feature of dolastatin 10 should be noted, namely that it is the first antimitotic natural product from an animal source. As far as we are aware, all other natural products causing mitotic arrest are derived from plants. The marine sea hare *D. auricularia* is a shell-less mollusk, and it will be of interest to determine whether dolastatin 10 is as cytotoxic with marine tissues (e.g. clam or sea urchin eggs) as it is with mammalian cells. Further, it will also be of interest to determine whether dolastatin 10 is a toxic agent for plant tisues, for there is little overlap between maximally active antimitotic agents in plant and animal cells, even with closely related compounds [18].

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