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STRUCTURE–ACTIVITY STUDIES WITH CHIRAL ISOMERS AND WITH SEGMENTS OF THE ANTIMITOTIC MARINE PEPTIDE DOLASTATIN 10

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Abstract—Eighteen configurational isomers of the antimitotic peptide dolastatin 10 (Bai *et al.*, *Biochem Pharmacol* **39**: 1941–1949, 1990) derived from *Dolabella auricularia*, together with segments obtained as precursors in its synthesis (Pettit *et al.*, *J Am Chem Soc* **111**: 5463–5465, 1989), were examined as inhibitors of tubulin polymerization and as inhibitors of growth of L1210 murine leukemia cells in culture. Dolastatin 10 consists of four amino acids (in order from the amino terminus: dolavaline, valine, dolaisoleuine, and dolaproine), three unique to *D. auricularia*, linked to an unusual primary amine (dolaphenine, probably derived from phenylalanine) at what would otherwise be its carboxyl terminus. Dolastatin 10 has nine asymmetric carbon atoms, and available isomers included alternate configurations at five positions (positions 9 and 10 in the dolaproine moiety and positions 18, 19 and 19a in the dolaisoleuine moiety). For tubulin polymerization, only alterations at positions 18 and 19 resulted in loss of inhibitory activity of the isomer. In addition, a tripeptide containing dolavaline, valine and dolaisoleuine with all asymmetric carbons identical configurationally to those in dolastatin 10 was found to be about 30% as effective as dolastatin 10 in inhibiting tubulin polymerization. Cytotoxic effects were much more sensitive to alterations in the dolastatin 10 structure. The only modification which did not lead to reduced cytotoxicity was reversal of configuration at position 19a in the dolaisoleuine moiety. Both this isomer and dolastatin 10 had IC_{50} values of less than 1 nM. Several other isomers had IC_{50} values with the L1210 cells in the range of 30–90 nM, but these did not correlate well with their inhibitory effects on tubulin polymerization. The tripeptide effective as an inhibitor of tubulin polymerization had no activity against the L1210 cells.

The sea hare *Dolabella auricularia* produces a number of cytotoxic peptides and depsipeptides containing unique amino acid residues [1–6]. The most potent of these has been named dolastatin 10 [1], and its absolute configuration has now been established by total synthesis [7] (structure presented in Fig. 1). Moreover, we have demonstrated that its mechanism of action is as a potent mitotic inhibitor [8]. Dolastatin 10 causes cells in culture to accumulate in metaphase arrest. Like virtually all drugs with this property [9], dolastatin 10 interacts with the protein tubulin and inhibits its polymerization into microtubules. The oligopeptide also strongly interferes with the binding of radiolabeled vinca alkaloids to tubulin [8], but it probably binds at a different site on the protein since kinetic studies have demonstrated a noncompetitive mode of inhibition [10].

The total synthesis of dolastatin 10 [7] resulted in many segments of the molecule as intermediates, and a substantial number of dolastatin 10 isomers at five of the nine chiral centers (see Fig. 1) were also prepared. Since such compounds should provide valuable information about the binding site of dolastatin 10 on the tubulin molecule, a structure–activity evaluation of their effects on tubulin polymerization was performed. The results, presented here, provided specific insights into which features of the complex dolastatin 10 structure are essential for its interaction with tubulin.

We also felt it would be important to determine whether there was any correlation between effects of the peptide analogs on tubulin polymerization and cell growth, particularly since another peptide antimitotic compound (phomopsin A) potently inhibits polymerization but has limited cytotoxicity [8]. Such an evaluation of the currently available analogs should indicate directions for further synthetic activity for the design of optimally active compounds. The structure–activity comparison therefore included an initial evaluation of the effects of the peptide isomers on the growth of L1210 murine leukemia cells in culture.

MATERIALS AND METHODS

Materials. Electrophoretically homogeneous bovine brain tubulin was prepared as described previously [11]. Synthetic dolastatin 10 was prepared as described previously, as were intermediates in its synthesis [7]. Dolastatin 10 isomers were prepared by analogous chemical routes [12]. Only limited amounts of most isomers (less than 500 μg) were available, and 1 mM stock solutions were prepared by dissolving the peptides in dimethyl sulfoxide.

Methods. Evaluation of drug effects on tubulin

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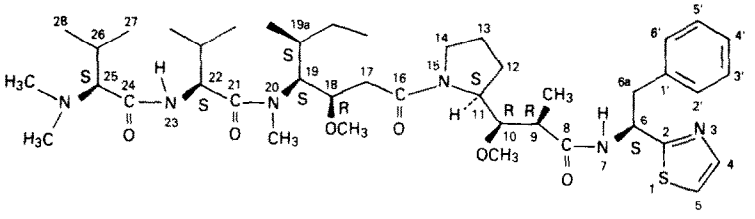
DOLASTATIN 10

Dolavaline Valine Dolaisoleuine Dolaproine Dolaphenine



TRIPEPTIDE A

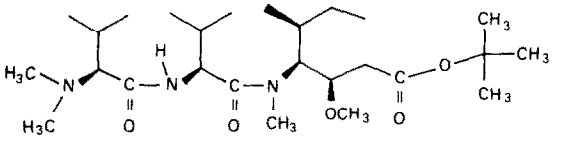


Fig. 1. Molecular formulas of dolastatin 10 and the analogous carboxyl terminal protected tripeptide. The individual residues and configurations of asymmetric carbon atoms are indicated in the figure.

polymerization in 1.0 M monosodium glutamate (pH 6.6 with HCl) and on the growth of L1210 murine leukemia cells was performed as before [8]. In the polymerization assay, reaction mixtures containing 1.0 mg/mL tubulin, 1.0 M glutamate, 1.0 mM $MgCl_2$, 4% (v/v) dimethyl sulfoxide, and various inhibitor concentrations were preincubated for 15 min at 37°. The reaction mixtures were placed on ice, GTP was added (final concentration, 0.4 mM), and the reaction mixtures were transferred to cuvettes held at 0° in a recording spectrophotometer. Reaction temperature was raised to 37° (over about 75 sec) with an electronic temperature controller, and the polymerization reaction was followed turbidimetrically for 20 min. Drug effects were evaluated by comparing the extent of the reaction to that in duplicate control reaction mixtures, with graphical determination of IC_{50} values. At least three independent determinations of each IC_{50} value were made, except that most inactive compounds ($IC_{50} > 40\ \mu M$) were examined only twice. The limited amount of material available restricted the analysis to concentrations up to 40 μM.

L1210 murine leukemia cells were grown in suspension culture in RPMI 1630 medium without glutamate and variable concentrations of drugs (dimethyl sulfoxide at 0.5%, v/v). Initital cell concentration was $1–2 \times 10^5$ cells/mL. After 24 hr at 37°, cell growth was evaluated and compared to control cultures without drug.

RESULTS

Table 1 presents a compilation of the currently available isomers of dolastatin 10, summarizing their configurations at asymmetric carbon atoms (since no isomers differ from the natural peptide at positions 6, 11, 22, or 25, these positions are not included in the table), their inhibitory effects on tubulin polymerization in terms of extent of reaction, and their inhibitory effects at 24 hr on the growth of L1210 murine leukemia cells in culture. The results of the two bioassays are not completely concordant, although no isomer which failed to inhibit tubulin polymerization was an effective cytotoxic agent with the L1210 cells. Table 1 also summarizes the geminal asymmetry present at the two pairs of neighboring chiral centers (10/11 and 18/19) with bonds to heteroatoms. In dolastatin 10 the groups with the heteroatoms are in the *erythro* configuration in both pairs.

Inhibition of tubulin polymerization. The pattern of activity in the polymerization assay was relatively clearcut. Reversal of configuration at positions 9, 10, and/or 19a had little effect on the potent inhibition of polymerization obtained with dolastatin 10: isomers 1, 2, 3, and 4 were as active as dolastatin 10 itself, and isomer 5 had only slightly reduced activity. Reversal of configuration at position 18 alone (isomer 6) or at both positions 18 and 19 (isomer 7) had more substantial impact on the ability of the peptide to inhibit tubulin polymerization, for the former isomer was about one-eighth and the latter one-fifteenth as inhibitory as dolastatin 10. If configuration was reversed at position 18 and/or 19 and one or more additional centers, inert peptides generally resulted. The sole exception to this generalization was the weakly inhibitory isomer 8, which suggests that position 19a is of less importance than either position 9 (cf. isomer 9) or position 10 (cf.

Table 1. Configurational isomers of dolastatin 10 and carboxy-terminal protected tripeptides: inhibition of tubulin polymerization and growth of L1210 leukemia cells*

Peptide	Position 9	10	18	19	19a	Geminal asymmetry‡ Position pair 10/11	18/19	Inhibition of tubulin polymerization IC_{50} (μM)	Inhibition of cell growth§ IC_{50} (M)
		configuration†							
Dolastatin 10	*R*	*R*	*R*	*S*	*S*	E	E	1.2 ± 0.08	9×10^{-10} (1.0)
Isomer 1		*S*				T	E	1.3 ± 0.2	9×10^{-8} (0.01)
Isomer 2					*R*	E	E	1.4 ± 0.06	8×10^{-10} (1.1)
Isomer 3	*S*				*R*	E	E	1.2 ± 0.1	6×10^{-7} (0.002)
Isomer 4	*S*	*S*				T	E	1.4 ± 0.3	3×10^{-7} (0.003)
Isomer 5	*S*					E	E	2.6 ± 0.4	3×10^{-8} (0.03)
Isomer 6			*S*			E	T	10 ± 0.8	$>10^{-6}$ (<0.001)
Isomer 7			*S*	*R*		E	E	18 ± 1	4×10^{-8} (0.02)
Isomer 8			*S*	*R*	*R*	E	E	28 ± 2	4×10^{-8} (0.02)
Isomer 9	*S*		*S*			E	T	>40	$>10^{-6}$ (<0.001)
Isomer 10	*S*			*R*		E	T	>40	$>10^{-6}$ (<0.001)
Isomer 11	*S*		*S*		*R*	E	T	>40	$>10^{-6}$ (<0.001)
Isomer 12	*S*	*S*	*S*			T	T	>40	$>10^{-6}$ (<0.001)
Isomer 13	*S*		*S*	*R*		E	E	>40	6×10^{-7} (0.002)
Isomer 14	*S*			*R*	*R*	E	T	>40	$>10^{-6}$ (<0.001)
Isomer 15	*S*		*S*	*R*	*R*	E	E	>40	6×10^{-7} (0.002)
Isomer 16	*S*	*S*		*R*	*R*	T	T	>40	$>10^{-6}$ (<0.001)
Isomer 17		*S*	*S*	*R*	*R*	T	E	>40	$>10^{-6}$ (<0.001)
Isomer 18	*S*	*S*	*S*	*R*	*R*	T	E	>40	$>10^{-6}$ (<0.001)
Tripeptide A	NA	NA				NA	E	4.2 ± 0.1	$>10^{-6}$ (<0.001)
Tripeptide B	NA	NA		*R*		NA	T	>40	$>10^{-6}$ (<0.001)
Tripeptide C	NA	NA	*S*	*R*		NA	E	>40	$>10^{-6}$ (<0.001)

* The IC_{50} values for the inhibition of tubulin polymerization were determined a minimum of three times, except for inactive compounds which were confirmed in a second assay, with the data plotted graphically. Average values with standard deviation (SD) are presented in the table. Reaction mixtures contained tubulin at 1.0 mg/mL, various concentrations of drugs, 4% (v/v) dimethyl sulfoxide, 1.0 M monosodium glutamate (pH 6.6 with HCl), and 1 mM $MgCl_2$. Following a 15-min preincubation at 37°, reaction mixtures were chilled, 0.4 mM GTP was added, and polymerization was followed turbidimetrically for 20 min at 37° at 350 nm. The IC_{50} values for the growth of L1210 murine leukemia cells were determined in two independent experiments (average values are presented in the table), with cell growth measured after 24 hr.

† 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, position numbers refer to the analogous positions in dolastatin 10 (NA, not applicable).

‡ Geminal asymmetry is described in terms of heteroatoms attached to two adjacent chiral center carbon atoms. Two such pairs of carbon atoms are present in dolastatin 10 (see Fig. 1). In the *erythro* (E in the table) configuration, the heteroatoms are on the same side of the molecule relative to its carbon backbone. In the *threo* (T in the table) configuration, the heteroatoms are on opposite sides of the molecule relative to its carbon backbone. In the tripeptides, the 10/11 pair of chiral centers is not present (NA, not applicable).

§ The numbers in parentheses represent cytotoxicity of the isomers and tripeptides relative to the cytotoxicity of dolastatin 10. The IC_{50} value of dolastatin 10 was divided by the IC_{50} value of each analog.

isomer 10). This is probably because reversal of configuration at position 19a results in only a minor structural modification in the peptide (inversion of ethyl and methyl groups). In summary, reversal of configuration at position 18 and probably 19 (also see below) adversely affected the inhibitory activity of a peptide analog of dolastatin 10, while reversal of configuration at positions 9, 10, and 19a was only significant if combined with a change at 18 or 19. Our findings with these isomers led to the conclusion that dolaisoleuine is the key residue involved in the binding of dolastatin 10 to tubulin.

This conclusion was further strengthened from results obtained with intermediates available from the synthesis of dolastatin 10 and of some of the isomers described above. The synthetic route [7] involved first coupling *N*-carbobenzoxy-L-valine to dolaisoleuine *tert*-butyl ester. The resulting protected dipeptide was then reacted with dolavaline pentafluorophenol ester to yield the protected tripeptide (tripeptide A) whose structure is presented in Fig. 1. In turn, the tripeptide was coupled to *N*-*tert*-butoxycarbonyl-dolaproyldolaphenine to yield dolastatin 10. Among the intermediates examined for effects on tubulin polymerization were dolavaline, completely protected dolaisoleuine, amino-blocked dolaproyldolaphenine, protected tripeptide A shown in Fig. 1, and the isomeric tripeptides B and C (structures summarized in Table 1). Only tripeptide A inhibited tubulin polymerization, and its activity was quite high, about 30% of that shown by dolastatin 10 (IC_{50} of 4.2 μM as compared to 1.2 μM for dolastatin 10). No inhibition of tubulin polymerization occurred

with either tripeptide B (configuration reversed only at the position analogous to position 19 in dolastatin 10) or tripeptide C (configurations reversed at positions analogous to both positions 18 and 19 in dolastatin 10) or any other fragment of dolastatin 10 or its isomers yet examined.

Inhibition of cell growth. The conclusions derived from the L1210 cytotoxicity studies are somewhat different, provided one assumes that there would be little difference in the interactions of the dolastatin 10 isomers and the protected tripeptides with murine tubulin as compared to bovine brain tubulin. No isomer which failed to inhibit tubulin polymerization had substantial inhibitory effects on the growth of the L1210 cells. Only isomer 2 (configuration reversed only at position 19a) was as cytotoxic as dolastatin 10. Dolastatin 10 and isomer 2 had IC_{50} values of 0.9 and 0.8 nM respectively. Except for isomer 2, there was a large loss of cytotoxic activity with any chiral center modification of the dolastatin 10 molecular structure. The next most active analog, isomer 5, had an IC_{50} value of 30 nM (i.e. it was less than one-thirtieth as inhibitory as dolastatin 10). The other three isomers highly active as inhibitors of tubulin polymerization (1, 3, and 4) were even less cytotoxic, with IC_{50} values ranging from 90 to 600 nM. Moreover, two feebly active inhibitors of polymerization (isomers 7 and 8) were more cytotoxic (IC_{50} values of 40 nM) than these three potent polymerization inhibitors. Especially surprising from the point of view of inhibitory effects on tubulin polymerization was the enhanced cytotoxicity obtained with isomers 7 and 8 as compared to isomer 6: while an increasing number of inversions at chiral centers resulted in progressive loss of antitubulin activity, for cytotoxicity reversal of configuration at position 19 appeared to counteract the deleterious effect of reversal of configuration at position 18.

If the five chiral centers for which configurational isomers are available are considered individually, almost all reversals of configuration led to significant loss of cytotoxic activity. Position 19a was almost the exception to this generalization, possibly because the methyl–ethyl inversion is relatively minor from a structural point of view. Of four isomer pairs in which the two members differ from each other only in configuration at position 19a, both members of three pairs had identical cytotoxic activity (dolastatin 10 and isomer 2; isomers 7 and 8; isomers 13 and 15); but in the fourth pair, isomer 5, with the natural *S* configuration, was twenty times as cytotoxic as isomer 3 with position 19a in the *R* configuration.

At positions 9 and 10, in all cases of isomer pairs differing in configuration at a single position, it was the isomer with the natural configuration which was more cytotoxic. For position 9, compare dolastatin 10 with isomer 5, isomer 2 with isomer 3, 1 with 4, 7 with 13, and 8 with 15. For position 10, compare dolastatin 10 with isomer 1, isomer 5 with isomer 4, 8 with 17, and 15 with 18.

This generalization is also true for many isomer pairs at positions 18 and 19. For position 18, compare dolastatin 10 with isomer 6, isomer 3 with isomer 11, 5 with 9, and 4 with 12. For position 19, compare isomer 3 with isomer 14 and 5 with 10.

But at both positions 18 and 19 there were isomer pairs in which the isomer with the unnatural configuration was the more active. For position 18, isomer 13 was more cytotoxic than isomer 10, and 15 than 14. For position 19, isomer 7 was more active than isomer 6, 13 than 9, and 15 than 11 (see Discussion).

The results obtained with the protected tripeptides also emphasize the importance of the carboxyl portion of the dolastatin 10 molecule for cytotoxicity, for none of the tripeptides inhibited the growth of L1210 murine leukemia cells in culture.

Because of the differences between the cell culture evaluation and the tubulin polymerization study, all dolastatin 10 isomers with IC_{50} values less than 100 nM with the L1210 cells were also examined specifically to determine whether they were mitotic inhibitors at cytotoxic concentrations. Metaphase figures were increased substantially with all isomers examined (1, 2, 5, 7, and 8) (data not presented).

DISCUSSION

Most of the dolastatin 10 isomers described here were prepared in the course of developing a total synthesis of the natural peptide and establishing the absolute configurations of its nine asymmetric centers [7]. With the demonstration that the natural peptide derives its potent cytotoxicity as a mitotic inhibitor that prevents tubulin polymerization [8], it was of interest to determine whether any of the isomers shared its biological properties. In addition, intermediates in the synthesis of dolastatin 10 were also evaluated for effects on tubulin polymerization, and, to a more limited extent, as inhibitors of cell growth.

The tubulin polymerization studies demonstrated that the middle (third) amino acid residue of dolastatin 10, dolaisoleuine [2], was the most critical for interaction of the peptide with tubulin *in vitro*. Of the three asymmetric carbons in this residue, the configurations of those at positions 18 and 19 (*R* and *S* respectively) were particularly important in the naturally occurring isomer, while configuration of the side chain asymmetric carbon (position 19a) did not affect inhibition of polymerization by the peptide. The relative unimportance of the fourth and fifth residues was emphasized, first, by the potent inhibitory effects of isomers (1, 4, and 5) with reversal of configuration at position 9 or at both positions 9 and 10 and, second, by the activity of tripeptide A, which lacks the fourth and fifth residues. (In addition, the segment containing only these last two residues was inactive.) The studies presented here strongly suggest that configurations corresponding to positions 18 and 19 in the dolaisoleuine residue should be left unaltered in further synthetic studies designed to yield potent dolastatin 10 analogs.

The effects of chiral center modifications on cytotoxicity of isomers towards L1210 cells were complex. There was only limited correlation between inhibitory effects on tubulin polymerization and cell growth, for, with the exception of reversal of configuration only at position 19a (isomer 2), any configurational modification resulted in a large loss in cytotoxic activity. The next most active compound, isomer 5, was only 3% as effective as dolastatin 10

as an inhibitor of cell growth. Moreover, two weak inhibitors of tubulin polymerization (isomers 7 and 8) were more cytotoxic than several stronger tubulin inhibitors (isomers 1, 3, 4, and 6).

These findings may be partially explained by considering vicinal asymmetric centers in dolastatin 10. At two locations in the molecule (positions 10/11 and positions 18/19) heteroatomic substituents are attached to neighboring chiral centers, giving rise to geminal asymmetry at these locations. In dolastatin 10 both these pairs of chiral centers are in an *erythro* configuration. Reversal of configuration at position 10 converts the 10/11 pair to a *threo* configuration. This always leads to a loss of cytotoxic activity in a dolastatin 10 isomer. Since no isomers have been prepared with reversal of configuration at position 11, we have no information on the alternate *erythro* and *threo* configurations.

For the 18/19 pair, isomers with both *erythro* and both *threo* configurations have been prepared. In no case was a *threo* isomer at this location cytotoxic, but several isomers with the unnatural *erythro* configuration (with inversions at both positions 18 and 19) were more cytotoxic than corresponding *threo* isomers with reversal of configuration only at position 18 or position 19. This is the case in all the examples cited above (see Results) where reversal of configuration resulted in enhanced cytotoxicity (i.e. isomer 13 as compared with isomer 10, 15 vs 14, 7 vs 6, 13 vs 9, and 15 vs 11). In addition, where reversal of configuration at position 18 or 19 does lead to reduced cytotoxicity, one of the *threo* configurations is generated (i.e. isomer 3 as compared with isomer 11, 5 vs 9, 4 vs 12, 3 vs 14, 5 vs 10, and dolastatin 10 vs isomer 6).

Such patterns of activity suggest that an important interaction is occurring between the peptides and a receptor, involving differential transport into cells, with the *erythro* configuration strongly favored over the *threo*. This appears to be as important as direct peptide–tubulin interactions in explaining the differential cytotoxicity of the isomers.

We cannot, however, exclude intracellular metabolism of the peptides as playing a role in their differential cytotoxicities. This possibility, too, must be considered in view of our preliminary results with another peptide derived from *D. auricularia*. Dolastatin 15 [5] is almost as cytotoxic as dolastatin 10, and the two compounds are structurally related. Dolastatin 15, too, causes the accumulation of cells arrested at metaphase and the disappearance of intracellular microtubules, but it has little effect on microtubule assembly *in vitro*. The mechanism of action of dolastatin 15 is being intensively investigated, including the possibility of its intracellular modification. If such metabolism can be demonstrated, it would raise the possibility that intracellular modification of dolastatin 10 and the isomers examined here could also occur.

We are also conducting comparative studies on cytotoxicity with the dolastatin 10 isomers in cell lines other than the L1210 murine leukemia line. So far results with L1210 cells seem characteristic and strongly suggest that it may not be simple to prepare analogs of dolastatin 10 which preserve or increase the cytotoxicity of the natural peptide. Nonetheless, finding simpler alternate amino acid residues for dolaproine and/or dolaphenine would be most worthwhile, for it would considerably simplify a relatively complex synthesis [7].

On the other hand, it may prove to be beneficial that alterations in the dolastatin 10 structure reduce, but do not eliminate, its cytotoxicity. In our previous study we found dolastatin 10 to be exceptionally cytotoxic as compared to most other antitubulin drugs binding in the vinca domain. Its IC_{50} value with L1210 cells was comparable to that of maytansine and much lower than that of vinblastine. If clinical studies indicate that dolastatin 10 needs to be modified for effective use in human subjects, isomers of intermediate cytoxicity (e.g. isomers 1, 5, 7, and 8 with IC_{50} values of 30–90 nM) should be investigated further. Analogously, even if reduced cytotoxicity occurs, it may be worthwhile preparing analogs with modified carboxyl termini to simplify the synthetic route to compounds with potential activity.

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