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DIFFERENTIAL EFFECTS OF ACTIVE ISOMERS, SEGMENTS, AND ANALOGS OF DOLASTATIN 10 ON LIGAND INTERACTIONS WITH TUBULIN

CORRELATION WITH CYTOTOXICITY

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Abstract—Dolastatin 10 is a potent antimitotic peptide isolated from the marine mollusk *Dolabella auricularia*. Four of its five residues are modified amino acids (in sequence, dolavaline, valine, dolaisoleuine, dolaproine, dolaphenine). Besides inhibiting tubulin polymerization, dolastatin 10 noncompetitively inhibits vinca alkaloid binding to tubulin, inhibits nucleotide exchange and formation of the β^s cross-link, and stabilizes the colchicine binding activity of tubulin. To examine the mechanism of action of dolastatin 10 we prepared six chiral isomers, one tri- and one tetrapeptide segment, and one pentapeptide analog of dolastatin 10, all of which differ little from dolastatin 10 as inhibitors of tubulin polymerization. However, only two of the chiral isomers were similar to dolastatin 10 in their cytotoxicity for L1210 murine leukemia cells and in their effects on vinblastine binding, nucleotide exchange, β^s cross-link formation, and colchicine binding. These were isomer 2, with reversal of configuration at position C(19a) in the dolaisoleuine moiety, and isomer 19, with reversal of configuration at position C(6) in the dolaphenine moiety. The pentapeptides with reduced cytotoxicity and reduced effects on tubulin interactions with other ligands were all modified in the dolaproine moiety at positions C(9) and/or C(10). The tripeptide and tetrapeptide segments which inhibited polymerization but not ligand interactions were the amino terminal tripeptide (lacking dolaproine and dolaphenine) and the carboxyl terminal tetrapeptide (lacking dolavaline). We speculate that strong inhibition of other ligand interactions with tubulin requires stable peptide binding to tubulin (i.e. slow dissociation), but that inhibition of polymerization requires only rapid binding to tubulin.

Dolastatin 10 (structure in Fig. 1) is a highly cytotoxic, five-subunit peptide with nine chiral centers originally isolated from the sea hare *Dolabella auricularia* [1]. Definitive stereochemistry was established by its total synthesis [2], which provided ample supplies of the compound for detailed mechanistic studies. We have found that dolastatin 10 inhibits the growth of cultured cells, with 50% growth inhibition occurring at subnanomolar concentrations [3]. Cells accumulate in apparent metaphase arrest at toxic drug concentrations, and, at sufficiently high levels of dolastatin 10, intracellular microtubules completely disappear [4]. Dolastatin 10 is a potent inhibitor of the polymerization of purified tubulin and its associated GTPase reaction and of microtubule assembly requiring both tubulin and microtubule-associated proteins [3]. The peptide noncompetitively inhibits the binding of vincristine to tubulin, and it does not inhibit the binding of colchicine to tubulin. On the contrary, dolastatin 10 prevents the time-dependent decay of the colchicine binding activity of tubulin [5]. Besides these effects on the interactions of other drugs with tubulin, dolastatin 10 affects nucleotide–tubulin interactions. At superstoichiometric concentrations, the peptide almost eliminates exchange of nucleotide at the exchangeable GTP site of tubulin without actually displacing nucleotide already bound to tubulin [5]. Moreover, dolastatin 10 strongly inhibits formation of a cross-link with *N*,*N*′-ethylenebis(iodoacetamide) (EBI‖) between two cysteine residues of β-tubulin [6] which occurs only when the protein is depleted of exchangeable site nucleotide [7]. This is the β^s cross-link between Cys^{12} and either Cys^{201} or Cys^{211} [8].

The development of the synthetic procedure for the preparation of dolastatin 10 also permitted us to perform an initial structure–activity analysis of requirements for the activity of the peptide [9], for a large number of chiral isomers and segments were prepared during the course of the synthesis. We found that reversal of configuration at positions C(18) or C(19) leads to loss of activity for the peptide as an inhibitor of tubulin polymerization, but that reversal of configuration at positions C(9) or C(10) does not reduce inhibitory effects on the reaction

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‖ Abbreviations: EBI, *N*,*N*′-ethylenebis(iodoacetamide); IC_{50}, the drug concentration required to inhibit a defined reaction parameter by 50%; and Mes, 4-morpholineethanesulfonate.

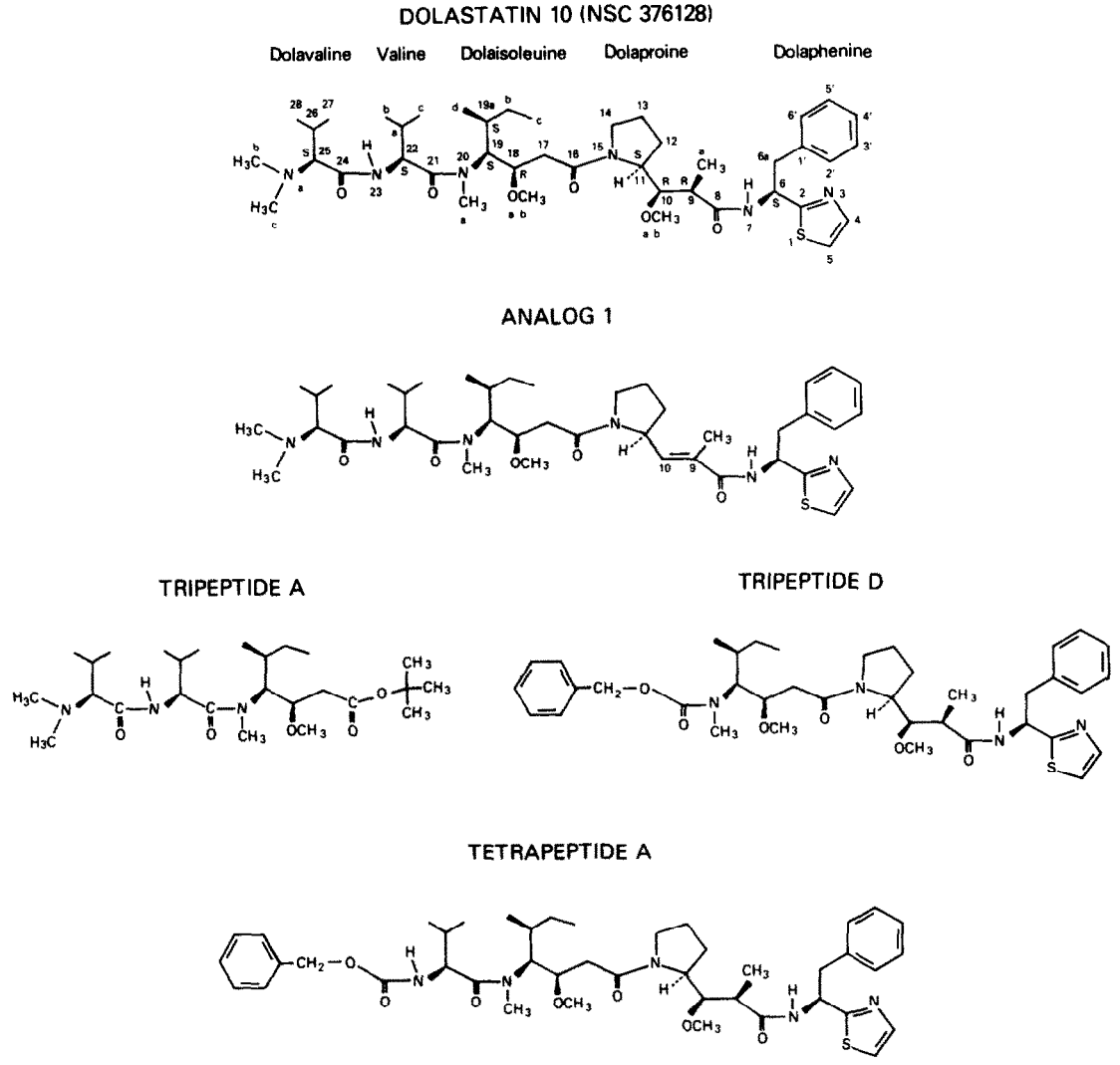

Fig. 1. Structures of dolastatin 10, analog 1, tripeptides A and D, and tetrapeptide A. Note the nine chiral centers in dolastatin 10. See Table 1 for details of the structures of the chiral isomers used in the present studies.

[9]. Cytotoxic activity, however, is greatly reduced by reversal of configuration at any of the four positions [9]. The amino terminal tripeptide segment (tripeptide A, see Fig. 1) also was found to be an effective, but noncytotoxic, inhibitor of tubulin polymerization [9]. The tripeptide, while it inhibits the GTP hydrolysis associated with polymerization, does not inhibit vinca alkaloid binding to tubulin nor nucleotide exchange, nor does it stabilize the colchicine binding activity of tubulin [5].

Based on the findings summarized above, we speculated that dolastatin 10 bound to tubulin in a site near the vinca alkaloid and exchangeable nucleotide sites. We proposed that binding occurred primarily through the amino terminal portion of the molecule, with access to the vinca and nucleotide sites sterically blocked by the carboxyl portion of the molecule (Fig. 2) [5].

We have continued to prepare chiral isomers, segments, and structural analogs of dolastatin 10, with several goals in mind. These include improving the synthetic procedure, preparation of radiolabeled dolastatin 10, and development of more or equally potent compounds for therapeutic use. As will be described here, reversal of configuration at position C(6) (isomer 19) yielded a highly cytotoxic agent which was an effective inhibitor of tubulin polymerization. Our first analog, modified in the dolaproine unit (see Fig. 1), inhibited polymerization, but, like the isomers with reversal of configuration at positions C(9) and C(10), had limited cytotoxicity. Somewhat analogously, newly prepared carboxyl tri- and tetrapeptide segments (tripeptide D* and

* For the new tripeptide segment and chiral isomer of dolastatin 10 described in this paper, we will resume at the letters and numbers used previously [9], which concluded with tripeptide C and isomer 18.

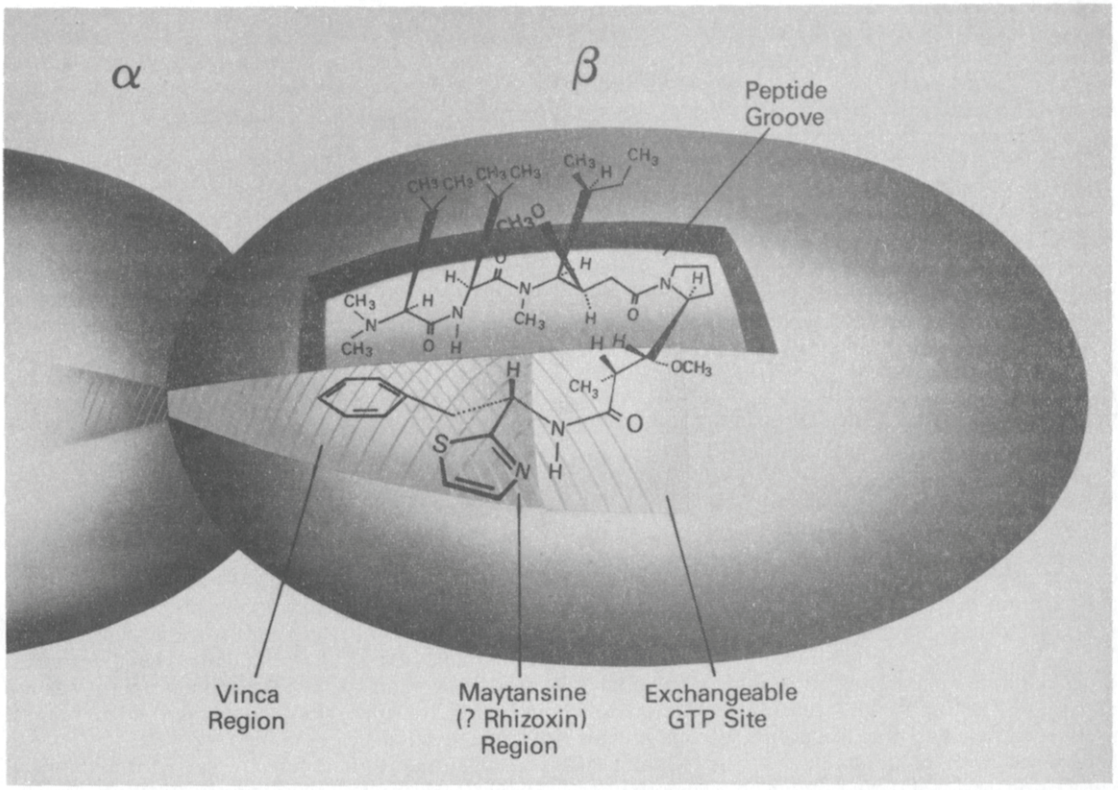

Fig. 2. Model for the binding of antimitotic peptides to tubulin. This model was previously presented elsewhere [5].

tetrapeptide A, see Fig. 1) had activity as inhibitors of polymerization, but little or no cytotoxicity relative to dolastatin 10.

These new results caused us to question our earlier assumption that all the isomers which inhibited tubulin polymerization would have similar effects on the *in vitro* interactions of tubulin with other ligands. We therefore examined both the newly synthesized compounds and those evaluated previously for effects on vinblastine and colchicine binding, on nucleotide exchange, and on formation of the β^s cross-link. All modifications in the dolaproine moiety caused a nearly coordinate loss of these activities of dolastatin 10, and the three segments of the molecule that inhibit polymerization also had little activity in these reactions. Only the cytotoxic isomers with reversal of configuration at either position C(6) or position C(19a) had a full range of activities comparable to those of dolastatin 10.

MATERIALS AND METHODS

Materials. Dolastatin 10 was synthesized as described previously [2], and tripeptide A is a segment prepared in this synthesis. Chiral isomers of dolastatin 10 and the dolastatin 10 "analog 1" were prepared by analogous routes from appropriate synthetic precursors. Isomer and peptide nomenclature used in a previous publication are continued here [9]. Tripeptide D and tetrapeptide A are successive precursors for a new synthetic procedure from carboxyl to amino terminus for the preparation of radiolabeled dolastatin 10 (manuscript in preparation). Electrophoretically homogeneous bovine brain tubulin containing approximately 0.85 mol/mol each of nonexchangeable site GTP and exchangeable site GDP was prepared as described previously, including gel filtration chromatography to remove unbound nucleotide [10]. This tubulin was used in the polymerization and ligand binding studies. The bovine brain tubulin used in the sulfhydryl cross-linking experiments was prepared by the method of Fellous *et al.* [11]. GDP, monosodium glutamate, and nonradiolabeled colchicine, GTP, and vinblastine were obtained from Sigma; [8-^{14}C]GTP was from Moravek; and [^{3}H]-vinblastine and [^{3}H]colchicine were from Amersham. All nucleotides were repurified by anion exchange chromatography by triethylammonium bicarbonate gradient elution.

Methods. The IC_{50} values for inhibition of glutamate-induced polymerization of purified tubulin were determined as before [3]. Drug and tubulin in 0.24 mL were preincubated for 15 min at 37° prior to addition of GTP in 10 μL. The IC_{50} values were determined for the extent of polymerization after 20 min at 37°. Reaction mixtures contained 1.0 mg/mL (10 μM) tubulin, 1.0 M monosodium glutamate

(pH 6.6 with HCl), various drug concentrations, 1 mM $MgCl_2$, 4% (v/v) dimethyl sulfoxide (as drug solvent; not required for polymerization in glutamate), and 0.4 mM GTP. Concentrations refer to the final reaction volume of 0.25 mL.

The binding of radiolabeled vinblastine and GTP to tubulin was followed by centrifugal gel filtration on Sephadex G-50 (superfine) microcolumns prepared in tuberculin syringes [12]. Triplicate 0.15-mL aliquots of each 0.5-mL reaction mixture were processed as described before [12], with radiolabel and protein in the column filtrates quantitated to determine binding stoichiometry. Reaction mixtures contained 0.1 M 4-morpholineethanesulfonate (Mes) (pH 6.9), 0.5 mM $MgCl_2$ and radiolabeled ligand, peptides, tubulin, and dimethyl sulfoxide as indicated in the individual experiments. Incubation in the vinblastine binding experiments was for 15 min at room temperature with centrifugal gel filtration performed at room temperature. In the vinblastine binding experiments tubulin was the last addition to the reaction mixtures. Incubation in the GTP binding experiments was for 15 min on ice with centrifugal gel filtration performed in a 4° cold room. In the GTP exchange experiments the [8-^{14}C]GTP was the last addition to the reaction mixtures, because prior drug addition maximizes the inhibitory effects observed. The 0.1 M Mes reaction condition was used in these experiments because of our greater experience with it in studies of the binding of vinblastine and GTP to tubulin. No experiments have been performed examining inhibition of the binding of these ligands by antimitotic peptides in 1.0 M glutamate. We have no reason to believe that significant qualitative differences would be observed with different peptides in 0.1 M Mes as opposed to 1.0 M glutamate. In addition, we have observed little quantitative difference in inhibitory effects of dolastatin 10 or vinblastine on polymerization in 1.0 M glutamate as opposed to 0.1 M Mes (with microtubule-associated proteins) [3].

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 [12]. Each 20-μL reaction mixture contained 1.0 mg/mL (10 μM) tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 100 μM [8-^{14}C]GTP, 10% (v/v) dimethyl sulfoxide, and peptides as indicated. Tubulin was the last component added to the reaction mixtures. Thus, in contrast to the polymerization assays, there was no drug–tubulin preincubation prior to addition of GTP. Incubation was for 5 min at 37°, and reactions were stopped by addition of 20 μL of 20% (v/v) acetic acid.

The binding of radiolabeled colchicine to tubulin was followed on DEAE-cellulose filters, using the reaction conditions of Ludueña *et al.* [13]. Each 0.1-mL reaction mixture contained 0.37 mg/mL tubulin, 50 μM [^{3}H]colchicine, peptides as indicated at 100 μM, 10% (v/v) dimethyl sulfoxide, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 1.0 mM GTP, 1.0 mM 2-mercaptoethanol, 0.1 mM EDTA, and 1.0 mM ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetate. Reaction mixtures were preincubated for 3 hr at 37° in the absence of [^{3}H]colchicine. After the colchicine was added, incubation resumed for 2 hr at 37°. In a 1.0 M glutamate reaction condition tubulin decay was too slow to obtain useful comparisons [cf. Ref. 14].

Quantitation of drug effects on β^s formation was performed as before [15]. Reaction mixtures (0.25 mL) contained 0.66 mg/mL tubulin, 0.2 mg/mL reduced and carboxymethylated conalbumin, 0.91 mM *N,N'*-ethylenebis(iodoacetamide) (EBI), 50 μM podophyllotoxin, 4% (v/v) dimethyl sulfoxide, and peptides as indicated and were incubated for 1 hr at 30°. The β^s tubulin was resolved from unreacted tubulin by polyacrylamide gel electrophoresis and quantitated by densitometric analysis of the stained gel.

Studies on drug effects on the growth of L1210 murine leukemia cells, including determination of the proportion of cells arrested in apparent metaphase, were performed as described previously [4]. The initial inoculum was 5×10^5 cells into a 5-mL suspension culture in RPMI 1640 medium containing 15% fetal bovine serum (both from Gibco-BRL), 0.3% L-glutamine, 0.01 mg/mL gentamicin sulfate, various drug concentrations, and 0.1% (v/v) dimethyl sulfoxide. The cells were grown in a 5% CO_2 atmosphere at 37°. Cell number was determined after 24 hr, and at this time point there was no decrease from the initial value even at the highest drug concentrations examined.

Only limited studies have been performed on cell viability. With 10^{-8} M dolastatin 10 there was no increase in cell number. If the drug was removed after 1 hr, cell number increased very slowly compared to growth in the control culture. After 2 hr of treatment, growth did not resume following removal of the drug (data not presented).

For determination of mitotic index, cells were harvested by centrifugation after 8–16 hr of drug treatment and washed in phosphate-buffered saline (pH 7.2). They were swollen in 0.08 M sodium phosphate (pH 7.2) for 10 min, recentrifuged, fixed with ice-cold 1.5%(v/v)–0.5%(v/v) acetic acid for 15 min, recentrifuged, suspended in 75% (v/v) ethanol–25% (v/v) acetic acid, transferred to a slide, stained with Giemsa, and examined by bright-field microscopy. With all peptides an increase in the mitotic index was observed at toxic drug concentrations.

RESULTS

Inhibition of tubulin polymerization and cell growth. Figure 1 and Table 1 summarize the structures, including configuration at chiral centers, of the compounds that were examined in the studies presented here. Isomers 1–5 were found previously [9] to differ little from dolastatin 10 in their abilities to inhibit tubulin polymerization at concentrations substoichiometric to the tubulin concentration (10 μM). Isomers 6 and 7 were much less potent inhibitors of polymerization, and an additional 11 isomers had little or no inhibitory effect on polymerization. In this earlier study tripeptide A was also found to inhibit polymerization, while two chiral isomers of the tripeptide at positions equivalent to C(18) and/or C(19) were noninhibitory. We had

Table 1. Effects of dolastatin 10 and related compounds on cell growth and tubulin-dependent reactions

Peptide	Position 6	9	10	18	19	19a configuration*	Inhibition of polymerization† IC_{50} (μM)	Inhibition of cell growth‡ IC_{50} (M)	Inhibition of vinblastine binding§ IC_{50} (μM)	Inhibition of nucleotide exchange‖ IC_{50} (μM)	Inhibition of GTP hydrolysis¶ IC_{50} (μM)
Dolastatin 10	S	R	R	R	S	S	1.2 ± 0.1	9×10^{-10}	6	10	4.0
Isomer 1			S				1.3 ± 0.2**	6×10^{-8}	80	50	3.2
Isomer 2						R	1.4 ± 0.06**	3×10^{-10}	4	7	2.2
Isomer 3		S				R	1.2 ± 0.1**	3×10^{-8}	75	120††	2.7
Isomer 4		S	S				1.4 ± 0.3**	4×10^{-7}	120‡‡	90	2.4
Isomer 5		S					2.6 ± 0.4**	4×10^{-8}	105‡‡	50	5.1
Isomer 6				S			10 ± 0.8**	$> 10^{-6}$**	NI	NI	14
Isomer 7				S	R		18 ± 1**	4×10^{-8}**	NI	NI	50
Isomer 19	R						1.9 ± 0.06	5×10^{-9}	8	7	5.7
Analog 1		NA	NA				4.9 ± 0.3	3×10^{-8}	140‡‡	290††	4.0
Tripeptide A	NA	NA	NA				4.2 ± 0.1**	$> 10^{-6}$	NI	NI	3.3
Tripeptide D							13 ± 0.8	$> 10^{-6}$	NI	NI	8.5
Tetrapeptide A							3.0 ± 0.2	2×10^{-7}	150‡‡	230††	4.2

* See Fig. 1 for structural details. Configurations are listed only for positions at which isomeric forms exist. Details are presented for all positions for dolastatin 10. Otherwise, only variants from the dolastatin 10 configuration are listed. For the tripeptides and tetrapeptide, position numbers refer to the analogous positions in dolastatin 10. NA, not applicable. In analog 1 the substituent at position C(10) is absent and the 9–10 bond has been changed to sp^2(olefin) (see Fig. 1).

† The IC_{50} values for inhibition of tubulin polymerization were determined a minimum of three times, with the data plotted graphically. Average values with standard deviation are presented in the table.

‡ The IC_{50} values for the growth of L1210 murine leukemia cells were determined in two independent experiments (average values presented in the table), with cell growth measured after 24 hr.

§ Data from Fig. 3. NI, noninhibitory.

‖ Data from Fig. 5. NI, noninhibitory.

¶ Data from Fig. 6.

** Data from Ref. 9.

†† Value extrapolated from data presented in Fig. 5.

‡‡ Value extrapolated from data presented in Fig. 3.

also found that all isomers and segments, except isomer 2, were considerably less cytotoxic than dolastatin 10 towards L1210 murine leukemia cells. To summarize this earlier work, isolated reversal of configuration at positions C(9) and/or C(10) in the dolaproine moiety did not greatly alter inhibitory effects on tubulin polymerization but did reduce cytotoxicity. In contrast, reversal of configuration at positions C(18) and/or C(19), but not C(19a), in the dolaisoleuine moiety significantly reduced inhibitory effects on tubulin polymerization and on cell growth. Reversal of configuration at chiral centers in both amino acids yielded compounds with negligible activity in both assays.

For the current study the relative inhibitory effects on tubulin polymerization of isomers 1–7 and tripeptide A were confirmed in single assays, but these compounds were not reevaluated in detail. The values presented in Table 1 for dolastatin 10, isomer 19, analog 1, tripeptide D, and tetrapeptide A were obtained in contemporaneous experiments. Since more variability can occur in cytotoxicity assays, all values, except those for isomers 6 and 7, presented in Table 1 for effects on the growth of L1210 murine leukemia cells were obtained in contemporaneous experiments. The values obtained for dolastatin 10 and for isomers 1–5 did not differ substantially from the IC_{50} values obtained earlier [9].

In view of the strong inhibitory effects on tubulin polymerization of isomers 1, 3, 4, and 5 and, especially, of the truncated tripeptide A, it was not surprising that isomer 19 and analog 1 were also good polymerization inhibitors. Isomer 19 had reversal of configuration only at position C(6) in the carboxyl terminal amine, dolaphenine, while analog 1 was modified at positions C(9) and C(10), with the C(10) substituent removed and the 9–10 bond changed from sp^3 to sp^2 (olefin). These latter changes resulted in a larger loss in activity (IC_{50} of 4.9 μM for analog 1) than reversal of configuration at position C(9) (isomer 5, IC_{50} of 2.6 μM) or at both C(9) and C(10) (isomer 4, IC_{50} of 1.4 μM), or even in removal of the carboxyl terminal dipeptide (tripeptide A, IC_{50} of 4.2 μM).

Consistent with the earlier findings with L1210 cells, analog 1 had substantially reduced cytotoxicity compared to dolastatin 10. The IC_{50} value obtained with analog 1 was similar to those obtained with the three chiral isomers modified only at positions C(9) and/or C(10) (isomers 1, 4, and 5). In contrast, isomer 19, with reversal of configuration only at position C(6) in the dolaphenine moiety, was only moderately less cytotoxic than dolastatin 10. Of all the unnatural peptides prepared thus far, its cytotoxicity towards L1210 cells is exceeded only by that of isomer 2, which has reversal of configuration only at position C(19a) in the dolaisoleuine side chain.

In our earlier study with dolastatin 10 segments [9], we found that *N-tert*-butoxycarbonyl-dolaproinyldolaphenine did not inhibit the polymerization of tubulin. In our current approach to the synthesis of radiolabeled dolastatin 10 we prepared *N-tert*-butoxycarbonyl-dolaisoleuinyldolaproinyldolaphenine (tripeptide D) and *N-tert*-butoxycarbonyl-valyldolaisoleuinyldolaproinyldolaphenine (tetrapeptide A) (Fig. 1), and both carboxyl terminal segments were analyzed for their effects on tubulin polymerization (Table 1). Addition of the dolaisoleuine moiety to the inert carboxyl terminal dipeptide yielded a tripeptide with modest inhibitory properties (IC_{50} of 13 μM for tripeptide D), about one-third as potent as the amino terminal tripeptide A. Addition of valine to tripeptide A to form the carboxyl terminal tetrapeptide A, however, yielded a strong inhibitor of polymerization (IC_{50} of 3.0 μM) even more potent than tripeptide A.* Thus it appears that the amino terminal dolavaline is less important for effective interaction with tubulin than is the carboxyl terminal dolaproinyldolaphenine. The carboxyl terminal tripeptide D was not cytotoxic, while tetrapeptide A weakly inhibited cell growth (IC_{50} value of 0.2 μM).

Effects on vinblastine binding to tubulin. We have shown previously that dolastatin 10 is a potent noncompetitive inhibitor of the binding of [^{3}H]-vincristine to tubulin and of GTP exchange [5]. Tripeptide A, in contrast, had negligible effects on the binding of these ligands to tubulin. These findings led us to propose that dolastatin 10 bound to tubulin in a different site than the vinca alkaloids, but in a site near the vinca and exchangeable nucleotide sites. We speculated that the dolaproine and/or dolaphenine moieties sterically block access to the other sites to account for the failure of tripeptide A to interfere with the binding of vinca alkaloids and GTP (Fig. 2). Such a model predicts that tetrapeptide A, and perhaps the less active tripeptide D, should inhibit GTP exchange and the binding of vinca alkaloids to tubulin.

When vinblastine binding was examined, however, we found that neither tripeptide D (data not presented) nor tetrapeptide A (Fig. 3) strongly inhibited the reaction. In the experiment presented in Fig. 3 about 25 times as much tetrapeptide A as dolastatin 10 was required to obtain equivalent inhibition of vinblastine binding (an extrapolated IC_{50} value of 150 μM was obtained for tetrapeptide A as compared with a value of 6 μM for dolastatin 10), even though only about 2.5 times as much tetrapeptide was required for a comparable inhibitory effect on tubulin polymerization.

These results led us to examine the effects of all dolastatin 10 chiral isomers and analog 1 on vinblastine binding to tubulin, and we found that potent inhibitory effects on polymerization did not indicate that there would be potent inhibition of vinblastine binding (Fig. 3). Besides dolastatin 10 strong inhibition occurred only with isomer 2, which was more potent than dolastatin 10, and isomer 19. These two isomers were also those which were most toxic to the L1210 cells. Weak or almost negligible inhibition was observed with tetrapeptide A, isomers 1, 3, 4, and 5, and analog 1. No significant inhibition was observed with 100 μM concentrations of isomers 6–18 or tripeptides A and D (data not presented).

* The deprotected tetrapeptide (valyldolaisoleuinyldolaproinyldolaphenine) differed little from tetrapeptide A in its activities as an inhibitor of tubulin polymerization and of L1210 murine leukemia cell growth.

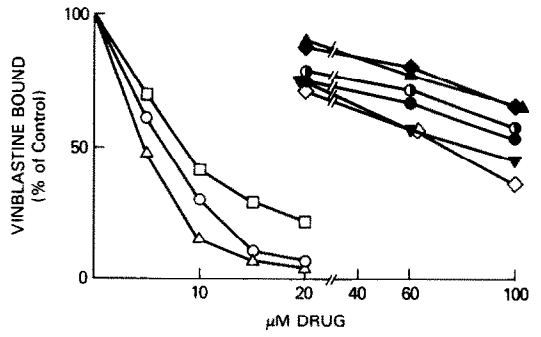

Fig. 3. Comparison of the effects of dolastatin 10 with those of isomers 1–5 and 19, analog 1, and tetrapeptide A on the binding of vinblastine to tubulin. Reaction mixtures contained 1.0 mg/mL (10 μM) tubulin, 10 μM [^{3}H]-vinblastine, 10% (v/v) dimethyl sulfoxide, and peptides as indicated. In the control reaction mixtures, in the absence of peptide, 0.49 pmol of [^{3}H]vinblastine was bound per pmol tubulin. Key: (○) dolastatin 10; (▼) isomer 1; (△) isomer 2; (◇) isomer 3; (◑), isomer 4; (●), isomer 5; (□) isomer 19; (◆) analog 1; and (▲) tetrapeptide A.

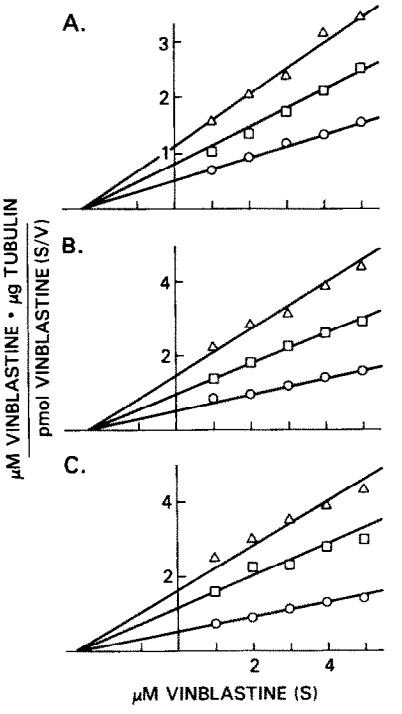

Fig. 4. Noncompetitive inhibition of the binding of vinblastine to tubulin by dolastatin 10, isomer 2, and isomer 19. Reaction mixtures contained 0.5 mg/mL (5 μM) tubulin, 2% (v/v) dimethyl sulfoxide, and the indicated concentrations of [^{3}H]vinblastine and peptides. (A) Inhibition by dolastatin 10. Concentrations of dolastatin 10: (○) none; (□) 2 μM; and (△) 4 μM. (B) Inhibition by isomer 2. Concentrations of isomer 2: (○) none; (□) 2 μM; and (△) 3 μM. (C) Inhibition by isomer 19. Concentrations of isomer 19: (○), none; (□) 5 μM; and (△), 7 μM.

The data presented in Fig. 3 are summarized in Table 1 in terms of IC_{50} values.

We confirmed that the pattern of inhibition of [^{3}H]vinblastine binding with dolastatin 10, as well as with isomers 2 and 19, was noncompetitive (Fig. 4), as it had been for [^{3}H]vincristine binding. These experiments are presented in the Haynes format in which noncompetitive inhibition is indicated when data obtained at different inhibitor concentrations yield curves which intersect on the abscissa (as in the Lineweaver–Burk format), in contrast to competitive inhibition which yields parallel curves (as posed to the ordinate intercept obtained in the neweaver–Burk format) [16]. Dixon analyses [16] of these data and a second set of similar experiments were performed and yielded the following apparent K_i values: 0.9 μM for dolastatin 10; 0.6 μM for isomer 2; and 2.8 μM for isomer 19.

Effects on tubulin-nucleotide interactions. As with inhibition of vinblastine binding, tetrapeptide A was disproportionately less effective than dolastatin 10 as an inhibitor of nucleotide exchange on tubulin (measured as inhibition of the binding of [8-^{14}C]-GTP to tubulin*), as compared with their relative inhibitory effects on polymerization (Fig. 5; Table 1). While comparable inhibition of polymerization required about 2.5 times as much tetrapeptide as dolastatin 10, equivalent inhibition of GTP exchange required 23 times as much tetrapeptide (extrapolated IC_{50} value of 230 μM for the tetrapeptide as compared with 10 μM for dolastatin 10).

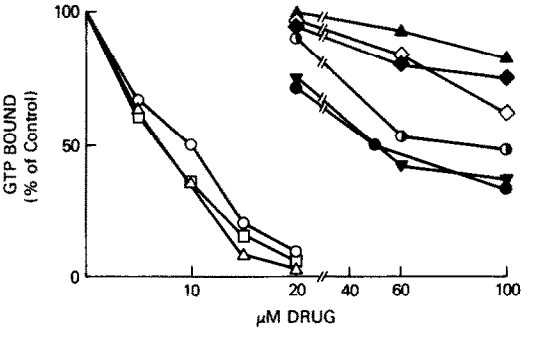

Fig. 5. Comparison of the effects of dolastatin 10 with those of isomers 1–5 and 19, analog 1, and tetrapeptide A on the binding of GTP to tubulin (nucleotide exchange). Reaction mixtures contained 1.0 mg/mL (10 μM) tubulin, 50 μM [8-^{14}C]GTP, 10% (v/v) dimethyl sulfoxide, and peptides as indicated. The peptides were always added prior to addition of the GTP, since maximal inhibition of nucleotide exchange requires that inhibitor be added first. In the control reaction mixtures, in the absence of peptide, 0.65 pmol of [8-^{14}C]GTP was bound per pmol tubulin. Symbols as in Fig. 3.

* Experiments presented previously demonstrated that no displacement of exchangeable site GDP from tubulin occurs when dolastatin 10 (as well as phomopsin A, maytansine, halichondrin B, or rhizoxin) interacts with the protein [5, 17, 18]. It is nucleotide *exchange* rather than nucleotide *binding* which is actually inhibited by these antimitotic compounds.

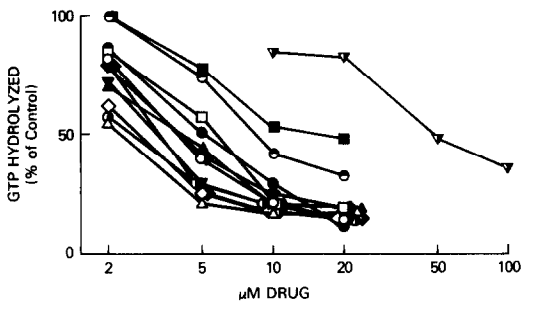

Fig. 6. Comparison of the effects of dolastatin 10 with those of isomers 1–7 and 19, analog 1, tripeptides A and D, and tetrapeptide A on tubulin-dependent GTP hydrolysis. Peptide concentrations were as indicated. In the control reaction mixtures, in the absence of peptide, 7.3 nmol of [8-^{14}C]GDP per mL of reaction was formed. Symbols as in Fig. 3, with the following additions: (■) isomer 6; (▼) isomer 7; (◆) tripeptide A; and (◓) tripeptide D.

When the chiral isomers, analog 1, and tripeptides A and D were examined to determine their effects on GTP exchange, results similar to their effects on vinblastine binding were obtained (Fig. 5; Table 1). Isomers 2 and 19 were both more effective than dolastatin 10. Isomers 5, 1, 4, and 3 and analog 1 were progressively weaker inhibitors of GTP exchange, despite their strong inhibitory effects on polymerization. As with vinblastine binding, no significant inhibition of GTP exchange occurred with the remaining 13 chiral isomers of dolastatin 10 or with tripeptides A and D when any of these compounds were present at 100 μM in the reaction mixture (data not presented).

The failure of peptides that inhibit tubulin polymerization to interfere strongly with GTP exchange and vinblastine binding raises the possibility that these compounds may interact with tubulin in a different site than dolastatin 10 (see Discussion). Thus far, all drugs which interfere with vinblastine binding to tubulin inhibit polymerization-associated GTP hydrolysis [3, 17, 19, 20]. In contrast, some colchicine site drugs inhibit polymerization-associated hydrolysis [19–22], while others induce a GTPase reaction uncoupled from polymerization with apparent stimulation of net GTP breakdown [19, 20, 23]. In addition, no colchicine site drug has yet been found to interfere with GTP exchange on tubulin [18]. It was therefore of interest to determine whether significant differences would be observed in the effects of dolastatin 10, its chiral isomers, analog 1, and the inhibitory segments on tubulin-dependent GTP hydrolysis. No such differences were observed (Fig. 6), and inhibition of GTP hydrolysis was closely linked to inhibition of polymerization. With all strong inhibitors of polymerization over 75% inhibition of hydrolysis occurred with a stoichiometric amount of drug relative to tubulin, and IC_{50} values for inhibition of GTP hydrolysis were more closely correlated with those for inhibition of polymerization than those for inhibition of nucleotide exchange (Table 1).* Only tripeptide D and isomers 6 and 7 were clearly distinguishable by weaker effects on the reaction. The hydrolysis results thus provide no evidence that the different peptides interfere with tubulin polymerization by different mechanisms even though there is no correlation between effects of this group of compounds on nucleotide exchange, as measured by binding of [8-^{14}C]GTP to tubulin, and their effects on GTP hydrolysis.

Effects on cross-link formation in nucleotide-depleted tubulin. EBI, a bivalent chemical that reacts with sulfhydryl groups, can induce formation of two independent cysteine–cysteine cross-links in β-tubulin. One of these, termed β^s between Cys^{12} and either Cys^{201} or Cys^{211} [8], is suppressed completely in the presence of exogenous guanine nucleotide, and demonstration of its formation requires the use of nucleotide-depleted tubulin [7]. Formation of the β^s cross-link is also strongly inhibited by sufficient concentrations of dolastatin 10 [6], maytansine [7], rhizoxin [24], phomopsin A [25], and halichondrin B [26], while vinblastine is only partially inhibitory [7]. There appears to be a close correlation between the ability of antimitotic agents to inhibit nucleotide exchange on tubulin [5, 17, 18] and their ability to inhibit β^s formation. We therefore examined the abilities of chiral isomers 1–7 and 19, analog 1, tripeptides A and D, and tetrapeptide A, in comparison with dolastatin 10, to inhibit formation of this intra-β cross-link (Table 2).

Relative to the strong inhibitory effect of dolastatin 10 on β^s formation, comparable activity was observed with only two compounds, isomer 2 (slightly more effective) and isomer 19 (slightly less effective). Weak inhibition of β^s cross-link formation occurred with isomer 3 and tetrapeptide A. The latter was again disproportionately less active in this assay than it had been as an inhibitor of polymerization. The remaining chiral isomers, analog 1, and the tripeptides had little or no inhibitory effect on β^s formation.

Effects on the colchicine binding activity of tubulin. Dolastatin 10, as well as phomopsin A, prevents the time-dependent denaturation of tubulin as measured by prevention of both increased binding of a fluorescent hydrophobic dye and loss of colchicine binding activity [5, 6, 13, 25]. We evaluated the dolastatin 10 chiral isomers, analog 1, and the peptide segments for their effects on the latter reaction (Table 3).

Under the conditions used in the experiments presented in Table 3, inclusion of dolastatin 10 in

* Note that there were several differences in reaction conditions between the experiments in which IC_{50} values were obtained for polymerization and for GTP hydrolysis. In the former experiments, but not the latter, a drug–tubulin preincubation was used, and this would tend to reduce the IC_{50} values obtained. In the GTPase experiments a higher dimethyl sulfoxide concentration was used (10 vs 4%), and this might lead to increased polymerization and GTP hydrolysis (i.e. higher IC_{50} values). In previous studies with dolastatin 10 and other vinca domain agents [3], we did not observe significant differences in extent of inhibition of GTP hydrolysis as a function of incubation time, but such a comparison has not been performed with the isomers, segments, or analog 1.

Table 2. Effects of dolastatin 10 and related compounds on β^s formation*

Peptide (μM)	Percent inhibition of β^s formation†
Experiment I	
Dolastatin 10 (5)	63
Dolastatin 10 (20)	100
Isomer 1 (20)	22
Isomer 2 (5)	87
Isomer 2 (20)	100
Isomer 3 (20)	41
Isomer 4 (20)	25
Isomer 5 (20)	26
Isomer 6 (20)	12
Isomer 7 (20)	31
Isomer 19 (5)	59
Isomer 19 (20)	90
Experiment II	
Dolastatin 10 (20)	87
Analog 1 (20)	0
Tripeptide A (100)	15
Tripeptide D (100)	14
Tetrapeptide A (20)	28
Tetrapeptide A (40)	41

* See text for experimental details.

† Data are expressed relative to the average of two control values obtained in each experiment in the absence of peptides. In Experiment I the control values were 32.1 and 43.2% of β_1-tubulin converted to the β^s form; in Experiment II the control values were 28.5 and 29.9%.

the reaction mixture resulted in enhanced binding of colchicine to tubulin relative to the nonpreincubated control. This also probably represents a stabilization effect of the peptide, which prevents decay during the 2-hr incubation of colchicine with tubulin. As in the other reactions described above, only isomer 19 and especially isomer 2 had activity comparable to that of dolastatin 10 itself. Relatively good stabilization, in which the preincubated tubulin had activity comparable to that of the nonpreincubated control tubulin, occurred in reaction mixtures containing isomers 1 and 3 and tetrapeptide A. Partial stabilization, in which the preincubated tubulin had activity between that of the preincubated and nonpreincubated controls occurred in reaction mixtures containing isomers 4, 5, and 7 and analog 1. No significant stabilizing effect was demonstrable with isomer 6 or tripeptides A and D, nor with isomers 9–18 (data not presented).*

DISCUSSION

Dolastatin 10 is a highly cytotoxic antimitotic agent that inhibits tubulin polymerization and the associated hydrolysis of GTP, noncompetitively inhibits the binding of vinca alkaloids to the protein, interferes with nucleotide exchange, prevents formation of the β^s cysteine–cysteine cross-link, and strongly stabilizes the native conformation of tubulin [3, 5, 6]. In this report we have extended our evaluation of eighteen chiral isomers of the peptide, particularly five isomers which are potent inhibitors of polymerization [9], to the other effects dolastatin 10 has on tubulin *in vitro*. We also report our results with a nineteenth chiral isomer, with an initial analog of dolastatin 10 with a major modification in the dolaproine residue, and with two carboxyl terminal segments of dolastatin 10.

Knowledge of the relative cytotoxic effects of dolastatin 10 and related peptides is of great importance in planning for the preclinical and clinical evaluation of these compounds as potential chemotherapeutic agents. Since only scant amounts of dolastatin 10 can be obtained from its natural source, these studies will require synthetic peptide. If a more active isomer or analog can be identified, or a more readily synthesized analog can be shown to have comparable potency, then the choice of peptide for development will need to be carefully considered.

Our earlier work with chiral isomers had indicated that while tubulin polymerization was not greatly affected by reversal of configuration at positions C(9) or C(10) in the dolaproine moiety, such changes produced isomers much less cytotoxic than dolastatin 10 [9]. Analog 1 was prepared to examine this further by removing the substituent at position C(10) and introducing a double bond between C(9) and C(10). Once again a compound was obtained that inhibited tubulin polymerization but that had considerably less cytotoxicity than dolastatin 10. Thus, the dolaproine moiety still seems to be an unpromising candidate for chemical modification.

Isomer 19 was prepared as an initial approach to

* With isomer 8 partial stabilization occurred (a value of 0.28 pmol colchicine bound was obtained). Isomer 8 has reversal of configuration at positions C(18), C(19), and C(19a) [9].

Table 3. Effects of dolastatin 10 and related compounds on colchicine binding*

Peptide added	pmol colchicine bound/pmol tubulin (% preincubated control)
None (no preincubation)	0.30 (NA†)
None (preincubation)	0.18 (100)
Dolastatin 10	0.41 (228)
Isomer 1	0.30 (167)
Isomer 2	0.42 (233)
Isomer 3	0.33 (183)
Isomer 4	0.24 (133)
Isomer 5	0.29 (161)
Isomer 6	0.19 (106)
Isomer 7	0.27 (150)
Isomer 19	0.37 (206)
Analog 1	0.26 (144)
Tripeptide A	0.18 (100)
Tripeptide D	0.17 (94)
Tetrapeptide A	0.31 (172)

* See text for experimental details. The preincubation without colchicine was for 3 hr at 37°. The incubation following colchicine addition was for 2 hr at 37°.

† NA, not applicable.

study the role of the dolaphenine moiety in the cytotoxicity of dolastatin 10. The high activity of this compound as an inhibitor of polymerization was not surprising in view of the inhibitory activity of tripeptide A, which lacks both the dolaproine and dolaphenine residues. The cytotoxicity of isomer 19, with reversal of configuration only at position C(6), was only moderately less than that of dolastatin 10, indicating that the dolaphenine moiety has at most only a minor role in the cytotoxicity of the natural peptide. This finding is of practical importance, for the preparation of dolaphenine is now the most difficult and expensive step in the synthesis of dolastatin 10. If an alternate residue could be placed at the carboxyl terminus with retention of activity, such an analog would merit careful study.

Tetrapeptide A, lacking only the dolavaline residue, had good activity as an inhibitor of tubulin polymerization, but this segment of dolastatin 10 had minimal cytotoxicity. This implies that synthetic modification in the amino terminal residue would be unrewarding in terms of analogs with clinical promise.

The other major aspect of the work presented here is the incomplete correlation of inhibitory effects of peptides on tubulin polymerization with their effects on the other *in vitro* tubulin reactions. Not only the amino terminal tripeptide A [5], but also the carboxyl terminal tetrapeptide A and pentapeptides with modifications in the dolaproine moiety (analog 1 and isomers 1, 3, 4, and 5) failed to have major effects on vinblastine binding, nucleotide exchange, and formation of the β^s cross-link despite activity as inhibitors of polymerization comparable to that of dolastatin 10. Only isomers 2 and 19 [with reversal of configuration at positions C(19a) and C(6), respectively] had properties in these reactions similar to those of dolastatin 10. These two isomers were also the only ones equivalent to dolastatin 10 in ability to stabilize the colchicine binding activity of tubulin, while a range of partial activities in this assay was observed with the other peptides active as inhibitors of polymerization.

Although these findings do not appear to support our earlier model [5] for the binding site on tubulin of peptide antimitotic agents, careful consideration of these and related data also does not provide convincing support for any of several alternatives. The earlier model (see Fig. 2) placed the binding site on tubulin for dolastatin 10 ("the peptide site") in close physical proximity to both the vinca site and the exchangeable GTP site with steric, as opposed to allosteric, factors accounting for the interference of the peptide with the interaction of tubulin with the other ligands. The model was based on nearly equivalent findings with dolastatin 10 and another peptide antimitotic agent, phomopsin A, both as noncompetitive inhibitors of vincristine binding and as inhibitors of nucleotide exchange that did not displace GDP from the exchangeable site, and on the failure of tripeptide A to affect these reactions [5]. The inhibitory effects of GTP, dolastatin 10, and phomopsin A, but not tripeptide A, on β^s formation further support such a model. Because the proposed peptide site was near the binding site for vinca alkaloids, we suggested that this region of the tubulin molecule be termed the "vinca domain" to distinguish it from the specific vinca binding site. The vinca domain was placed primarily on β-tubulin because the exchangeable GTP site has been localized to this subunit [27, 28].

One alternative interpretation is that the effects of dolastatin 10, and of phomopsin A, on the interactions of other ligands with tubulin are due to

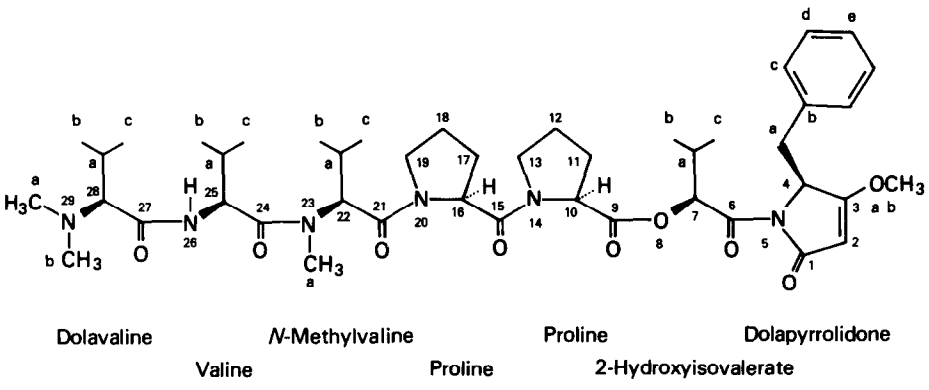

Fig. 7. Structure of dolastatin 15.

allosteric factors, with the peptides binding in a truly distinct site on the protein, whether distant or close to the sites of other ligands. Such a model readily accounts for the noncompetitive inhibition of vincristine and vinblastine binding obtained with dolastatin 10, isomers 2 and 19, and phomopsin A on the one hand and the negligible inhibition obtained with the dolaproine-modified isomers and the segments on the other. The latter compounds when bound to tubulin would inhibit polymerization but would not have adequate structural features to alter the conformation of the protein enough to interfere with its interactions with other ligands. However, this model does not adequately account for the nearly identical effects of dolastatin 10, isomers 2 and 19, and phomopsin A on interactions of tubulin with such diverse ligands and on β^s formation. Nor is it consistent with the fact that all natural products which interfere with vinca alkaloid binding (maytansine, rhizoxin, dolastatin 10, phomopsin A, halichondrin B) inhibit both nucleotide exchange and β^s formation, whether the inhibition of vinca binding is competitive or noncompetitive. Conversely, drugs that do not inhibit vinca binding neither affect GTP exchange [23] nor inhibit β^s formation [29].

A third model which merits consideration is that more than one binding site exists for dolastatin 10. If this were the case, then binding at either site could inhibit polymerization, while binding at one of the two sites might interfere with interactions with other ligands. Li *et al.* [30] recently described Scatchard data obtained with radiolabeled phomopsin A that indicated two classes of binding site. Timasheff *et al.* [31] have argued persuasively, however, that if a ligand causes tubulin–tubulin interactions such Scatchard results must be interpreted with great caution and that only a single binding site may actually exist. While Li *et al.* [30] reported that phomopsin A did not cause tubulin–tubulin interactions in their system, Tonsing *et al.* [32] described extensive interactions induced by phomopsin A. Their work included a well-documented morphological description of the oligomer formed in the presence of the drug as a consequence of microtubule disassembly. We have observed formation of tubulin oligomers induced by both phomopsin A and dolastatin 10 from unpolymerized tubulin [33; unpublished observations], and, in the presence of microtubule-associated proteins, these were morphologically identical to those described by Tonsing *et al.* [32].

Lacking evidence that clearly supports either of these two alternative models, we still prefer our original steric interference model [5]. Under our experimental conditions the binding of both radiolabeled vinca alkaloids and GTP is so rapid that binding of these ligands is near maximal at the earliest time points we have examined even at 0°. In evaluating the effects of dolastatin 10 and its analogs, or of other compounds, on inhibition of the binding of vinca alkaloids to tubulin or on nucleotide exchange, we are not measuring inhibition of the rate of binding of the radioligand. In the case of the vinca alkaloids we seem to be measuring equilibria involving formation of a mixture of tubulin–inhibitor and tubulin–radioligand complexes. Measurement of inhibition of nucleotide exchange by vinca domain drugs is even more complicated, for maximal effects require that tubulin and drug be mixed prior to addition of radiolabeled nucleotide to the reaction mixture. Further, extent of inhibition varies with reaction temperature. Maytansine is nearly as potent as phomopsin A and dolastatin 10 in inhibiting exchange at 0° [17], but its inhibitory potency progressively diminishes as reaction temperature rises [5,18]. A similar loss of activity occurs with rhizoxin [5]. This probably results from differential effects of temperature on the tubulin–nucleotide equilibrium as opposed to the tubulin–maytansine (or rhizoxin) equilibrium. In contrast, only a small loss of inhibitory effects on exchange occurs with dolastatin 10 and phomopsin A when reaction temperature increases, and this led us to predict that these antimitotic agents would bind rapidly and tightly to tubulin, especially as compared to GTP [5]. Such tight binding appears to occur with phomopsin A [30]. We therefore believe that the

dolaproine-modified dolastatin 10 isomers and analog, as well as tripeptide A and tetrapeptide A, bind much less tightly to tubulin than does dolastatin 10 but rapidly enough to inhibit the polymerization reaction.* As a consequence the equilibria with vinblastine and GTP were not greatly altered in their presence, nor was the binding of these peptides to tubulin sufficiently stable to prevent β^s cross-link formation or some loss of colchicine binding activity. Assuming this model is essentially correct, the portions of the dolastatin 10 molecule which sterically interfere with nucleotide exchange and vinca binding to tubulin are not yet as clearly established as implied in the original scheme presented in Fig. 2.

We should note that thus far our studies on effects on tubulin polymerization of the series of peptides presented here have been limited to one reaction condition and to a single parameter (turbidity at 20 min) of that condition. Although the apparent inhibitory effects of dolastatin 10, isomers 1–5 and 19, analog 1, tripeptide A, and tetrapeptide A did not differ greatly in our experiments, altering the reaction conditions and/or the reaction parameter could change the relative activities of the peptides and bring them into greater accord with the cytotoxicity/ligand interaction data.† This could be especially true if more subtle effects on tubulin polymerization were examined at submicromolar peptide concentrations. Examples of such experimental approaches would be evaluation of peptide effects on the flux of tubulin subunits through the microtubule and on association–dissociation reactions at microtubule ends [37, 38] or of peptide effects on microtubule dynamics (examples with other antimitotic agents are summarized in Ref. 39). If our conclusion that the more cytotoxic peptides bind more tightly to tubulin (i.e. dissociate more slowly) than the less cytotoxic peptides is correct, then a significant difference between the two groups should appear in the concentration required to alter tubulin flux or dynamics.

The data presented here show a good correlation for the dolastatin 10 peptides between interference with ligand interactions and cytotoxicity for L1210 cells. If the above model is correct, this indicates that only peptides that bind tightly to tubulin will be cytotoxic and suggests that analysis of ligand binding effects are essential in evaluating this class of agent. At present such an interpretation must be viewed with caution, particularly in terms of predictive value. On the one hand, phomopsin A potently inhibits vincristine binding and nucleotide exchange [5] despite limited cytotoxicity (IC_{50} value of 7 μM for L1210 cells, Ref. 3). On the other, the depsipeptide dolastatin 15 (Fig. 7), somewhat structurally related to dolastatin 10, is a potent antimitotic agent, with an IC_{50} value for L1210 cells of 3 nM [4], comparable to that of isomer 19, and causes the disappearance of intracellular microtubules. Not only is it a weak inhibitor of tubulin polymerization (IC_{50} value of 23 μM), but we could detect no inhibition by dolastatin 15 of vinblastine binding or GTP exchange. If dolastatin 15 binds weakly to the dolastatin 10 site (an assumption based only on structural analogy), then interference of a peptide with other ligand interactions is not totally reliable as a predictor of cytotoxicity.

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* We recently described a class of compounds, derivatives of 2-styrylquinazolin-4(3*H*)-one, that strongly inhibited tubulin polymerization [34]. Even though they bound in the colchicine site of tubulin, their dissociation reactions were so rapid that inhibition of the binding of radiolabeled colchicine by this group of compounds was difficult to demonstrate [35].

† In the case of a series of colchicine-site ligands, relative inhibitory activities did change when reaction conditions were altered [36].

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