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Spongistatin 1, a Highly Cytotoxic, Sponge-Derived, Marine Natural Product that Inhibits Mitosis, Microtubule Assembly, and the Binding of Vinblastine to Tubulin

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SUMMARY

A highly cytotoxic macrocyclic lactone polyether has been isolated from a *Spongia* species and named spongistatin 1. With L1210 murine leukemia cells an IC_{50} value for cell proliferation of 20 pM was obtained, and an increase in the mitotic index concordant with the decrease in cell number was observed. Kangaroo rat kidney PtK1 cells were examined by indirect immunofluorescence with a spongistatin 1 concentration that caused 50% reduction in cellular protein (0.3 nM) and with a 10-fold higher concentration. These cells displayed mitotic and nuclear aberrations at both concentrations, and intracellular microtubules were reduced in number at the lower concentration and disappeared at the higher. Similar changes in PtK1 cells were observed after treatment with equivalent toxic concentrations of the antimitotic agents colchicine, vinblastine, halichondrin B, and dolastatin 10. Spongistatin 1 inhibited the glutamate-induced polymerization of purified tubulin (IC_{50} value of 3.6 μM versus 2.1 μM for dolastatin 10 and vinblastine and 5.2 μM for halichondrin B). Spongistatin 1 had no effect on the binding of colchicine to tubulin, but it was a potent inhibitor of the binding of vinblastine and GTP to tubulin. In initial experiments with 5 μM tubulin and 5 μM vinblastine, spongistatin 1 and dolastatin 10 both had IC_{50} values of 2 μM, whereas halichondrin B had an IC_{50} value of 5 μM. Spongistatin 1 thus represents a new member of the group of complex natural products that inhibit mitosis by binding in the *Vinca* alkaloid domain of tubulin.

The major component of microtubules, the protein tubulin, is the target of many naturally occurring toxic compounds that cause cells to arrest in mitosis. The first such agents isolated were obtained from higher plants and include colchicine, podophyllotoxin, steganacin, the *Vinca* alkaloids, maytansine, and taxol (see Ref. 1 for a review). Subsequently, the ansamitocins (2), phomopsin A (3), and rhizoxin (4) were obtained from fungal organisms. Most recently marine animals have been found to be a rich source of new, structurally unusual, antimitotic agents. The peptide dolastatin 10 and the depsipeptide dolastatin 15 were obtained from the mollusk *Dolabella auricularia* (5–8), and the macrolide lactone polyethers halichondrin B, homohalichondrin B, halistatin 1, and halistatin 2 were isolated from a variety of sponges (9–13).

Spongistatin 1, a particularly cytotoxic compound, has recently been isolated from a member of the genus *Spongia* (14). Like the halichondrins, spongistatin 1 is a macrolide polyether that contains a lactone group, but there are considerable differences in their structures. These are compared in Fig. 1. Although its relative and absolute configurations are presently unknown, spongistatin 1 has 24 chiral centers, compared with the 32 chiral centers of halichondrin B. The "nucleus" of spongistatin 1 is smaller than that of halichondrin B. There are 41 atoms along the outside of the spongipyran nucleus (see Fig. 1), compared with 51 atoms along the outside of the halipyran nucleus (see Fig. 1). Spongistatin 1 also lacks groups comparable to the complex of bridging oxygens linking C(11) with C(14), C(9) with C(12), and C(8) with C(14) that occurs in the halichondrins.

Upon its purification and characterization, we initiated studies with spongistatin 1 to determine whether it was an antimitotic agent. We confirmed that the compound caused the accumulation of cells arrested in mitosis and the disappearance of intracellular microtubules. With L1210 murine leukemia cells spongistatin 1 was exceptionally potent, inasmuch as we obtained an IC_{50} value for cell growth of 20 pM. The studies presented here also summarize our initial studies on interactions of spongistatin 1 with tubulin. The compound was more active than halichondrin B as an inhibitor of tubulin polymerization but less active than dolastatin 10. Like both halichon-

ABBREVIATIONS: DAPI, 4,6-diamidino-2-phenylindole; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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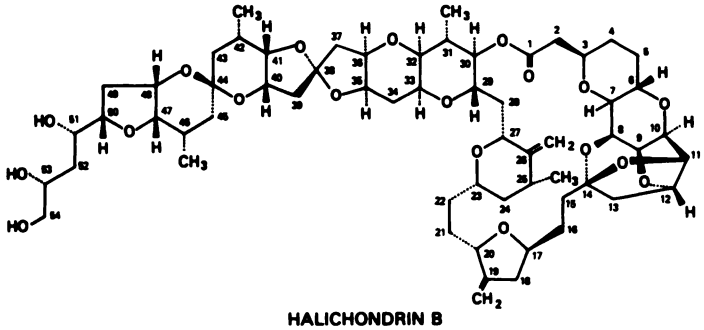
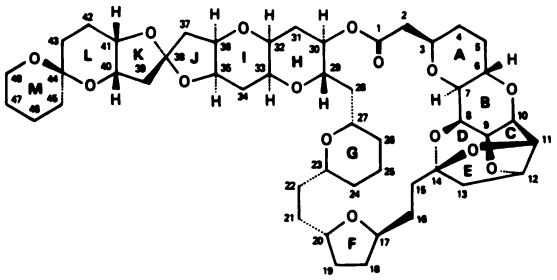
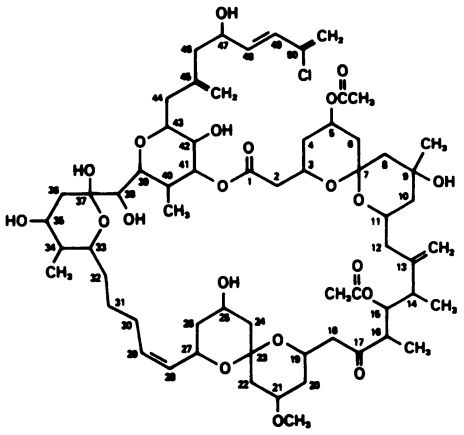


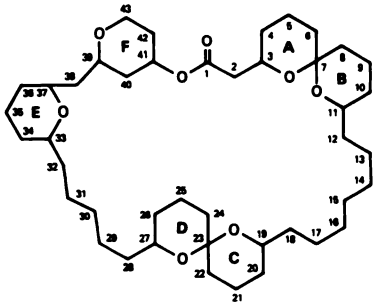
HALICHONDRIN B



HALIPYRAN RING SYSTEM



SPONGISTATIN 1



SPONGIPYRAN RING SYSTEM

Fig. 1. Comparison of the structure of spongistatin 1 with that of halichondrin B. The figure also presents the halipyran and spongipyran skeletons proposed by Pettit *et al.* (12, 14).

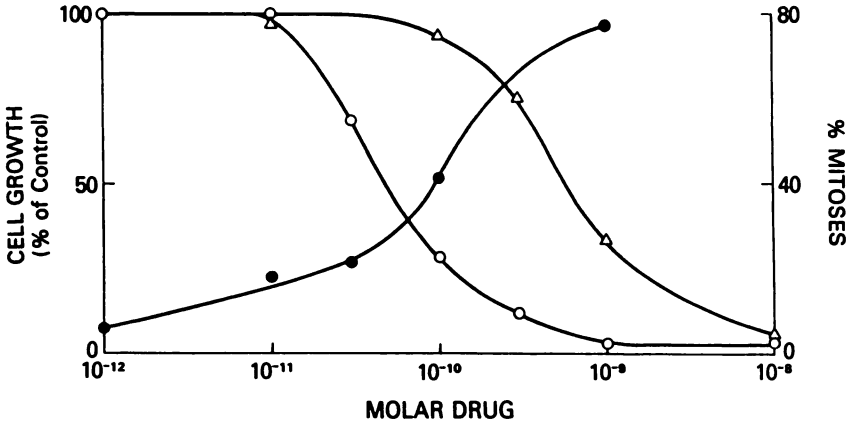


Fig. 2. Comparison of the effects of spongistatin 1 and dolastatin 10 on the growth of L1210 murine leukemia cells. Cells were grown at 37° for 16 hr in 5-ml suspension cultures, in a 5% CO_2 atmosphere. Drug concentrations were as indicated. The initial inoculum was 10^5 cells/ml. At the end of the incubation cells were either counted (*open symbols*) or harvested, fixed, and stained with Giemsa for determination of mitotic figures (*closed symbols*). *Circles*, spongistatin 1; *triangles*, dolastatin 10.

TABLE 1

IC_{50} values of antimitotic drugs

IC_{50} values were determined as described in the text. Values presented represent averages of at least two independent determinations.

Drug	IC_{50}	
	PtK1 cells	L1210 cells
	M	
Spongistatin 1	3×10^{-10}	2×10^{-11}
Halichondrin B	5×10^{-9}	3×10^{-10}
Dolastatin 10	3×10^{-9}	6×10^{-10}
Vinblastine	6×10^{-7}	2×10^{-8}
Colchicine	8×10^{-7}	5×10^{-8}

drin B and dolastatin 10, spongistatin 1 inhibited the binding of radiolabeled vinblastine and GTP to tubulin, with a potency similar to that of dolastatin 10 and greater than that of halichondrin B.

Experimental Procedures

Materials. Spongistatin 1 was isolated from a sponge species of the genus *Spongia*, as described elsewhere (14). Halichondrin B was isolated from a species of *Axinella* (a marine sponge) (10). Dolastatin 10 was synthesized as described previously (15). Nonradiolabeled vinblastine and colchicine were obtained from Sigma, [^{3}H]vinblastine from Amersham, [^{3}H]colchicine from DuPont, and [8-^{14}C]GTP from Moravek Biochemicals. The radiolabeled GTP was repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephacel. Bovine brain tubulin bearing GDP in the exchangeable site was prepared as

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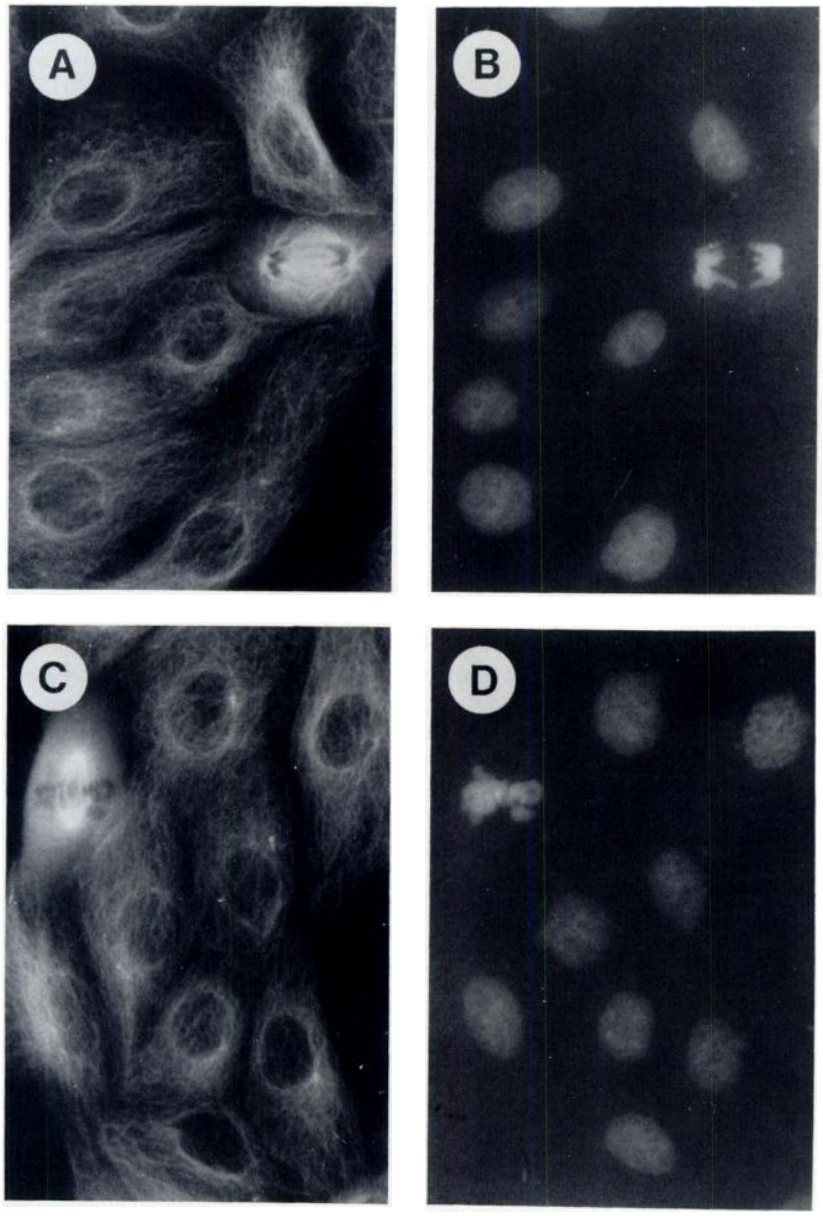

Fig. 3. Microtubule networks of untreated PtK1 cells. A and C, Indirect immunofluorescence images in which the primary antibody was directed against β-tubulin. B and D, Images of the same cells showing fluorescence of DAPI bound to DNA. × 430.

described elsewhere (16), as was tubulin bearing [8-^{14}C]GDP in the exchangeable site (17). PtK1 cells (CCL 35; normal kidney cells of the kangaroo rat *Potorous tridactylis*) were obtained from and maintained in culture as recommended by the American Type Culture Collection.

Methods. Drug effects on the growth of L1210 murine leukemia cells (cell number was the parameter followed) and on their mitotic index were measured as described elsewhere (18). Measurement of drug effects on the growth of PtK1 cells (protein production was the parameter followed), examination of these cells by DAPI stain for DNA, and indirect immunofluorescence with an anti-β-tubulin antibody were performed as described previously for Chinese hamster ovary cells (8).

Drug effects on tubulin polymerization were evaluated by determination of the concentration required to inhibit the extent of the reaction by 50% (IC_{50} value). Reaction mixtures (0.24 ml) contained 1.0 mg/ml (10 μM) tubulin, 1.0 M monosodium glutamate (2.0 M stock solution, adjusted to pH 6.6 with HCl), 1.0 mM $MgCl_2$, 4% (v/v) dimethylsulfoxide (the drug solvent), and varying concentrations of drug. All concentrations refer to a final reaction volume of 0.25 ml. The reaction mixtures were incubated at 37° for 15 min and chilled to 0°, and 10 μl of 10 mM GTP were added to each reaction mixture. The reaction mixtures were transferred to cuvettes held at 0° in Gilford 250 and 2400S spectrophotometers equipped with electronic temperature controllers. Polymerization, monitored at 350 nm, was initiated by a temperature jump to 37°, which took about 75 sec. The extent of polymerization after 20 min was quantitated. Each experiment consisted of 16 samples, including two control samples without drug. Each drug was examined in a minimum of three independent experiments.

The binding of [^{3}H]vinblastine or [8-^{14}C]GTP to tubulin was measured by the centrifugal gel filtration technique, as described in detail previously (19, 20). Reaction mixtures (0.3 ml) contained 0.5 mg/ml (5 μM) tubulin, 0.1 M MES, pH 6.9, 0.5 mM $MgCl_2$, 2% (v/v) dimethylsulfoxide, drug as indicated, and either 5 μM [^{3}H]vinblastine or 50 μM [8-^{14}C]GTP. Incubation was for 10 min at room temperature for vinblastine binding or on ice for GTP binding. Triplicate 0.1-ml aliquots were placed on syringe columns, which were processed at room temperature for vinblastine binding or at 4° for GTP binding. Radioactivity and protein in the filtrates were quantitated to determine the amount of vinblastine or GTP bound to tubulin. In nucleotide binding experiments, drug was always added to the reaction mixture before addition of nucleotide.

Colchicine binding to tubulin was measured by the DEAE-cellulose filter assay (21), using conditions that stabilize tubulin (22) to deter-

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mine whether spongistatin 1 is an inhibitor of colchicine binding or the conditions of Ludueña *et al.* (23) to determine whether spongistatin 1 itself stabilizes tubulin.

Results and Discussion

Interactions with cells. The activity-based purification of spongistatin 1 and its evaluation in the National Cancer Institute human cancer cell line drug evaluation program had shown that it was a highly cytotoxic compound (14). This observation was confirmed with L1210 murine leukemia cells in our laboratory, in a study in which spongistatin 1 was compared with the potent antimitotic peptide dolastatin 10 (Fig. 2). As in earlier studies (7, 24) a subnanomolar IC_{50} value (0.6 nM) was obtained for dolastatin 10 (in multiple experiments with dolastatin 10, IC_{50} values ranging from 0.3 to 0.9 nM have been obtained). Spongistatin 1 was even more potent; in the experiment presented in Fig. 2, the drug yielded an IC_{50} value of 50 pM. In this experiment the new agent was thus 12-fold more cytotoxic than dolastatin 10. The average IC_{50} value obtained for spongistatin 1 in seven experiments was 20 pM (range, 8–50 pM). As shown in Fig. 2, the number of cells arrested in mitosis increased as cell growth was inhibited, strongly indicating that inhibition of cell division is the primary toxic effect of spongistatin 1 on cell growth.

The high potency of spongistatin 1 as an inhibitor of mitosis, coupled with a rather standard effect on tubulin polymerization (see below), led us to examine the effects of the drug on intracellular microtubules by indirect immunofluorescence. Initial experiments were performed with Chinese hamster ovary cells, because we wished to compare the effects of spongistatin 1 with those that we had previously observed with dolastatin 10 (8). Although increasing concentrations of spongistatin 1 clearly caused progressive disruption of the microtubule network, the high toxicity of the drug caused extensive loss of cells from the culture and major distortions of cellular morphology.

We therefore examined the effect of spongistatin 1 on other cell lines, and we found that PtK1 cells could be readily examined by indirect immunofluorescence following treatment with the drug. Because this line was new to our laboratory, we first obtained IC_{50} values for cell growth at 48 hr for spongistatin 1 and four other antimitotic natural products that inhibit tubulin polymerization (halichondrin B, dolastatin 10, vinblastine, and colchicine). With all drugs we obtained IC_{50} values with PtK1 cells that were about 10-fold higher than those we obtained with L1210 cells after growth for 24 hr. Both sets of values are summarized in Table 1. Besides the incubation time, there were other significant differences between the assay conditions used with the two cell lines. The L1210 cells were in suspension culture, and cell number was determined. The PtK1 cells were in monolayer culture, and protein content was determined (25). With the latter cells, progressively lower IC_{50} values were obtained as incubation time increased, so that still higher IC_{50} values would have been obtained at 24 hr.

The IC_{50} values were obtained for this series of drugs so that their effects on intracellular microtubules could be evaluated at comparably toxic concentrations. Cells were also stained with DAPI to evaluate nuclear morphology/chromosome display. Control cells are presented in Fig. 3, including a cell in anaphase (Fig. 3, A and B) and one in metaphase (Fig. 3, C and D). Fig. 4 presents cells treated with spongistatin 1 at the IC_{50} dose (Fig. 4, A and B) and at a 10-fold higher drug concentration (Fig. 4, C and D). Fig. 5 shows tubulin immunofluorescence patterns for cells treated with IC_{50} concentrations of halichondrin B (Fig. 5A), dolastatin 10 (Fig. 5B), vinblastine (Fig. 5C), and colchicine (Fig. 5D) (DAPI patterns not presented). Fig. 6 presents tubulin immunofluorescence and DAPI patterns for cells treated with the same drugs at 10-fold higher drug concentrations (Fig. 6, A and B, halichondrin B; C and D, dolastatin 10; E and F, vinblastine; G and H, colchicine).

Compared with the extensive microtubule network in nonmitotic control cells (Fig. 3, A and C), the microtubules in interphase cells (those with decondensed chromosomes) treated at the IC_{50} concentration of spongistatin 1 (Fig. 4A) seem sparser and more disorganized. These interphase cells treated with spongistatin 1 (Fig. 4A) do not differ significantly in appearance from those treated with IC_{50} concentrations of halichondrin B (Fig. 5A) or colchicine (Fig. 5D). The residual microtubule network in interphase cells treated with IC_{50} concentrations of dolastatin 10 (Fig. 5B) or vinblastine (Fig. 5C) seem somewhat sparser than those in cells treated with the other drugs.

At the IC_{50} concentration all of the antimitotic drugs caused the appearance of increased numbers of cells with condensed chromosomes, presumably representing cells arrested in mitosis, and of cells with major perturbations of nuclear morphology. The latter include cells with two nuclei, multiple nuclei or nuclear fragments, or highly lobulated nuclei. Such cells probably represent cells that have failed to undergo a normal mitotic cycle.[1] These effects are compared quantitatively in Table 2, in which the percentages of cells with two or more nuclear fragments or with highly lobulated nuclei are tabulated as "multinucleate" and those with condensed chromosomes as "mitotic." Except in the case of colchicine, normal mitotic events were rarely seen at the IC_{50} concentrations. In arrested cells stained using the anti-β-tubulin antibody, brightly stained foci, perhaps representing centrosomes or centrosomal fragments, were seen with all agents. With spongistatin 1, halichondrin B, and colchicine (Figs. 4A, 5A, and 5D, respectively) these foci were few in number and had a stellate appearance, suggesting that they had nucleated many short microtubules, whereas with dolastatin 10 and vinblastine (Fig. 5, B and C, respectively) the foci were usually multiple and punctate. In two independent experiments with 0.8 μM colchicine, many of the mitotic cells were essentially normal in appearance, despite a significant increase in their number; besides the abnormal arrested cell shown in Fig. 5D, there is an anaphase cell and a late telophase cell pair with midbody.

At the present time we have not accumulated enough independent observations (either determination of IC_{50} values or immunofluorescence experiments at IC_{50} concentrations) to be certain that the differences described above for IC_{50} drug treatments represent significant differences in drug effects, especially considering the experimental variability in determining IC_{50} values (see above for the ranges obtained for spongistatin 1 and dolastatin 10 with L1210 murine leukemia cells). Thus, it seems most likely that the sparser interphase microtubules seen with dolastatin 10 and vinblastine, compared with spon-

[1] With L1210 murine leukemia cells the appearance of multinucleated cells after treatment with antimitotic drugs is often associated with the appearance of hyperploid (greater than tetraploid) cells, as determined by flow cytometric analysis for DNA content.

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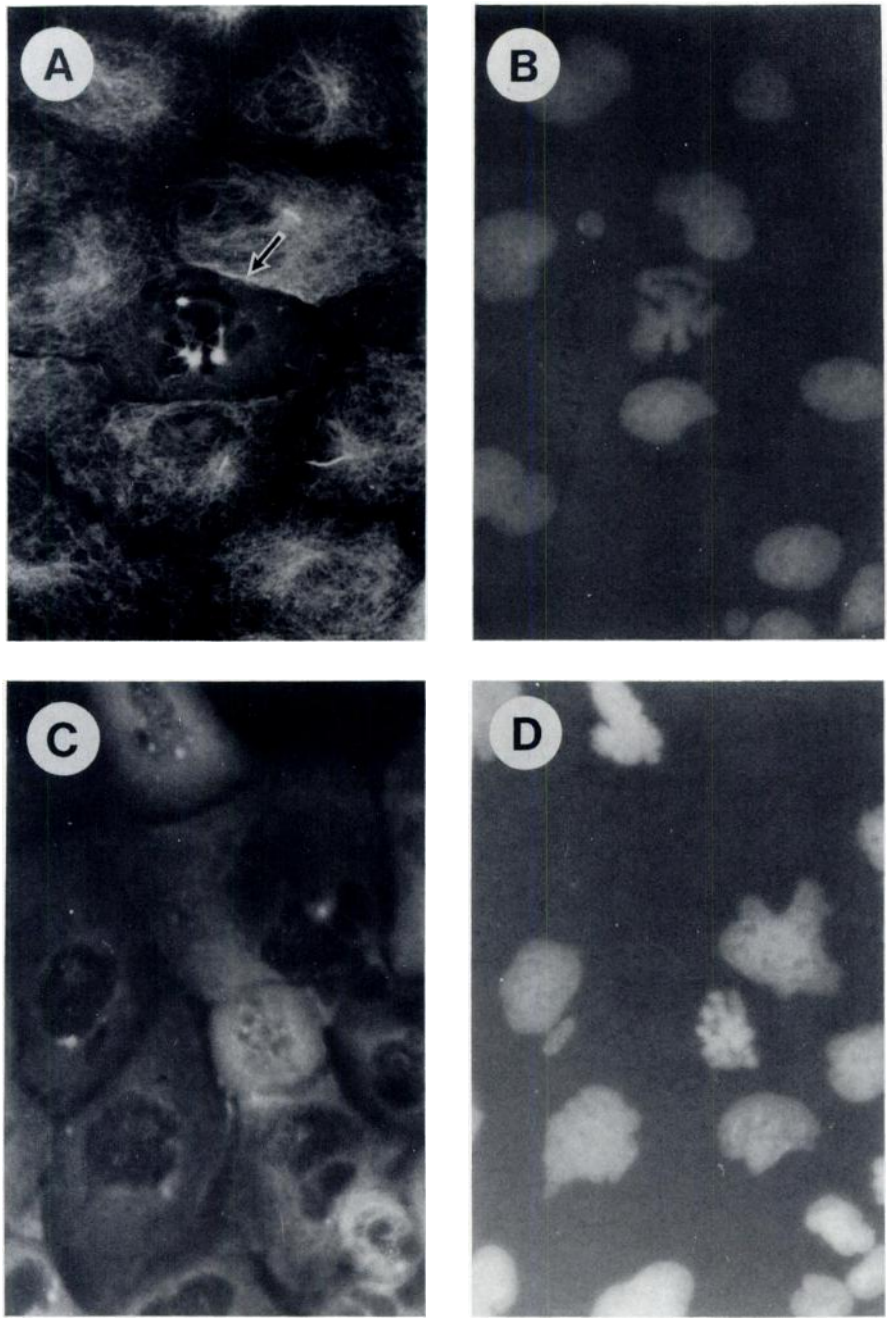

Fig. 4. Microtubule networks of PtK1 cells treated with spongistatin 1 at the IC_{50} concentration (A and B) and 10 times the IC_{50} concentration (C and D). The spongistatin 1 concentrations used were 3×10^{-10} M (A and B) and 3×10^{-9} M (C and D). A and C, Indirect immunofluorescence images in which the primary antibody was directed against β-tubulin. B and D, Images of the same cells showing fluorescence of DAPI bound to DNA. *Arrow*, mitotic cell. × 470.

gistatin 1, halichondrin B, and colchicine, indicate that higher relative concentrations of the former agents were used in the immunofluorescence experiments (e.g., an IC_{60} concentration). Similarly, the persistance of an increased number of normal (in appearance) mitotic events in the colchicine-treated cells probably indicates a lower relative concentration of this drug in these experiments (e.g., an IC_{40} concentration). We conclude that cells treated with the IC_{50} concentration of spongistatin 1 differ little from those treated with IC_{50} concentrations of other agents that inhibit tubulin polymerization, whether the cells are in interphase or arrested in mitosis.

At 10 times the IC_{50} concentration (Figs. 4, C and D, and 6), all the inhibitors of microtubule assembly, including spongistatin 1 (Fig. 4, C and D), caused the complete loss of microtubules both in interphase cells and in cells arrested in mitosis. In addition, with vinblastine (Fig. 6E) paracrystals were prominent both in cells in interphase and in cells arrested in mitosis.

Although we have not yet studied the effects of any of these antimitotic agents on PtK1 cells over a wide range of concentrations, these initial findings suggest the following progression of effects on cellular microtubules as drug concentration increases. At the lowest concentrations (e.g., IC_{10} to IC_{40}) the major effects are probably on microtubule assembly and/or disassembly rates or microtubule dynamics, as has been suggested by Jordan and co-workers (26, 27) in their model of effects of antimitotic drugs on spindle function. A major result at these lower drug concentrations would be an increased time in mitosis, producing the higher mitotic index with morphologically normal mitotic cells observed with 0.8 μM colchicine. The nearly complete disappearance of microtubules in some mitotic cells but no interphase cells in these colchicine-treated cultures

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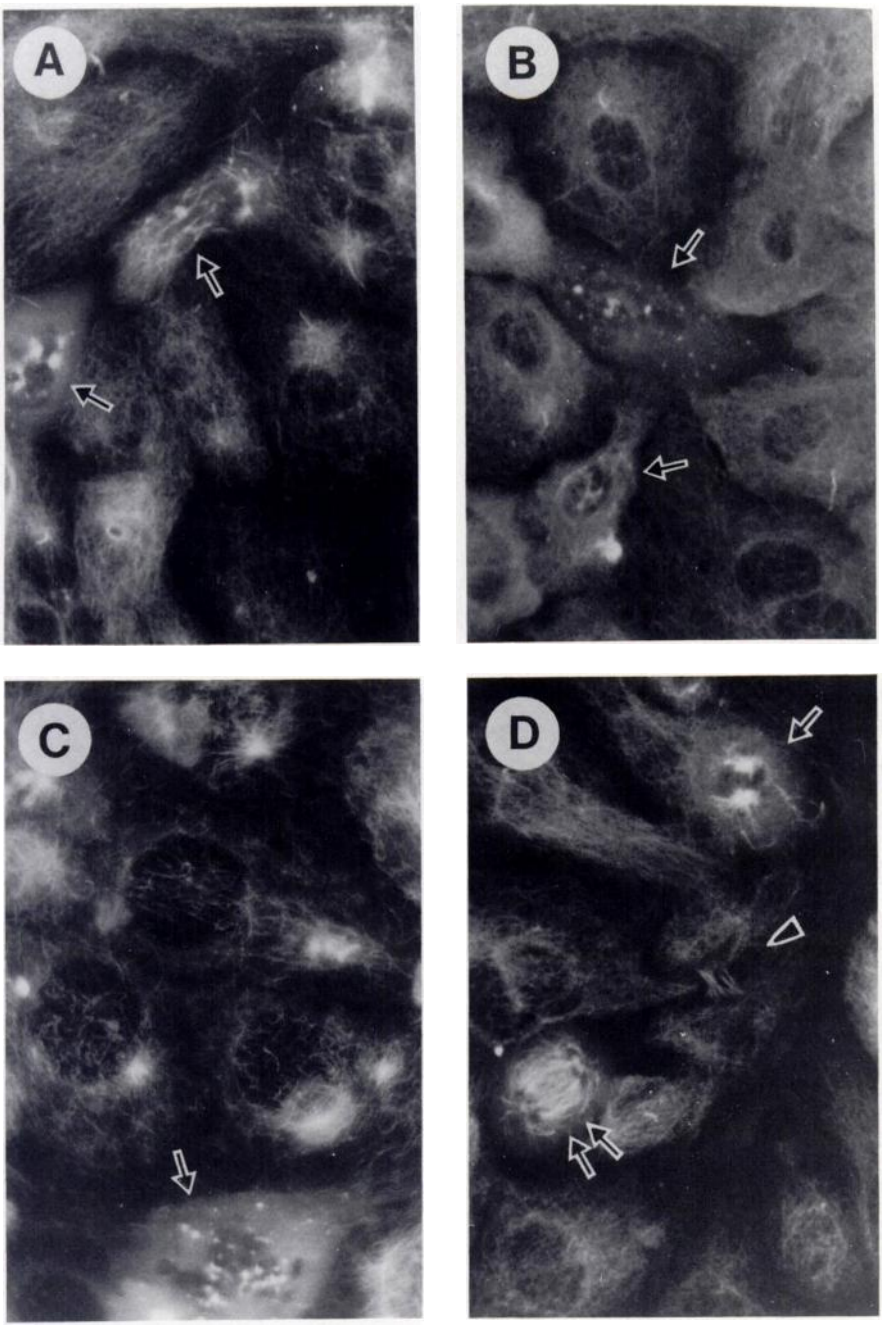

Fig. 5. Microtubule networks of PtK1 cells treated with halichondrin B (A), dolastatin 10 (B), vinblastine (C), and colchicine (D) at concentrations that inhibited cell growth by 50% (IC_{50} values). The drug concentrations are summarized in Table 1. All panels present indirect immunofluorescence images in which the primary antibody was directed against β-tubulin. *Single arrows*, mitotic cells. D, *Double arrow*, anaphase cell; *arrowhead*, telophase cell pair. × 470.

indicates that spindle microtubules are more sensitive to low drug concentrations than are cytoskeletal microtubules. At higher drug concentrations (e.g, IC_{50} to IC_{70}) the spindle microtubules disappear, and the interphase microtubules become noticeably depleted. Although we have no quantitative data, the ready observation of microtubule loss by indirect immunofluorescence probably indicates at least a 50% loss of microtubule mass near the IC_{50} concentration. It is of interest that the PtK1 cells seem to differ from HeLa cells in this regard, because Jordan *et al.* (27) found minimal loss of microtubule mass, as well as many cells with well formed spindles, at the IC_{50} concentration of vinblastine in the latter cell line. Finally, in PtK1 cells, at the highest drug concentrations examined microtubules completely disappeared from both mitotic and interphase cells. This could occur either by induction of depolymerization of preformed microtubules or by inhibition of assembly. Insight into these processes could probably be obtained by studying the time course of drug action on PtK1 microtubules.

Interactions with tubulin. As with most potent antimitotic drugs (1), spongistatin 1 was found to inhibit the glutamate-induced polymerization of purified tubulin in a concentration-dependent manner (Fig. 7). In the series of experiments summarized in Table 3, spongistatin 1 yielded an IC_{50} value of 3.6 μM, intermediate between the lower values obtained with dolastatin 10 and vinblastine (2.1 μM for both compounds) and the higher values obtained with maytansine (3.9 μM) and halichondrin B (5.2 μM).

In initial experiments to define the class of inhibitory agent to which spongistatin 1 belongs, we examined its effects on the binding of radiolabeled colchicine and vinblastine to tubulin. We could demonstrate no inhibitory effect of this new agent on the binding of radiolabeled colchicine to tubulin (Table 4,

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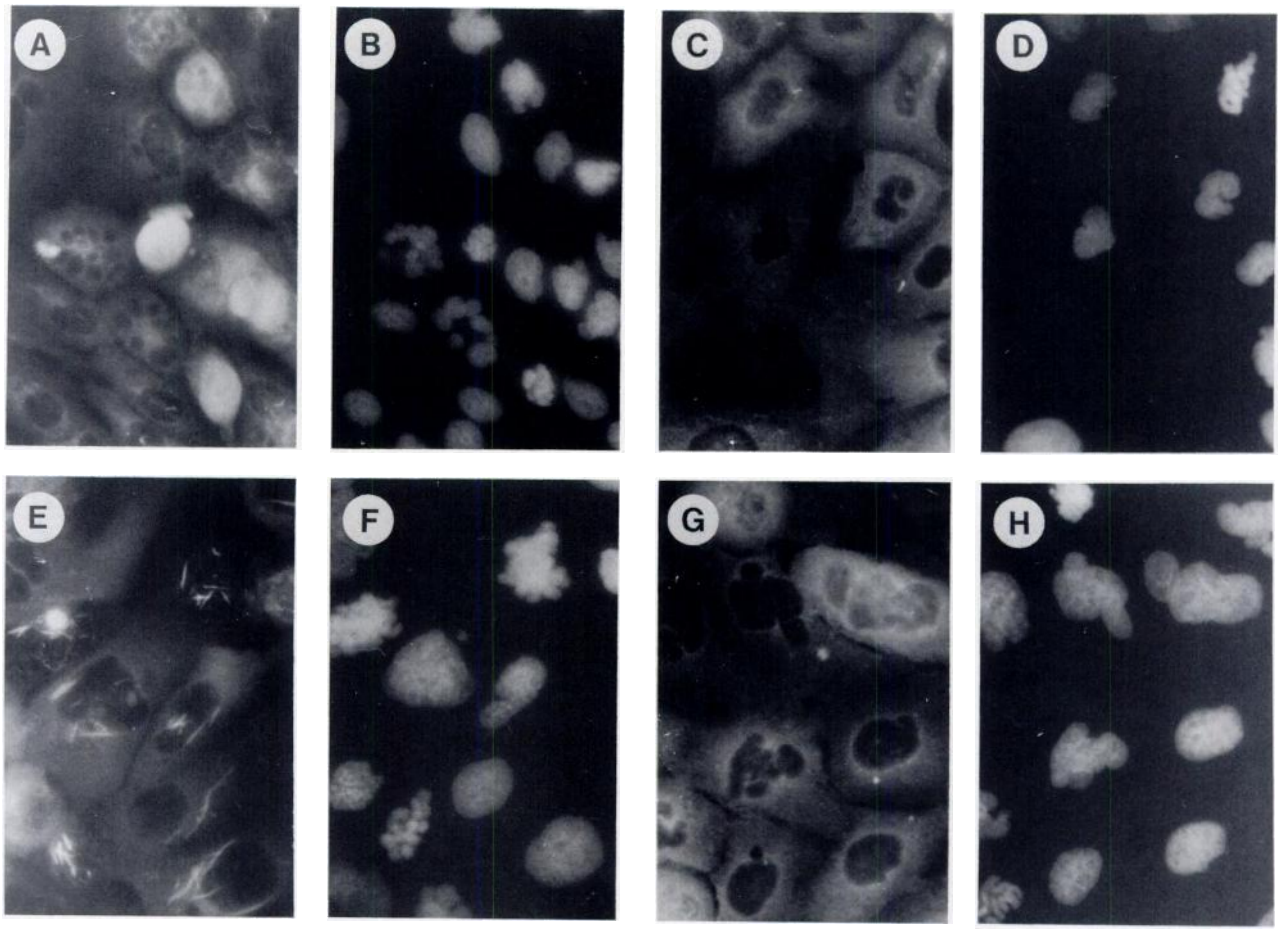

Fig. 6. Microtubule networks of PtK1 cells treated with halichondrin B (A and B), dolastatin 10 (C and D), vinblastine (E and F), and colchicine (G and H) at concentrations 10 times the IC_{50} values, which are summarized in Table 1. A, C, E, and G, Indirect immunofluorescence images in which the primary antibody was directed against β-tubulin. B, D, F, and H, Images of the same cells showing fluorescence of DAPI bound to DNA. × 330.

TABLE 2

Effects of antimitotic drugs on the nuclear morphology of PtK1 cells

PtK1 cells stained with DAPI were examined by fluorescence microscopy to evaluate nuclear morphology after treatment with the indicated drug concentrations (see Table 1) for 48 hr. "Mitotic" cells were those with condensed chromosomes. "Multinucleate" cells were those with multiple small nuclei, two nuclei, or highly lobulated nuclei, compared with the ovoid nuclei seen in control cells (see Fig. 3). Examples of such abnormal nuclear morphologies can be seen in Figs. 4 and 6. Two hundred cells were counted at each drug concentration. Control values were 2% mitotic cells and 5% multinucleated cells. No effort was made to recover cells that had become detached from the coverslips.

Drug	IC_{50} concentration		10× IC_{50} concentration	
	Mitotic cells	Multinucleate cells	Mitotic cells	Multinucleate cells
	%	%	%	%
Spongistatin 1	7	22	18	20
Halichondrin B	6	28	12	20
Dolastatin 10	9	28	13	27
Vinblastine	13	26	28	22
Colchicine	8	29	13	31

experiment I), nor did spongistatin 1 appear to stabilize the colchicine-binding activity of tubulin (Table 4, experiment II), as occurs with the peptide antimitotic agents dolastatin 10 and phomopsin A (20, 23). In contrast, spongistatin 1 was a potent inhibitor of the binding of radiolabeled vinblastine to tubulin (Fig. 8; average values from three experiments). Under the reaction conditions used in these experiments, with both tubulin and vinblastine at 5 μM, the inhibitory effects of spongis-

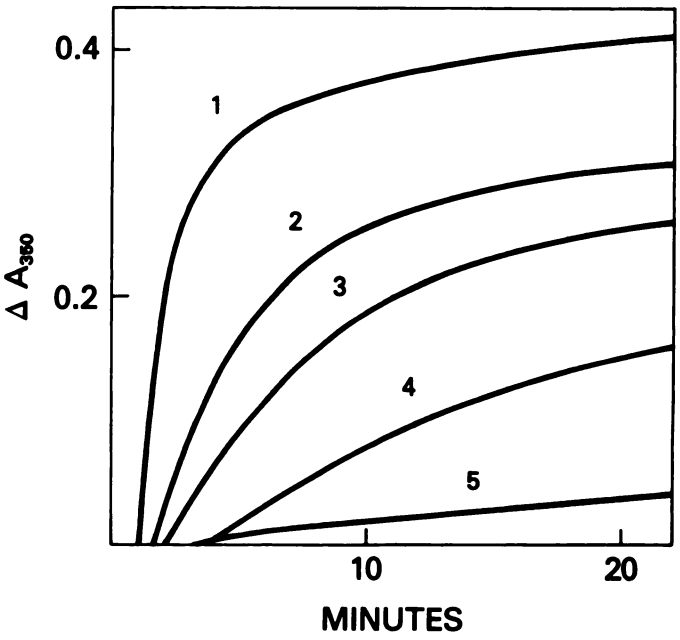

Fig. 7. Progressive inhibition of glutamate-induced tubulin polymerization by increasing concentrations of spongistatin 1. Experimental conditions were as described in the text. *Curve 1*, no drug; *curves 2, 3, 4,* and *5*, spongistatin 1 at 2, 3, 4, and 5 μM, respectively.

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TABLE 3

Inhibition of tubulin polymerization by spongistatin 1 and related compounds

A minimum of three independent determinations of the drug concentration required to inhibit the extent of tubulin polymerization by 50% (20-min incubation at 37°) were performed as described in the text. Tubulin and drug were preincubated for 15 min before addition of the GTP required to initiate polymerization. Values are mean ± standard deviation.

Drug	IC_{50}
	μM
Spongistatin 1	3.6 ± 0.4
Vinblastine	2.1 ± 0.3
Dolastatin 10	2.1 ± 0.4
Maytansine	3.9 ± 0.5
Halichondrin B	5.2 ± 0.4
Rhizoxin	12 ± 0.7

TABLE 4

Evidence that spongistatin 1 does not inhibit colchicine binding or stabilize the colchicine-binding activity of tubulin

In experiment I (inhibition of colchicine binding) (triplicate samples), each 0.1-ml reaction mixture contained 0.1 mg/ml tubulin, 5.0 μM [^{3}H]colchicine, 0.5% (v/v) dimethylsulfoxide, 1.0 M monosodium glutamate, pH 6.6, 0.1 M glucose 1-phosphate, 0.5 mg/ml bovine serum albumin, 1 mM GTP, 1 mM $MgCl_2$, and inhibitor, as indicated. Incubation was for 10 min at 37°. (Tubulin is highly stable under these reaction conditions.) In experiment II (stabilization of colchicine-binding activity) (triplicate samples), each 0.1-ml reaction mixture contained 0.37 mg/ml tubulin, 0.1 M MES (pH 6.4 with NaOH), 0.1 mM EDTA, 1 mM EGTA, 1 mM GTP, 0.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 5% (v/v) dimethylsulfoxide, and the indicated drug at 50 μM. Except for one set of control reactions, samples were preincubated for 3 hr at 37°. [^{3}H]Colchicine was added to the reaction mixtures to a concentration of 57 μM, and the samples were incubated for 2 hr at 37°.

Expt. I Drug added	Inhibition of [^{3}H]colchicine binding
	% of control
Podophyllotoxin (5 μM)	7
Podophyllotoxin (50 μM)	0
Spongistatin 1 (50 μM)	99
Expt. II Drug added	**Colchicine bound**
	% of nonpreincubated control
None	58
Spongistatin 1	54
Dolastatin 10	116
Halichondrin B	52

tatin 1, dolastatin 10, and maytansine were virtually indistinguishable (IC_{50} values of about 2 μM; summarized in Table 5). The nearly identical quantitative effects of these three drugs as inhibitors of vinblastine binding differ from the 2-fold range observed for the inhibitory effects of the drugs on polymerization. Halichondrin B and rhizoxin were significantly less potent as inhibitors of vinblastine binding, with IC_{50} values of 5 and 9 μM, respectively.

Because all drugs previously found to inhibit the binding of vinblastine to tubulin also inhibit nucleotide exchange on tubulin (11, 20, 28), we studied the effects of spongistatin 1, in comparison with dolastatin 10 and halichondrin B, on the binding of radiolabeled GTP to tubulin (Fig. 9, with averages from two experiments presented; Tables 5 and 6). When incubation was on ice, spongistatin 10 strongly inhibited GTP binding to tubulin, with activity comparable to that obtained with dolastatin 10 and maytansine and significantly greater than that of halichondrin B (IC_{50} values from the duplicate experiments summarized in Table 5).

Although we previously showed that dolastatin 10 and maytansine had quantitatively similar inhibitory effects on the

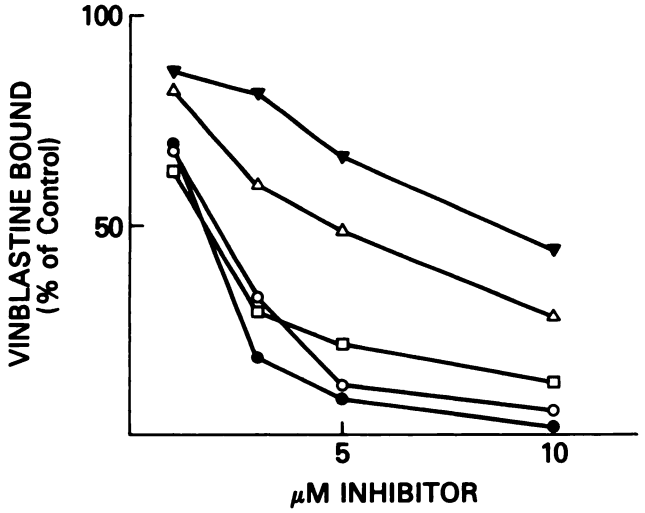

Fig. 8. Inhibitory effects of spongistatin 1, dolastatin 10, maytansine, halichondrin B, and rhizoxin on the binding of radiolabeled vinblastine to tubulin. Each 0.5-ml reaction mixture contained 0.5 mg/ml (5 μM) tubulin, 5 μM [^{3}H]vinblastine, 0.1 M MES (1 M stock solution, adjusted to pH 6.9 with NaOH), 0.5 mM $MgCl_2$, 2% (v/v) dimethylsulfoxide, and the indicated concentration of inhibitor (●, spongistatin 1; ○, dolastatin 10; □, maytansine; △, halichondrin B; ▼, rhizoxin). Incubation was for 10 min at room temperature. Triplicate 0.15-ml aliquots of each reaction mixture were processed by centrifugal gel filtration on syringe-columns of Sephadex G-50 (superfine), as described previously (19, 20). Protein and radioactivity in the filtrates were quantitated. In control reaction mixtures without drug, 0.37 mol of vinblastine was bound per mol of tubulin. Average values from three independent experiments are presented, except that rhizoxin was evaluated only twice in the current series of experiments.

TABLE 5

IC_{50} values for inhibition of vinblastine and GTP binding

The individual experiments summarized in Fig. 8 (vinblastine binding) and Fig. 9 (GTP binding) were analyzed graphically to yield IC_{50} values for each inhibitor studied. These were averaged; standard deviations are presented for the vinblastine inhibition values (three experiments) and differences from the average are presented for the GTP inhibition values (two experiments). Inhibition of vinblastine binding by rhizoxin was evaluated only two times in the current series of experiments, and difference from the average is presented. Inhibition of GTP binding by maytansine was evaluated only once in the current series of experiments.

Drug	IC_{50}	
	Vinblastine binding	GTP binding
	μM	
Spongistatin 1	1.8 ± 0.2	6.0 ± 0.3
Halichondrin B	5.0 ± 0.3	15.8 ± 0.6
Dolastatin 10	2.1 ± 0.2	5.6 ± 1.4
Maytansine	2.1 ± 0.4	7.7
Rhizoxin	9.2 ± 0.2	

binding of GTP to tubulin at 0° (11), only the former was able to inhibit nucleotide binding at 37° (20, 28). It was therefore of interest to compare the effects of spongistatin 1 on GTP binding at the higher temperature with those of dolastatin 10, maytansine, and halichondrin B (Table 6, experiment I). Like dolastatin 10, spongistatin 1 retained its inhibitory effect on nucleotide binding at 37°. In contrast, halichondrin B, like maytansine, had no apparent inhibitory effect at 37°, perhaps due in part to its weaker inhibitory effect on ligand interactions with tubulin (see Table 5).

Spongistatin 1, like the other antimitotic agents in this group (20, 28), also inhibited displacement of [8-^{14}C]GDP from the exchangeable site of tubulin (Table 6, experiment II) at 0°. This demonstrates that spongistatin 1, like the other drugs, does not directly bind in the exchangeable site but rather

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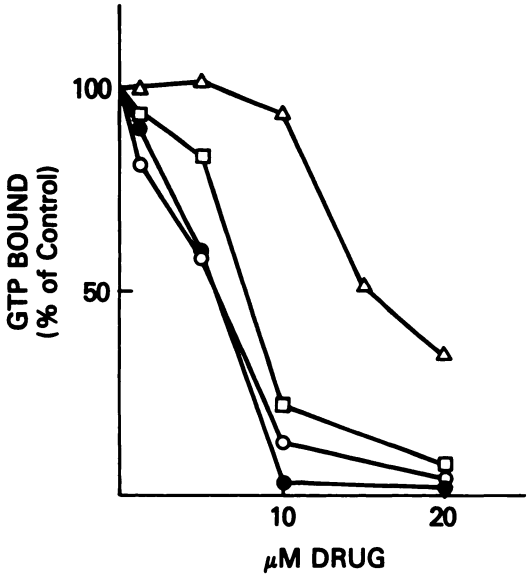

Fig. 9. Inhibitory effects of spongistatin 1, dolastatin 10, maytansine, and halichondrin B on the binding of radiolabeled GTP to tubulin. Each 0.5-ml reaction mixture contained 0.5 mg/ml (5 μM) tubulin, 50 μM [8-^{14}C]GTP, 0.1 M MES (1 M stock solution, adjusted to pH 6.9 with NaOH), 0.5 mM $MgCl_2$, 2% (v/v) dimethylsulfoxide, and the indicated concentration of inhibitor (●, spongistatin 1; ○, dolastatin 10; □, maytansine; △, halichondrin B). Incubation was for 10 min at 0°. Triplicate 0.15-ml aliquots of each reaction mixture were processed by centrifugal gel filtration on syringe-columns of Sephadex G-50 (superfine) at 4°, as described previously (19, 20). Protein and radioactivity in the filtrates were quantitated. In control reaction mixtures without drug, 0.70 mol of GTP was bound per mol of tubulin. Average values from two independent experiments are presented, except that maytansine was evaluated only once in the current series of experiments.

TABLE 6

Drug effects on nucleotide exchange

Reaction components and conditions were as described in the legend to Fig. 9, except as indicated. In experiment I (inhibition of nucleotide exchange at 37°), incubation was for 10 min at 37°, and centrifugal gel filtration was performed at room temperature (the microcolumns were at 37° until centrifugation). Drug concentration was 20 μM. In experiment II (drug inhibition of GTP displacement of [8-^{14}C]GDP from tubulin), instead of tubulin bearing nonradiolabeled GDP in the exchangeable site and [8-^{14}C]GTP, the reaction mixtures contained tubulin bearing [8-^{14}C]GDP in the exchangeable site and nonradiolabeled GTP. Thus, displacement of GDP from the exchangeable site by exogenous GTP was quantitated. Drug concentration was 20 μM.

Expt. I Drug added	[8-^{14}C]GTP bound
	mol/mol of tubulin
None	0.68
Spongistatin 1	0.06
Halichondrin B	0.70
Dolastatin 10	0.13
Maytansine	0.65
Expt. II Drug added	**[8-^{14}C]GDP bound**
	mol/mol of tubulin
None (no GTP)	0.72
None	0.05
Spongistatin 1	0.80
Halichondrin B	0.74
Dolastatin 10	0.67

inhibits the nucleotide exchange reaction. Although we cannot exclude an allosteric mechanism for this inhibition, we believe it is more likely that the structurally diverse *Vinca* alkaloid domain drugs sterically interfere with nucleotide exchange.

Conclusion. In summary, spongistatin 1 would be the most cytotoxic antimitotic compound yet isolated if its high cytotoxicity for L1210 cells and PtK1 cells is representative of its behavior with most cell lines. Data obtained in the National Cancer Institute human cancer cell line drug screen are consistent with our observations here (14). Spongistatin 1 is a new member of the group of complex natural products that interfere with the binding of *Vinca* alkaloids to tubulin and with nucleotide exchange. Although it has a strong inhibitory effect on tubulin polymerization, reflected in its causing the disappearance of microtubules in cells in culture, less cytotoxic compounds, such as vinblastine and dolastatin 10, inhibit tubulin polymerization more strongly than does spongistatin 1. Although there are many potential reasons for these differences, one possibility would be that spongistatin 1 has a high affinity for γ-tubulin, the minor tubulin variant that is localized at microtubule organizing centers (29). However, the similar effects of spongistatin 1, compared with those of four other inhibitors of tubulin polymerization, at the IC_{50} concentrations, argues against this possibility. We feel that the most likely explanation for the high cytotoxicity of spongistatin 1 will prove to be highly efficient cellular uptake of the drug. We are currently comparing cellular levels of vinblastine and dolastatin 10, and initial data indicate that the 30–200-fold higher (Table 1) cytotoxicity of the latter compound is derived from a higher penetration of cells, compared with vinblastine.

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