The Spongistatins, Potently Cytotoxic Inhibitors of Tubulin Polymerization, Bind in a Distinct Region of the Vinca Domain

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ABSTRACT: The highly cytotoxic, sponge-derived, antimitotic macrolide polyether spongistatin 1 has been previously shown to inhibit microtubule assembly, the binding of vinblastine and GTP to tubulin, and displacement of GDP bound in the exchangeable site of tubulin. We have now examined in detail inhibition by spongistatin 1 of both [^{3}H]vinblastine and [^{3}H]dolastatin 10 binding to tubulin. We found spongistatin 1 to be a noncompetitive inhibitor of the binding of both radiolabeled drugs to tubulin, in contrast to competitive patterns obtained with vincristine versus [^{3}H]vinblastine and with a chiral isomer of dolastatin 10 versus [^{3}H]dolastatin 10. Since dolastatin 10 is itself a noncompetitive inhibitor of vinca alkaloid binding to tubulin, this implies at least three distinct binding sites for the structurally complex and diverse natural products that interfere with each others binding to tubulin and with nucleotide exchange. Spongistatin 1, in contrast to both vinca alkaloids and peptide antimitotic agents like dolastatin 10, does not induce formation of a GTP-independent, morphologically distinctive polymer ("aggregate"). We also examined eight compounds closely related structurally to spongistatin 1 (spongistatins 2−9). The most distinctive in their properties were spongistatins 6 and 8. These two compounds, despite activity comparable to spongistatin 1 as inhibitors of tubulin polymerization and [^{3}H]vinblastine binding, had much reduced activity as inhibitors of nucleotide exchange and [^{3}H]dolastatin 10 binding. Spongistatins 1 and 6 were compared for effects on dolastatin 10-induced aggregate formation in conjunction with effects on [^{3}H]-dolastatin 10 binding. Spongistatin 6 was about 4-fold less active than spongistatin 1 as an inhibitor of aggregation and over 20-fold less active as an inhibitor of dolastatin 10 binding.

The major component of microtubules, the protein tubulin, interacts with a large number of structurally diverse drugs, many obtained from natural sources, which cause cells to arrest in mitosis. The key characteristics of these cells are the presence of condensed chromosomes and the absence of a nuclear membrane and spindle. We have recently described interactions with tubulin of two structurally complex macrocyclic lactone polyethers, halichondrin B (Bai et al., 1991) and spongistatin 1 (Bai et al., 1993a), obtained from marine sponges, and a structurally unusual peptide, dolastatin 10, obtained from a marine mollusk (Bai et al., 1990ab). These compounds are highly cytotoxic (IC_{50} values with L1210 murine leukemia cells range from 0.02 to 0.6 nM), cause the disappearance of intracellular microtubules, and inhibit in vitro microtubule assembly, nucleotide exchange, and the binding of vinca alkaloids to tubulin. With halichondrin B and dolastatin 10, inhibition of vinca binding was noncompetitive, as opposed to competitive inhibition observed with maytansine, rhizoxin, and an alternate vinca alkaloid.[1]

We now report that spongistatin 1 (structure in Figure 1) also is a noncompetitive inhibitor of the binding of [^{3}H]-vinblastine to tubulin. The recent preparation of [^{3}H]-dolastatin 10 (Bai et al., 1995) has provided us with an additional probe to explore interactions of this group of drugs with tubulin. We found that spongistatin 1 is an exceptionally strong inhibitor of the binding of [^{3}H]dolastatin 10 to tubulin. This inhibition also yielded a noncompetitive pattern on detailed analysis, in contrast to a competitive pattern obtained with a chiral isomer of dolastatin 10.

In addition to these detailed studies with spongistatin 1, we also will describe here initial studies with eight related compounds obtained from the marine sponges *Spongia* sp. (spongistatins 2 and 3; Figure 1; Pettit et al., 1993a) and *Spirastrella spinispirulifera* (spongistatins 4−9; Figure 1; Pettit et al., 1993c,d, 1994). With the exception of spongistatin 3, which had reduced activity in all tubulin-dependent assays, these agents were all similar to spongistatin 1 in their inhibitory effects on polymerization and vinblastine binding. More variable effects were observed on inhibition of nucleotide exchange and inhibition of [^{3}H]dolastatin 10 binding. Spongistatins 6 and 8 differed from the remaining compounds in being significantly less potent than spongistatin 1 as inhibitors of nucleotide exchange. Relatively weak inhibition

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[1] That is, as controls we showed that vincristine competitively inhibited the binding of [^{3}H]vinblastine to tubulin, and vinblastine competitively inhibited the binding of [^{3}H]vincristine.

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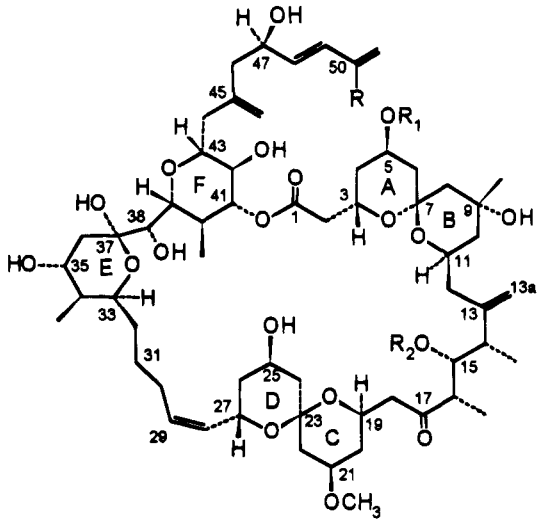
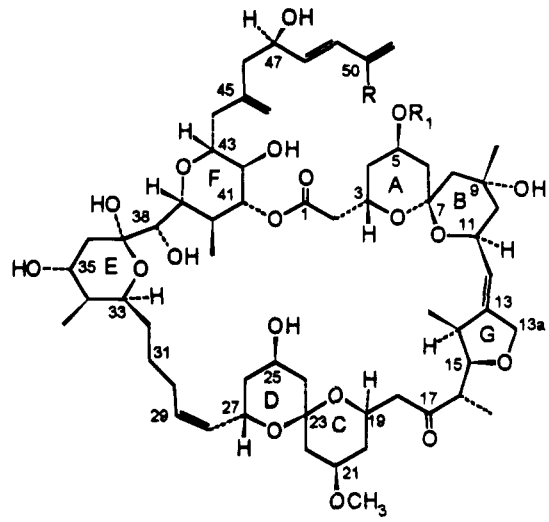


Spongistatin 1, R = Cl, $R_1 = R_2 = COCH_3$
Spongistatin 2, R = H, $R_1 = R_2 = COCH_3$
Spongistatin 3, R = Cl, R_1 = H, $R_2 = COCH_3$
Spongistatin 4, R = Cl, $R_1 = COCH_3$, R_2 = H
Spongistatin 6, R = H, $R_1 = COCH_3$, R_2 = H



Spongistatin 5, R = Cl, R_1 = H
Spongistatin 7, R = H, R_1 = H
Spongistatin 8, R = H, $R_1 = COCH_3$
Spongistatin 9, R = Cl, $R_1 = COCH_3$

FIGURE 1: Molecular structures of spongistatins 1–9, the macrocyclic polyether lactones isolated from a *Spongia* species (spongistatins 1–3) and from *Spirastrella spinispirulifera* (spongistatins 4–9).

of dolastatin 10 binding was observed with spongistatins 2 and 7 as well as 6 and 8.

MATERIALS AND METHODS

Materials. The spongistatins were isolated from the sponges of origin (Pettit et al., 1993a–d, 1994), and electrophoretically homogeneous bovine brain tubulin (Hamel & Lin, 1984), nonradiolabeled dolastatin 10 (Pettit et al., 1989), isomer 2 [nomenclature as in Bai et al. (1990c)] of dolastatin 10 (Pettit et al., 1990), [^{3}H]dolastatin 10 (Bai et al., 1995), and halichondrin B (Pettit et al., 1991) were prepared as described previously. Rhizoxin and maytansine were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, nonradiolabeled vinblastine from Sigma, [^{3}H]vinblastine from DuPont, and [8-^{14}C]-GTP from Moravek Biochemicals.

Methods. The binding of [^{3}H]vinblastine, [^{3}H]dolastatin 10, and [8-^{14}C]GTP to tubulin was measured by centrifugal gel filtration chromatography as described elsewhere (Bai et al., 1991, 1995). Reaction mixtures contained 0.1 M Mes (pH 6.9 with NaOH in 1 M stock solution), 0.5 mM $MgCl_2$, and tubulin, radiolabeled ligand, inhibitory drugs, and dimethyl sulfoxide (the drug solvent) as indicated in the individual experiments. When drug mixtures were examined, tubulin was the last addition to the reaction mixture, except as indicated. When [8-^{14}C]GTP binding was examined, [8-^{14}C]GTP was the last addition to the reaction mixture.

Inhibition of L1210 murine leukemia cell growth was performed in 5 mL cultures growing at 37 °C under 5% CO_2 in RPMI 1640 medium supplemented with 17% fetal bovine serum and 2 mM L-glutamine. Cultures were inoculated with 5×10^5 cells and growth evaluated after 16 h. Inhibition of glutamate-induced tubulin polymerization was evaluated as described previously (Bai et al., 1990b), following a 15 min drug–tubulin preincubation in the absence of GTP. Electron microscopic studies were performed on samples negatively stained with 0.5% uranyl acetate and examined in a Zeiss Model 10CA microscope.

HPLC analysis of spongistatin effects on dolastatin 10-induced tubulin aggregation was performed on a TSK G3000SW column (7.5 × 300 mm) with a Waters system equipped with a Ramona 5-LS flow detector and Digital 380 computer. Samples were injected after the indicated incubation at room temperature. The column was equilibrated and developed at room temperature with 0.1 M Mes (pH 6.9)–0.5 mM $MgCl_2$. The flow rate was 1.0 mL/min.

RESULTS

Noncompetitive Inhibition of [^{3}H]Vinblastine Binding by Spongistatin 1. In initial studies, we found that spongistatin 1 was a highly potent inhibitor of the binding of [^{3}H]-vinblastine to tubulin (Bai et al., 1993a), with effects quantitatively similar to those of maytansine and dolastatin 10. As the latter two compounds differ in that the former is a competitive inhibitor of vinca alkaloid binding to tubulin and the latter a noncompetitive inhibitor (Bai et al., 1990a), we examined spongistatin 1 versus [^{3}H]vinblastine in further detail at a variety of drug concentrations.

An example of such a study is presented in Figure 2A, with the data presented in the Hanes format. For comparison, Figure 2B presents an analogous study in which vincristine was used to inhibit the binding of [^{3}H]vinblastine to tubulin. In the Hanes format, a competitive inhibitor yields parallel lines, and a noncompetitive inhibitor, lines that intercept on the abscissa to the left of the origin (Dixon et al., 1979). By these criteria, vincristine, as expected, yielded data consistent with competitive inhibition, while spongistatin 1, like the peptide dolastatin 10 (Bai et al., 1990a) and the lactone polyether halichondrin B (Bai et al., 1991), yielded data consistent with noncompetitive inhibition. Apparent K_i values (averages of two independent experiments) of 1.3 and 1.8 μM were obtained for spongistatin 1 and vincristine, respectively.

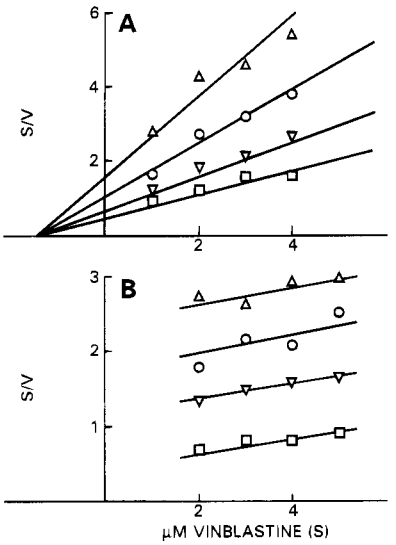


FIGURE 2: Comparison of the inhibitory effects of spongistatin 1 (A) and vincristine (B) on the binding of [^{3}H]vinblastine to tubulin, as analyzed by the Hanes method. Reaction mixtures (0.35 mL) contained 0.5 mg/mL (5.0 μM) tubulin, 4% (v/v) dimethyl sulfoxide, and [^{3}H]vinblastine as indicated. Incubation was for 20 min at room temperature. Aliquots (0.15 mL) were applied to duplicate syringe-columns of Sephadex G-50 (superfine) and processed by centrifugal gel filtration. Ordinate units for *S/V*: μM vinblastine × μg of tubulin/pmol of vinblastine. Abscissa units for S: μM vinblastine. (A) Spongistatin 1 concentrations: (□) none; (▽) 2.0 μM; (○) 3.0 μM; (△) 4.0 μM. (B) Vincristine concentrations: (□) none; (▽) 3.0 μM; (○) 9.0 μM; (△) 12 μM.

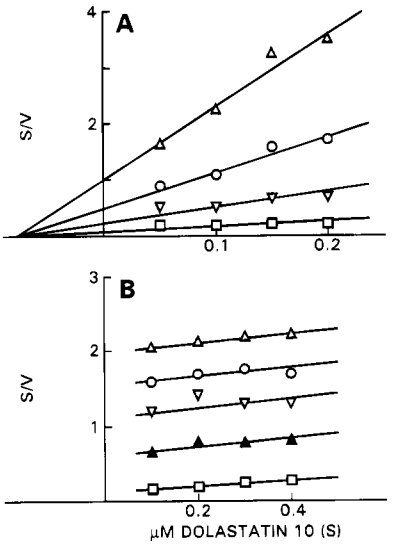


FIGURE 3: Comparison of the inhibitory effects of spongistatin 1 (A) and isomer 2 (a chiral isomer of dolastatin 10) (B) on the binding of [^{3}H]dolastatin 10 to tubulin, as analyzed by the Hanes method. Reaction mixtures (0.35 mL) contained 0.25 mg/mL (2.5 μM) tubulin, 4% (v/v) dimethyl sulfoxide, and [^{3}H]dolastatin 10 as indicated. Incubation was for 20 min at room temperature. Aliquots (0.15 mL) were applied to duplicate syringe-columns of Sephadex G-50 (superfine) and processed by centrifugal gel filtration. Ordinate units for *S/V*: μM dolastatin 10 × μg of tubulin/pmol of dolastatin 10. Abscissa units for *S*: μM dolastatin 10. (A) Spongistatin 1 concentrations: (□) none; (▽) 2.5 μM; (○) 2.75 μM; (△) 3.0 μM. B. Isomer 2 concentrations: (□) none; (▲) 6.0 μM; (▽) 8.0 μM; (○) 10 μM; (△) 12 μM.

Table 1: Inhibition of Binding of [^{3}H]Dolastatin 10 to Tubulin by Vinca Domain Drugs[a]

drug added	% inhibition
spongistatin 1	99
phomopsin A	99
isomer 2 of dolastatin 10	86
halichondrin B	60
maytansine	56
vincristine	54
rhizoxin	35
vinblastine	23

[a] Reaction mixtures (0.5 mL) contained 0.25 mg/mL (2.5 μM) tubulin, 2.5 μM [^{3}H]dolastatin 10, 4% (v/v) dimethyl sulfoxide, and the indicated vinca domain drug at 10 μM. Incubation was for 15 min at room temperature. Aliquots (0.2 mL) were applied to duplicate syringe-columns of Sephadex G-50 (superfine) and processed by centrifugal gel filtration. The stoichiometry of binding in samples taken from the control reaction mixture (no inhibitor) was 0.53 mol of dolastatin 10 bound/mol of tubulin.

Noncompetitive Inhibition of [^{3}H]Dolastatin 10 Binding by Spongistatin 1. The preparation of [^{3}H]dolastatin 10 (Bai et al., 1995) provided another probe for examining drug interactions with tubulin. In an initial survey of vinca domain drugs spongistatin 1 proved to be a powerful inhibitor of the binding of the radiolabeled peptide to tubulin (Table 1). The chiral isomer of dolastatin 10 with reversal of configuration at position C-19a [termed "isomer 2"; structure in Bai et al. (1990c)] also strongly inhibited [^{3}H]dolastatin 10 binding. In studies at various drug concentrations, inhibition by spongistatin 1 (Figure 3A) and by isomer 2 (Figure 3B) was examined in detail, with evaluation by the Hanes method. Data obtained with the dolastatin 10 isomer were consistent with competitive inhibition, while spongistatin 1 yielded data consistent with noncompetitive inhibition. Apparent K_i values (averages of two independent experiments) of 0.6 and 0.8 μM were obtained for spongistatin 1 and isomer 2, respectively.

Spongistatins 2–9. Besides spongistatin 1, the *Spongia* species and *S. spinispirulifera* yielded smaller amounts of eight related, highly cytotoxic compounds (Table 2) (Pettit et al., 1993a–d, 1994). With L1210 murine leukemia cells, IC_{50} values ranged from 0.1 nM for spongistatin 4 to 3 nM for spongistatin 8,[2] as compared with 30 pM for spongistatin 1. Except for spongistatin 3, inhibitory effects on glutamate-induced tubulin polymerization were also similar, with IC_{50} values ranging from 4.2 to 6.7 μM. Spongistatin 3 was clearly less active with an IC_{50} value of 13 μM. Spongistatins 2–9 were compared to spongistatin 1 as inhibitors of the binding of vinblastine, GTP, and dolastatin 10 to tubulin.[3] In agreement with the polymerization assay, spongistatins 4, 5, and 9 strongly inhibited binding of the three ligands, while spongistatin 3 was a relatively weak inhibitor. The remaining four compounds strongly inhibited vinblastine binding but otherwise fell into two groups. Spongistatins 6

[2] In the National Cancer Institute screen against as many as 60 human tumor cell lines, the following average IC_{50} values were obtained: spongistatin 1, 0.13 nM (Pettit et al., 1993a,d); spongistatins 2 and 3, 0.85 and 0.83 nM (Pettit et al., 1993a); spongistatins 4 and 5, 0.10 and 0.12 nM (Pettit et al., 1993d); spongistatins 6 and 7, 1.1 and 1.0 nM (Pettit et al., 1993c); and spongistatins 8 and 9, 0.23 and 0.04 nM (Pettit et al., 1994).

[3] In the case of GTP, inhibition of binding requires that drug be added prior to the radiolabeled GTP. With spongistatin 1 (as well as maytansine, dolastatin 10, phomopsin A, rhizoxin, and halichondrin B), using tubulin with [8-^{14}C]GDP bound in the exchangeable site, we have shown that drug does not displace nucleotide from the exchangeable site. Thus, it is actually nucleotide exchange, rather than nucleotide binding per se, that is inhibited by this group of drugs. Analogous studies with spongistatins 2–9 and tubulin-[8-^{14}C]GDP have not yet been performed.

Table 2: Comparison of Properties of Spongistatins 1−9[a]

spongistatin	inhibition of L1210 cell growth (IC_{50}, nM)	inhibition of tubulin polymerization (IC_{50}, μM)	inhibition of vinblastine binding (%)	inhibition of GTP binding (%)	inhibition of dolastatin 10 binding (%)
1	0.03	5.3	75	87	80
2	2.0	4.6	73	74	43
3	1.0	13	32	35	34
4	0.1	5.1	78	75	62
5	0.2	6.7	86	75	74
6	0.8	4.4	67	10	14
7	2.0	5.3	71	61	22
8	3.0	5.5	62	11	13
9	0.3	4.2	85	72	66

[a] L1210 murine leukemia cells were grown for 16 h as described in the text. Dimethyl sulfoxide concentration was 0.5%. IC_{50} values presented are averages obtained in two independent experiments. Drug effects on glutamate-induced tubulin polymerization were determined as described in the text. With spongistatins 1−5, three independent determinations were made, and the values obtained were within 10% of the average values shown; for spongistatins 6−9, a single determination was made with each agent (confirming ranges obtained in preliminary experiments) to conserve drug. Inhibition of binding of [^{3}H]vinblastine, [8-^{14}C]GTP, and [^{3}H]dolastatin 10 to tubulin was determined by centrifugal gel filtration as described in the text. Incubation was for 20 min at room temperature (drugs) or 0 °C (GTP). In the vinblastine binding experiments, reaction mixtures contained 0.5 mg/mL (5.0 μM) tubulin, 5.0 μM [^{3}H]vinblastine, the indicated spongistatin at 10 μM, and 4% dimethyl sulfoxide. Values presented are averages obtained in two independent experiments. Centrifugal gel filtration was performed at room temperature. In the GTP binding experiment, reaction mixtures contained 0.5 mg/mL (5.0 μM) tubulin, 50 μM [8-^{14}C]GTP, the indicated spongistatin at 10 μM, and 4% dimethyl sulfoxide. Values presented are averages obtained in two independent experiments. Centrifugal gel filtration was performed at 4 °C. In the dolastatin 10 binding experiments, reaction mixtures contained 0.25 mg/mL (2.5 μM) tubulin, 2.5 μM [^{3}H]dolastatin 10, the indicated spongistatin at 5.0 μM, and 2% dimethyl sulfoxide. Values presented are averages obtained in two independent experiments. Centrifugal gel filtration was performed at room temperature. Control values: 0.44 mol of [^{3}H]vinblastine bound/mol of tubulin; 0.50 mol of [8-^{14}C]GTP bound/mol of tubulin; 0.45 mol of [^{3}H]dolastatin 10 bound/mol of tubulin.

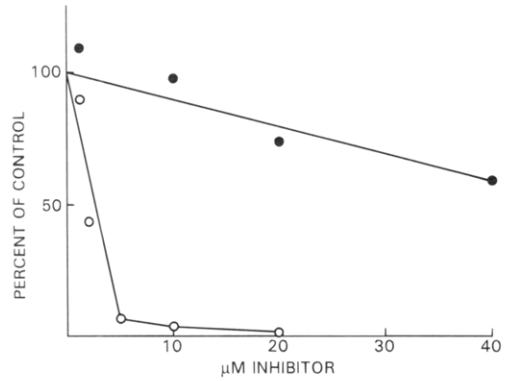


FIGURE 4: Comparison of the inhibitory effects of spongistatins 1 and 6 on the binding of [^{3}H]dolastatin 10 to tubulin. Reaction mixtures (0.35 mL) contained 0.25 mg/mL (2.5 μM) tubulin, 10 μM [^{3}H]dolastatin 10, 4% (v/v) dimethyl sulfoxide, and the indicated concentrations of spongistatin 1 (○) or spongistatin 6 (●). Incubation was for 15 min at room temperature. Aliquots (0.15 mL) were applied to duplicate syringe-columns of Sephadex G-50 (superfine) and processed by centrifugal gel filtration. In the control without inhibitor, 0.62 mol of dolastatin 10 was bound per mole of tubulin.

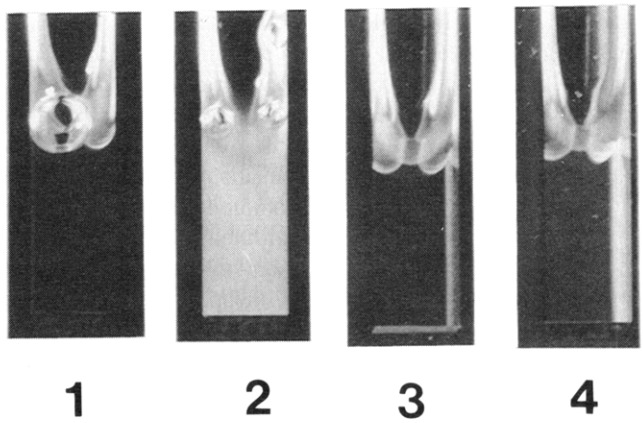


FIGURE 5: Inhibition of dolastatin 10-induced turbidity development by spongistatin 1 and spongistatin 6. The 200 μL reaction mixtures contained 4.0 mg/mL (40 μM) tubulin and 2% (v/v) dimethyl sulfoxide. Cuvette 1 contained no further addition; cuvette 2 also contained 40 μM dolastatin 10; cuvette 3 also contained 40 μM dolastatin 10 and 20 μM spongistatin 1; cuvette 4 also contained 40 μM dolastatin 10 and 20 μM spongistatin 6. The photograph was taken after the samples had been at room temperature (including time under illumination) for about 1 h.

and 8 weakly inhibited both GTP and dolastatin 10 binding as compared with spongistatin 1, while spongistatins 2 and 7 had substantially reduced effects on dolastatin 10 but not GTP binding. Of particular note is that the inhibitory effects of spongistatins 6 and 8 on the binding of GTP and dolastatin 10 were substantially less than those obtained with spongistatin 3, despite the latter's relatively weak activity in the polymerization and vinblastine binding assays.

Spongistatin 6 was selected for further study (see below), and detailed comparison of the inhibitory effects of spongistatins 1 and 6 on [^{3}H]dolastatin 10 binding (Figure 4) showed at least a 20-fold difference between the two agents.

Effects of Spongistatins 1 and 6 on Dolastatin 10-Induced Tubulin Aggregation. Dolastatin 10 causes extensive tubulin "aggregation," a GTP-independent assembly reaction yielding polymer of abnormal morphology (Bai et al., 1995). This can produce marked visual turbidity in reaction mixtures compared to controls without drug. All the spongistatins prevented this turbidity development from occurring, even when there was minimal inhibition of the binding of [^{3}H]-dolastatin 10 to tubulin, as with spongistatins 6 and 8. Since larger amounts of spongistatin 6 had been isolated (Pettit et al., 1993c, 1994), this compound was selected for more detailed study.

Figure 5 demonstrates aggregation of 40 μM tubulin induced by 40 μM dolastatin 10 (second sample; compare to first sample with no drug). This photograph was taken after 1 h at 22 °C, but the tubulin solution had become intensely turbid within 5 min. Equivalent turbidity develops on ice within 60−90 min following addition of dolastatin 10. This reaction did not occur when either spongistatin 1 (third sample) or spongistatin 6 (fourth sample) was present at 20 μM, together with 40 μM dolastatin 10. Relative inhibitory effects of the spongistatins on this aspect of

Table 3: Comparison of the Effects of Spongistatins 1 and 6 on Different Aspects of Dolastatin 10-Induced Tubulin Aggregation[a]

[tubulin] (μM)	[dolastatin 10] (μM)	spongistatin concn (μM) required for max inhibition of aggregation evaluated by: visual turbidity S1	visual turbidity S6	electron microscopy S1	electron microscopy S6	HPLC S1	HPLC S6
40	40	2.5	10	20	>40		
5	10			5	20	10	>40

[a] Reaction mixtures contained the indicated concentrations of tubulin and dolastatin 10 and varying concentrations of spongistatins 1 or 6. For the turbidity and microscopy evaluations, after 1 h at room temperature reaction mixtures were examined visually and compared to controls without drug or with dolastatin 10 only; or aliquots were placed on 200 mesh carbon-coated Formavar-treated copper grids, stained with 0.5% uranyl acetate, and examined electron microscopically for the presence of morphologically distinct aggregates presumed to be induced by the dolastatin 10. "Maximum" inhibition for evaluation of turbidity means the sample had the same appearance as a reaction mixture containing tubulin but no drug; and for microscopic evaluation, that no discrete aggregates were observed on the grid, or that such aggregates were seen in less than 5% of examined fields. In the HPLC evaluations (Figure 6; injections after 15 min at room temperature) at the highest spongistatin 1 concentration examined (10 μM), 2−5% of the added [^{3}H]dolastatin 10 remained associated with a 200 kDa peak. With 40 μM spongistatin 6, 30−40% of the [^{3}H]dolastatin 10 was oligomer-associated, a much higher proportion than with 5 μM spongistatin 1 (Figure 6C).

dolastatin 10-induced aggregation were estimated by visually examining samples containing 40 μM tubulin + 40 μM dolastatin 10 and different amounts of spongistatin 1 or 6 (Table 3). Spongistatin 6 appeared to be about one-fourth as potent as spongistatin 1 in preventing development of visual turbidity. We are presently attempting to develop a more quantitative spectrophotometric assay for this reaction.

Reaction mixtures were examined by electron microscopy in two separate experiments for the persistence of dolastatin 10-induced tubulin aggregate in the presence of spongistatins 1 and 6 (Table 3). We found that rings and spirals (Bai et al., 1995) were still present in nonturbid reaction mixtures, although they became increasingly difficult to find as the spongistatin concentrations increased, disappearing at about a 4-fold lower concentration of spongistatin 1 as compared with spongistatin 6. There was no evidence for morphologically discrete oligomers induced by high concentrations of spongistatin 1 or 6.

We previously showed that dolastatin 10-induced aggregation can be evaluated by HPLC analysis of reaction mixtures (Bai et al., 1995). In these studies, we were unable to demonstrate binding of [^{3}H]dolastatin 10 to 100 kDa tubulin. The smallest discrete radiolabeled species appeared to consist of two molecules each of tubulin and drug. Figure 6 presents experiments examining spongistatin effects on the incorporation of [^{3}H]dolastatin 10 into tubulin oligomers (5 μM tubulin, 10 μM dolastatin 10). Panel A shows the chromatographic appearance of the tubulin only, with only a small portion of protein in the void volume, and panel B the effect of 10 μM dolastatin 10. With drug, all the protein eluted from the column in the void volume (the arrow indicates the position of the 100 kDa peak), with about half the radiolabel bound to protein and half eluting as an unbound peak at 18 min.

Addition of substoichiometric amounts of spongistatin 1 caused progressive disappearance of the void volume peak

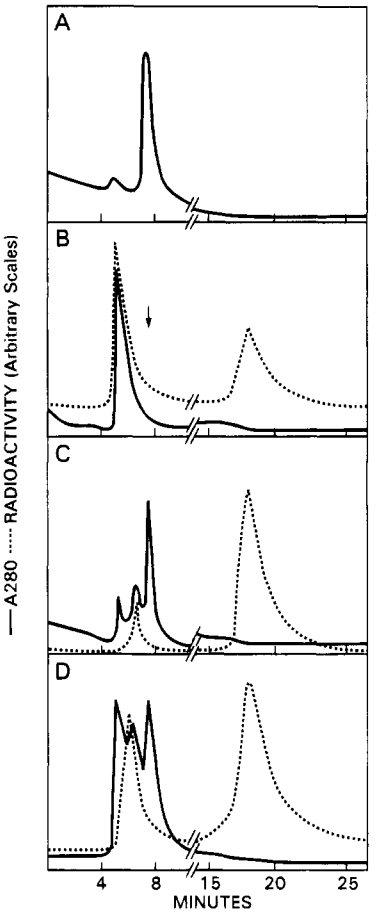


FIGURE 6: HPLC analysis of the effect of spongistatins 1 and 6 on dolastatin 10-induced oligomer formation. Reaction mixtures contained 0.5 mg/mL (5.0 μM) tubulin and 2% (v/v) dimethyl sulfoxide. A 100 μL injection was made after 15 min at room temperature for each run. The sample shown in panel A had no further addition, while those shown in panels B, C, and D also contained 10 μM [^{3}H]dolastatin 10. The reaction mixture of panel C also contained 5.0 μM spongistatin 1; and of panel D, 20 μM spongistatin 6. The arrow in panel B indicates the expected position of the 100 kDa tubulin heterodimer peak. It should be emphasized that the scales determined by the HPLC software differ for each panel. Consequently, relative peak sizes apply within each panel but not between panels.

and release of [^{3}H]dolastatin 10 from protein, until at 5 μM spongistatin 1 (Figure 6C) most of the protein had shifted from the void into the included volume and a large 100 kDa peak reappeared. Residual protein-bound [^{3}H]dolastatin 10 coeluted with a small oligomeric species whose elution time coincided with that of a 200 kDa standard [cf. Bai et al. (1995)]. Further increase in the spongistatin 1 concentration yielded a protein profile nearly indistinguishable from the control without drug, with almost all radiolabel in the free drug peak.

Spongistatin 6 at 5 μM yielded absorbance and radioactivity profiles indistinguishable from the pattern obtained with [^{3}H]dolastatin 10 alone, and with 10 μM spongistatin 6 the radioactivity profile was little changed, but the protein profile became slurred into the included volume of the column. The shift of protein into the included volume was increased when the spongistatin 6 concentration was doubled to 20 μM (Figure 6D), and about 30% of the protein was in the 100 kDa peak. The highest concentration of spongistatin 6 examined was 40 μM, and the absorbance and radioactivity HPLC profiles were nearly identical to the 20 μM pattern.

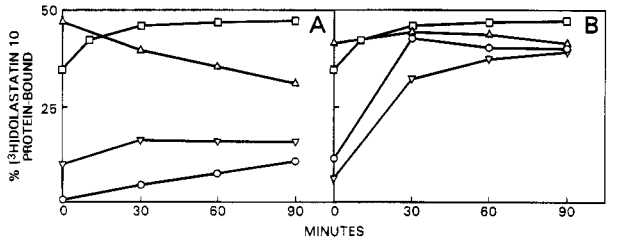


FIGURE 7: Effect of incubation time and reaction mixture addition order on the inhibitory effects of 10 μM spongistatin 1 (panel A) and 40 μM spongistatin 6 (panel B) on the binding of [^{3}H]dolastatin 10 to tubulin as measured by HPLC. All reaction mixtures contained 0.5 mg/mL (5.0 μM) tubulin, 10 μM [^{3}H]dolastatin 10, and 2% (v/v) dimethyl sulfoxide. The areas of the radiolabeled peaks associated with protein and with unbound drug (cf. Figure 6) were determined for each injection, and the data are expressed as the percentage of [^{3}H]dolastatin 10 bound to protein. Note that stoichiometric binding would be 50% of radiolabel in the bound peak. In each experiment, a 100 μL injection was made as soon as the reaction mixture was complete and every 30 min thereafter for 90 min, except that in the dolastatin 10 only experiment (□) a separate reaction mixture was prepared to obtain the 10 min point. Incubations and HPLC were at room temperature. Symbols as follows: (□) (same data in both panels), tubulin + [^{3}H]dolastatin 10 mixed at 0 °C; (○) tubulin + spongistatin 1 or 6 preincubated 10 min at room temperature prior to addition of [^{3}H]dolastatin 10; (△) tubulin + [^{3}H]dolastatin 10 preincubated 10 min at room temperature prior to addition of spongistatin 1 or 6; (▽) spongistatin 1 or 6 mixed with [^{3}H]dolastatin 10 prior to addition of tubulin at 0 °C.

At these higher spongistatin 6 concentrations, there was some inhibition of the total binding of [^{3}H]dolastatin 10 to tubulin, with protein-bound radioactivity peaking between the void volume and an included oligomer peak. In terms of this HPLC assay, both for persistence of oligomeric species (absorption pattern) and for retention of [^{3}H]dolastatin 10 by protein (radioactivity profile), spongistatin 6 was over 8-fold less potent than spongistatin 1. Addition of neither spongistatin 1 nor spongistatin 6 resulted in the appearance of a distinct [^{3}H]dolastatin 10 peak coeluting with 100 kDa tubulin.

Kinetic Effects on Inhibition. In the course of the HPLC studies with spongistatin 6, we noted that specific results depended on the incubation time, an effect not initially appreciated with spongistatin 1. Results were also affected by the order of addition of reaction components. Figure 7 presents studies that examine such effects with 5 μM tubulin, 10 μM [^{3}H]dolastatin 10, and 10 μM spongistatin 1 (panel A) or 40 μM spongistatin 6 (panel B).

When [^{3}H]dolastatin 10 and tubulin were mixed on ice, incubation resulted in only a slight increase in protein-bound dolastatin 10 [probably because of rapid warming of the "zero time" sample in the injection loop; cf. Bai et al. (1995)], and maximum stoichiometry of binding was about 0.95. Effects of the two spongistatins on this reaction were examined in three ways. First, spongistatin and tubulin were mixed and incubated for 10 min at room temperature, followed by addition of [^{3}H]dolastatin 10. Second, [^{3}H]-dolastatin 10 and tubulin were mixed and incubated for 10 min at room temperature, followed by addition of spongistatin. Third, the two drugs were mixed, and tubulin was added. A "zero time" injection was made upon completion of the reaction mixtures, followed by subsequent injections at 30 min intervals.

With spongistatin 1 (Figure 7A), profound inhibition of dolastatin 10 binding occurred when the polyether and tubulin were preincubated prior to addition of [^{3}H]dolastatin 10, but slow binding of dolastatin 10 to tubulin occurred as the incubation proceeded. Inhibition of binding that was nearly as extensive occurred when the two drugs were mixed prior to addition of protein. If [^{3}H]dolastatin 10 and tubulin were preincubated prior to addition of spongistatin 1, the polyether was initially noninhibitory, but there was a slow subsequent displacement of dolastatin 10 from tubulin.

With spongistatin 6 (Figure 7B), an essentially analogous pattern was observed, except that extensive inhibition of [^{3}H]-dolastatin 10 binding was only observed at the "zero time" points when spongistatin 6 and tubulin were preincubated and when the drugs were mixed prior to addition of tubulin. The subsequent time points imply substantial displacement of polyether from tubulin by the radiolabeled peptide, and this displacement was largely complete within 30 min. When [^{3}H]dolastatin 10 and tubulin were preincubated, spongistatin 6 had no inhibitory effect.

DISCUSSION

With [^{3}H]vinblastine as the "substrate", spongistatin 1, yielded data consistent with noncompetitive inhibition (Dixon et al., 1979). Previous studies indicated that the peptide antimitotics dolastatin 10 and phomopsin A and the sponge polyether halichondrin B are also noncompetitive inhibitors of vinca alkaloid binding to tubulin, while competitive patterns were obtained with maytansine, rhizoxin, and an alternate vinca alkaloid (Bai et al., 1990a, 1991).

The synthesis of [^{3}H]dolastatin 10 (Bai et al., 1995) permitted detailed analysis of inhibitory effects of spongistatin 1 on binding of the antimitotic peptide to tubulin. We found that spongistatin 1 noncompetitively inhibited the binding of dolastatin 10 to tubulin. Control experiments with an isomer of dolastatin 10 (Figure 3B), and with phomopsin A (data not presented), under the same reaction conditions yielded competitive patterns.

Interpreting these data in terms of classic models for competitive and noncompetitive inhibition (Dixon et al., 1979) leads to the conclusion that spongistatin 1, and presumably its analogs spongistatins 2−9, binds in a site on the tubulin molecule distinct from those where the vinca alkaloids and the peptide antimitotic agents bind. Tentatively, based on perhaps superficial similarities in molecular structure, we would propose that the halichondrins bind in the same site as the spongistatins, but the current scarcity of halichondrin B combined with its weaker inhibitory effect has not yet permitted us to determine the mode of inhibition of dolastatin 10 binding by halichondrin B.

We previously proposed (Bai et al., 1990a) that the peptide antimitotic site is physically near, perhaps even adjacent, to the "vinca site" where vinblastine and vincristine (and, presumably, the competitive inhibitors maytansine and rhizoxin) bind. To emphasize this proposed physical proximity together with the distinct peptide binding site implied by the noncompetitive mode of inhibition, we called this region the "vinca domain". We also proposed that the vinca domain must be primarily located on the β-tubulin subunit because, with the exception of vinblastine, all agents examined, if present at an adequate concentration, extensively inhibit *both* nucleotide exchange (Huang et al., 1985; Bai et al., 1990a, 1991, 1993ab) and formation of an intra-β-tubulin cross-link (Ludueña & Roach, 1981; Ludueña et al., 1989,

1992, 1993; Sullivan et al., 1990; Bai et al., 1993b) between Cys-12 and Cys-201/211 (Little & Ludueña, 1987). Further, cross-link formation is strongly inhibited by GTP, with maximal cross-link formation requiring use of tubulin depleted of exchangeable site nucleotide (Ludueña & Roach, 1981). Moreover, GTP itself will form a covalent bond with Cys-12 when it is photoactivated (Shivanna et al., 1993; Jayaram & Haley, 1994). We proposed that peptide antimitotic agents inhibited vinca alkaloid binding and nucleotide exchange primarily by sterically interfering with ligand access to the specific binding sites.

These same considerations apply to both the spongistatins and the halichondrins, with the additional finding that spongistatin 1 noncompetitively inhibits [^{3}H]dolastatin 10 binding to tubulin. Spongistatin 1 and halichondrin B inhibit [^{3}H]vinblastine binding to tubulin noncompetitively and inhibit GTP binding without displacing GDP from the exchangeable site (Bai et al., 1991, 1993a). Halichondrin B (Ludueña et al., 1993) and spongistatin 3 (Ludueña et al., 1995) both inhibit formation of the Cys-12-Cys-201/211 cross-link. Thus, it appears that the vinca domain contains at least three distinct drug binding regions, with the spongistatins and perhaps the halichondrins binding in a "polyether site" distinct from the "peptide site" and the "vinca site."

Two further aspects of these inhibitory effects merit further mention. First, the vinca alkaloids and the peptides dolastatin 10 and phomopsin A induce extensive aggregation reactions that probably occur under the conditions in which we examined drug binding (Tonsing et al., 1984; Bai et al., 1990b, 1995; Himes, 1991; Hamel, 1992). There appears to be no correlation between the pattern of inhibition of vinca alkaloid binding obtained with an inhibitory drug and whether or not the inhibitor can itself induce aggregation. Thus, with [^{3}H]vincristine and [^{3}H]vinblastine, we obtained competitive patterns with aggregating agents (another vinca alkaloid) and with the nonaggregating agents maytansine and rhizoxin (Bai et al., 1990a, 1991); and noncompetitive patterns with the aggregating agents dolastatin 10 and phomopsin A (Bai et al., 1990a) and with the nonaggregating agents halichondrin B and spongistatin 1 (Bai et al., 1991). With [^{3}H]dolastatin 10, we have less extensive data. Thus far we have obtained a noncompetitive pattern with the nonaggregating agent spongistatin 1 and competitive patterns with the aggregating agents isomer 2 and phomopsin A.

Second, in enzyme studies noncompetitive inhibition generally results in only partial, not total, inhibition of the reaction being studied. Such data are often interpreted as indicating binding at a distant site, with an allosteric effect causing an altered conformation of the substrate binding site and reduced substrate affinity. However, we generally find near-total inhibition of radiolabeled ligand binding together with graphical patterns most consistent with noncompetitive inhibition. This supports our concept of adjacent binding sites with these relatively large inhibitors sterically interfering with each others binding to tubulin, as opposed to distant allosteric effects. Such a conclusion is most convincing when inhibition of GTP binding to tubulin is considered. None of these agents bind in the exchangeable site, even though all except vinblastine can completely inhibit the binding of exogenous nucleotide to tubulin. First, [8-^{14}C]-GDP bound in the exchangeable site is not displaced by any vinca domain drug, but its displacement by GTP is prevented by the drugs. Second, inhibition of [8-^{14}C]GTP binding requires that drug and tubulin be added to the reaction mixture *prior* to addition of nucleotide. Inhibition is minimal if GTP and tubulin are mixed prior to drug addition (Huang et al., 1985; Bai et al., 1990a, 1991, 1993a,b). These findings are complemented by the observations that GTP and vinca domain drugs inhibit Cys-12-Cys-201/211 cross-link formation.[4]

Differences between spongistatins 1 and 6 on [^{3}H]-dolastatin 10 binding (Figure 4) and dolastatin 10-induced turbidity (Figure 5) had caused us to anticipate that spongistatin 6 might cause a shift of tubulin-bound [^{3}H]dolastatin 10 to the 100 kDa HPLC peak. This would imply simultaneous binding of spongistatin 6 and dolastatin 10 to tubulin. We found, however, that spongistatin effects on dolastatin 10-induced aggregation were more complex than expected. Drug-containing solutions indistinguishable in visual appearance from the control without drug contained aggregates that were readily detected by both electron microscopy and size-exclusion HPLC. Progressively higher concentrations of the spongistatins were required to inhibit turbidity development, aggregation products visualized by electron microscopy, and aggregation products detected by HPLC. This probably reflects an increase in sensitivity to progressively smaller aggregates by the three techniques. Disruption of the largest aggregates (visual turbidity) required very little spongistatin relative to either the tubulin or the dolastatin 10 concentrations, a substoichiometric inhibitory effect.[5] Disruption of the smallest aggregates (HPLC method) required superstoichiometric concentrations of both spongistatins relative to tubulin, and of spongistatin 6 relative to dolastatin 10. Moreover, at the highest spongistatin 6 concentrations examined, particularly when samples were rapidly analyzed (Figure 7B), there was inhibition of [^{3}H]-dolastatin 10 binding to tubulin, but we never observed discrete binding of [^{3}H]dolastatin 10 to the tubulin 100 kDa α-β-heterodimer. We thus have no evidence for simultaneous binding of spongistatin 6 and dolastatin 10 to tubulin to bolster the conclusion derived from the noncompetitive inhibition data that the peptide and polyether antimitotic agents bind at distinct sites on tubulin.

The HPLC studies indicate the spongistatins bind to tubulin more rapidly than dolastatin 10, considering the strong initial inhibition when tubulin was added to drug mixtures (Figure 7). This conclusion must be tempered, however, because of the aggregation reaction that accompanies dolastatin 10 binding. The data of Figure 7 establish that both spongistatin 1 and dolastatin 10 dissociate slowly from tubulin, although the rate-limiting step for the peptide could be disaggregation of multimeric structures. Spongistatin 6, in contrast, appears to dissociate relatively rapidly from tubulin. The differential effects of the spongistatins on inhibition of vinblastine, GTP, and dolastatin 10 binding probably derive from differences in association and dissociation rates of both the radiolabeled ligands and the spongistatins.

Finally, we should note that the differing activities of spongistatins 1–9 occur despite mostly minor structural differences. No compelling structure–activity theme is

[4] Note that inhibition of neither nucleotide exchange nor cross-link formation correlates with drug-induced tubulin aggregation.

[5] Substoichiometric effects on vinblastine-induced aggregation were observed with maytansine (Fellous et al., 1985) and rhizoxin (Takahashi et al., 1987).

apparent since the available spongistatins provide no definitive evidence of essential functionalities. The G ring (see Figure 1) is not predictive of activity, as it is present or absent in both more active and less active agents. Similarly, the C-5 and C-15 acetyl groups are found in both more active and less active spongistatin derivatives. While the C-50 chloride residue may be essential for maximal activity, being present in the four most active spongistatins (1, 4, 5, and 9), a C-50 chloride is not sufficient for high activity. The least active member of the group, spongistatin 3, also possesses a C-50 chloride substituent, and this agent differs from spongistatin 1 only in replacement of the C-5 acetyl group with a hydroxyl function.

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