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Biochemical Pharmacology 63 (2002) 707–715

Biochemical Pharmacology

Inhibition of tubulin polymerization by vitilevuamide, a bicyclic marine peptide, at a site distinct from colchicine, the vinca alkaloids, and dolastatin 10

Michael C. Edler[a], Annette M. Fernandez[b], Peter Lassota[c,1], Chris M. Ireland[b], Louis R. Barrows[a,*]

[a]*Department of Pharmacology and Toxicology, University of Utah, 30 S. 2000 E. Rm. 201, Salt Lake City, UT 84112-5820, USA*
[b]*Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112-5820, USA*
[c]*Wyeth Ayerst Research, Pearl River, NY 10965, USA*

Received 23 October 2000; accepted 6 July 2001

Abstract

Vitilevuamide, a bicyclic 13 amino acid peptide, was isolated from two marine ascidians, *Didemnum cuculiferum* and *Polysyncranton lithostrotum*. Vitilevuamide was cytotoxic in several human tumor cell lines, with LC_{50} values ranging from 6 to 311 nM, and analysis in a 25-cell line panel revealed a weak correlation with several taxol analogs. Vitilevuamide was strongly positive in a cell-based screen for inhibitors of tubulin polymerization. Vitilevuamide at 9 μg/mL (5.6 μM) had an effect equivalent to the maximal effect of colchicine at 25 μg/mL (62.5 μM). Vitilevuamide was active *in vivo* against P388 lymphocytic leukemia, increasing the lifespan of leukemic mice 70% at 30 μg/kg. We hypothesized that at least part of the cytotoxic mechanism of vitilevuamide was due to its inhibition of tubulin polymerization. Vitilevuamide was found to inhibit polymerization of purified tubulin *in vitro*, with an IC_{50} value of approximately 2 μM. Cell cycle analysis showed that vitilevuamide arrested cells in the G_2/M phase with 78% of treated cells tetraploid after 16 hr. Therefore, vitilevuamide was tested for its ability to inhibit binding of known tubulin ligands. Vitilevuamide exhibited non-competitive inhibition of vinblastine binding to tubulin. Colchicine binding to tubulin was stabilized in the presence of vitilevuamide in a fashion similar to vinblastine. Dolastatin 10 binding was unaffected by vitilevuamide at low concentrations, but inhibited at higher ones. GTP binding was also found to be weakly affected by the presence of vitilevuamide. These results suggest the possibility that vitilevuamide inhibits tubulin polymerization via an interaction at a unique site. © 2002 Published by Elsevier Science Inc.

Keywords: Tubulin inhibitor; Marine natural product; Antitumor agent; Vitilevuamide; Cytoskeletal poison

1. Introduction

Many structurally diverse molecules interact with tubulin, the major component of microtubules. Molecules that interfere with tubulin polymerization can cause cells to arrest in metaphase. Several agents with this mechanism of action are useful as antineoplastic drugs. Terrestrial plants have provided the tubulin inhibitors used in the clinic today, the vinca alkaloids, the taxols, and colchicine [1]. However, other organisms produce tubulin inhibitors. For instance, fungi have produced the tubulin active compounds phomopsin A [2], ustiloxins A–F [3], rhizoxin [4], and the ansamitocins [5]. Marine organisms also have provided many structurally diverse antimitotic compounds. Spongistatin 1, halichondrin B, halistatin 1 and 2, homohalichondrin D, and hemiasterlin were isolated from a variety of sponges [6–13]. Curacin A was isolated from a blue-green algae [14]. Dolastatin 10 and the depsipeptide dolastatin 15 are peptides discovered from the sea mollusk *Dolabella auricularia* [15–18].

Vitilevuamide, which we present here, is an especially cytotoxic compound with an average 50% lethal concentration of drug (LC_{50}) in mammalian cell lines of ~100 nM.

* Corresponding author. Tel.: +1-801-581-4547; fax: +1-801-585-5111.
E-mail address: lbarrows@deans.pharm.utah.edu (L.R. Barrows).
[1] Present address: Novartis Pharmaceuticals Corporation, Oncology, 556 Morris Avenue, Summit, NJ 07901-1398, USA.

Abbreviations: MTPs, tubulin plus microtubule-associated proteins; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LC_{50}, 50% lethal concentration of drug; α-MEM, minimum essential medium alpha modification; db-cAMP, dibutyryl-cAMP; PIPES, 1,4-piperazine-diethanesulfonic acid; MES, 4-morpholinoethanesulfonate; FACS, fluorescence-activated cell sorting; ILS, increased life-span; MAPs, microtubule-associated proteins.

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PII: S0006-2952(01)00898-X

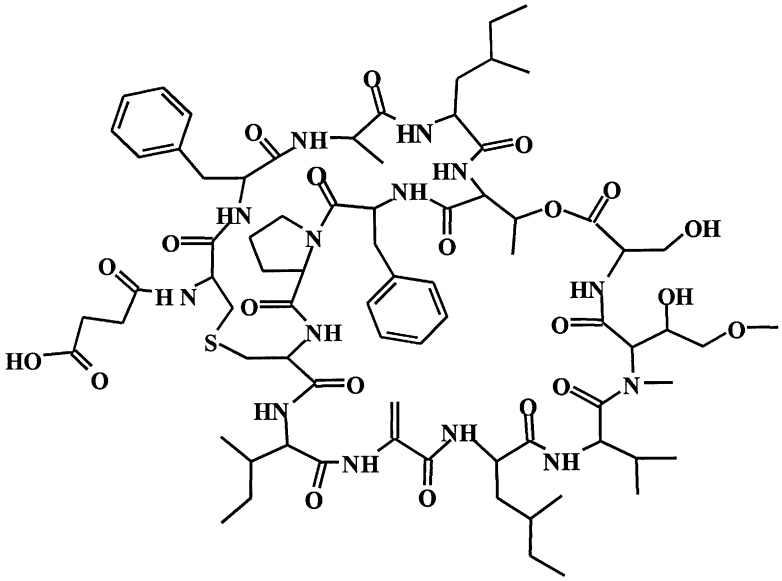

Fig. 1. Structural formula of vitilevuamide.

It was isolated from two species of marine ascidians, *Didemnum cuculiferum* and *Polysyncranton lithostrotum*. Structurally, vitilevuamide is a bicyclic, 13 amino acid peptide, with a molecular formula of $C_{77}H_{114}N_{14}O_{21}S$ (MrH+ 1603.8117) (Fig. 1). Amino acids were identified as Ala, Ser, Val, Thr, Ile, 2 Phe, Pro and modified amino acids as 2 homoisoluecine, lanthionine, dehydroalanine, and *N*-methyl methoxinine (and an additional succinate unit). Vitilevuamide also contains an ester linkage that is necessary for activity.

Due to the cytotoxic potency of vitilevuamide we evaluated it in a 25-cell line panel. Analysis of the varying sensitivities in these cell lines suggested a weak similarity to several taxol analogs. Upon further testing, vitilevuamide scored strongly positive in a cell-based screen for inhibitors of tubulin polymerization [19]. We therefore hypothesized that vitilevuamide was an inhibitor of tubulin polymerization. We present here our studies testing the ability of vitilevuamide to bind and interfere with tubulin function.

2. Materials and methods

2.1. Chemicals

The isolation and purification of vitilevuamide from *D. cuculiferum* and *P. lithostrotum* have been described elsewhere [20]. [^{3}H]Vinblastine was obtained from the Amersham and [^{3}H]colchicine from NEN. Non-radiolabeled vinblastine, vincristine, colchicine, and GTP were obtained from Sigma. [^{3}H]Dolastatin was a gift of R.-D. Haugwitz (Drug Synthesis and Chemistry Branch, National Cancer Institute), and non-radiolabeled dolastatin was a gift of D.-J. Newman (Natural Products Branch, National Cancer Institute). GTP and [8-^{3}H]GTP were obtained from Sigma and Amersham Pharmacia Biotech, respectively. Electrophoretically homogeneous bovine brain tubulin containing tubulin plus microtubule-associated proteins (MTPs) were isolated and purified with minor modifications according to methods described elsewhere [21,22]. This MTP was used in all tubulin-utilizing experiments described below. All drugs were dissolved in DMSO prior to being used.

2.2. Cell cytotoxicity

Cytotoxicity was established in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as performed by Mosmann [23] and modified by others [24,25]. Vitilevuamide was dissolved in 100% DMSO at an initial concentration of 10 mg/mL and serially diluted. The final concentration of DMSO in the cell culture wells was 1% or less. Each human cell line was seeded (20,000 cells per well) in 200 μL of growth medium in Corning 96-well microtiter plates. Cells were routinely cultured in minimum essential medium alpha modification (α-MEM) (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 10,000 U/L of penicillin, and 10,000 U/L of streptomycin (Sigma). Four hours after seeding, cells were treated in quadruplicate with 1 μL of drug. On day 3 of the assay, cultures were refed with fresh medium. Then each well was treated with 11 μL of MTT solution (5 mg/mL in PBS, pH 7.4) and incubated for 4 hr. Viable cells reduced the MTT to a purple formazan product that was solubilized by the addition of 100 μL of DMSO to aspirated culture wells. The absorbance at 540 nm was measured for each well using a Bio-Rad MP450 plate reader. Average absorbance for each set of drug-treated wells was compared to the average absorbance of the

control wells to determine the fractional survival at any particular drug concentration. The LC_{50} was defined as the drug concentration that yielded a fractional survival of 0.5.

2.3. Inhibition of tubulin polymerization in a cell-based assay

Inhibition of tubulin polymerization was determined using a cell-based assay that measures morphological changes of rat glioma cells treated with dibutyryl-cAMP (db-cAMP) [19]. C6 rat glioma cells were seeded on a 96-well plate at 5×10^4 cells per well in 200 μL of α-MEM and allowed to incubate for 24 hr. Vitilevuamide was added in quadruplicate wells ranging from 2.5 ng/mL to 50 μg/mL final concentration. Colchicine at a final concentration of 25 μg/mL was used as a positive control. Four hours later 1 μL of 0.5 M db-cAMP was added to the wells, and the cultures were incubated for 45 min to allow the cells to undergo a morphological change. This morphologic change is due to db-cAMP-induced tubulin polymerization and results in partial cell detachment from the culture substrate. Inhibition of tubulin polymerization prevented this detachment. The wells were then jet-aspirated using a customized tool in order to remove loosely attached cells. Each well was then supplied with 100 μL of McCoy's medium and 11 μL of 5 mg/mL stock MTT in PBS. Cell activity was measured as above in the cytotoxicity assay. Data were plotted as a fraction of colchicine absorbance at 25 μg/mL.

2.4. Cell cycle analysis

AA8 Chinese hamster ovary (CHO) cells (2–3 million) were seeded in 25 mL flasks and grown for 4 hr in α-MEM. Cells were then grown for 16 hr in an IC_{50} concentration of vitilevuamide to allow all cells time to traverse at least one cell cycle. Medium was removed, and the cells were trypsinized. Then cells were pelleted and resuspended in PBS and fixed by adding 2 mL of cold methanol drop-wise while mixing. Fixed cells were pelleted at 400 *g* for 5 min at room temperature and washed once with PBS. Cell suspensions were centrifuged as above and then resuspended in 0.5 mL of 100 μg/mL of propidium iodide solution in PBS. Then 0.5 mL of PBS containing 200 U/mL of RNase A was added. Cells were incubated at room temperature for 30 min in the dark. DNA content was determined by flow cytometry (University of Utah, FACS core facility). The results were analyzed using the Modfit cell cycle analysis program (Varity Software).

2.5. Inhibition of tubulin polymerization in a purified tubulin assay

Inhibition of tubulin polymerization was determined using a light-scattering assay [26,27] modified to use a 96-well microtiter plate. Tubulin (MTP) isolated as described above [21,22] was incubated with polymerization buffer (80 mM 1,4-piperazinediethanesulfonic acid (PIPES) (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 10% DMSO) and inhibitory drug. A base line (depolymerized) reading was measured at 4°. Then the plate was warmed to 37° for 45 min and read again. An increase in absorbance indicated tubulin polymerization. Reading was done on a 96-well plate reader at 595 nm.

2.6. Competitive binding of vitilevuamide with other tubulin ligands

Binding of [^{3}H]dolastatin and [^{3}H]vinblastine to tubulin was measured using centrifugal gel filtration chromatography as described elsewhere [28,29]. All reactions contained 0.1 M 4-morpholinoethanesulfonate (MES) (pH 6.9), 0.5 mM $MgCl_2$, tubulin, radiolabeled ligand, vitilevuamide, and DMSO (the drug solvent). Tubulin (1 mg/mL) was added last in every experiment and allowed to incubate for 45 min. Reaction mixtures were then added to 1 mL microspin columns of bio-gel P-30 and centrifuged at room temperature for 1 min in a tabletop centrifuge. The filtrate was collected, and radioactivity was determined by liquid scintillation counting, allowing calculation of a mole ratio of drug to tubulin for each experiment. Controls determined that no radioactivity passed through the column in the absence of tubulin.

2.7. Assay for GTP binding to tubulin

Each 0.5 mL reaction mixture contained 0.5 mg/mL (5 μM) of tubulin, 50 μM [8-^{3}H]GTP, 0.1 M MES (pH 6.9), 0.5 mM $MgCl_2$, 2% (v/v) DMSO, and different concentrations of inhibitor. Incubation was for 10 min at 4°. 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 [29,30]. Protein and radioactivity in the filtrates were quantified. Then values were normalized to the control reaction mixtures.

2.8. Stabilization of colchicine binding

Stabilization of colchicine binding activity to tubulin was observed using the method described by Ludueña *et al.* [31]. Reaction mixtures were filtered through a stack of three DEAE-cellulose filters.

2.9. In vivo activity against P388 lymphocytic leukemia

CDF1 mice were injected i.p. on day 0 with 1×10^6 P388 lymphocytic leukemia cells. Five animals were then randomly selected and placed into one of two groups, placebo or drug-treated. On days 1, 5, and 9 of post-tumor implantation, each mouse was treated with either vehicle (10% methylcellulose in Eagle's balanced pH solution) or

vitilevuamide by i.p. injection. Animals were checked twice daily, and the day of death for every mouse post-tumor implantation was recorded. A positive drug response was defined as a greater than 25% increase in the mean life-span (% increased life-span (ILS)) relative to the placebo control.

3. Results

The activity-based purification of vitilevuamide had shown that it was a highly cytotoxic compound. Further analysis in several human tumor cell lines revealed cytotoxic activity in the low nanomolar range. The LC_{50} values (graphs not shown) were obtained for HCT 116 human colon tumor (6 nM), A5249 lung cancer (124 nM), SK MEL-5 melanoma tumor (311 nM), and A498 kidney cancer (311 nM) cell lines. An LC_{50} value of 3.1 μM was obtained for CHO cells treated with vitilevuamide for only 16 hr instead of 72 hr. This was done to calculate the proper concentrations to be used in the cell cycle analysis. The low nanomolar toxicity of vitilevuamide in these cells suggested utility as an anticancer agent. Analysis in a proprietary cell panel (Wyeth Ayerst Research) suggested a mechanism of cytotoxicity similar to that of taxol analogs, anticancer agents that stabilize and promote tubulin polymerization.

3.1. Inhibition of tubulin polymerization in glioma cells

Although natural products with potent antimitotic activity strongly suggest an interaction with tubulin [32], other mechanisms of action are possible. Therefore, vitilevuamide was tested in a cell-based system capable of identifying tubulin interactive compounds. Vitilevuamide was found to inhibit the cAMP-stimulated differentiation of rat C6 glioma in a manner similar to colchicine. Fig. 2 depicts the absorbance of solution in the wells treated with vitilevuamide expressed as a function of the positive control, colchicine. Vitilevuamide had an effect at 9 μg/mL (5.6 μM) similar to the positive control colchicine at 25 μg/mL (62.5 μM). Vitilevuamide had an IC_{50} of 2.5 μM that compared favorably to both vinblastine (8.2 μM) and vincristine (6.2 μM) in this system [19]. Another marine natural product, curacin A, was more potent than vitilevuamide (curacin A, IC_{50} 510 nM).

3.2. Effect of vitilevuamide on cell cycle progression

AA8 CHO cells (2–3 million) were seeded in 25 mL flasks and treated with 15 μM vitilevuamide for 16 hr. Fluorescence-activated cell sorting (FACS) analysis (Fig. 3B) showed that vitilevuamide produced an accumulation of cells in the G_2/M phase of the cell cycle. In the presence of 15 μM vitilevuamide, 51.6% of the cells accumulated in G_2/M, compared with 17.0% in controls.

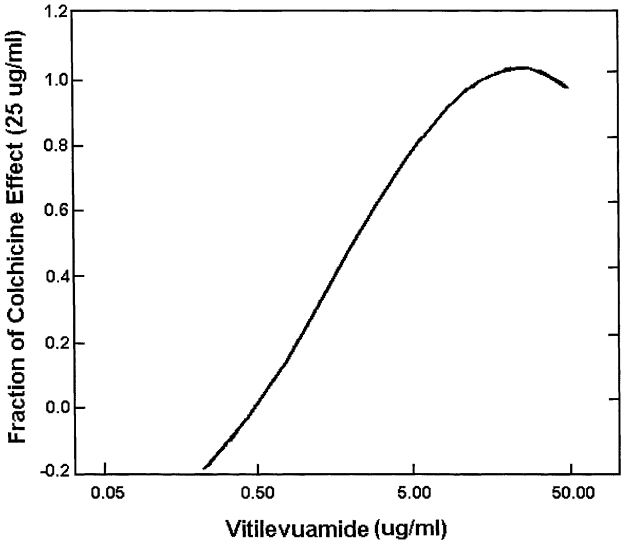

Fig. 2. Effect of vitilevuamide on C6 rat glioma cells treated with db-cAMP. The absorbance of the solution in the wells treated with vitilevuamide is expressed as a fraction of the absorbance of the solution in wells treated with 25 μg/mL of colchicine. Vitilevuamide expressed an IC_{50} activity at 4 μg/mL. These results are from one of two virtually identical experiments.

Correspondingly, the fraction of cells in G_0/G_1 was reduced from 40.8 to 7.9% in treated cells. Cells treated with vitilevuamide also demonstrated an increase in polyploidy. The drug-treated population contained 19% diploid cells, 78% tetraploid cells, and 3% octaploid cells. These results showed that, in cultures of cycling CHO cells, vitilevuamide arrests cell cycle progression in G_2/M.

3.3. Inhibition of tubulin polymerization in vitro

The ability of vitilevuamide to inhibit the polymerization of tubulin was confirmed *in vitro* in a purified protein system. Vitilevuamide was an effective inhibitor with an *in vitro* IC_{50} of 2 μM (data not shown). Additionally, higher concentrations yielded the paradoxical increase in absorbance indicative of tubulin aggregation as seen with another tubulin active peptide, dolastatin [28]. This aggregation was confirmed later by light microscopy (data not shown).

3.4. Effects of vitilevuamide on the drug-binding domains of tubulin

Several sites of drug interaction with tubulin have been described. Competitive binding assays using colchicine, vinblastine, and dolastatin 10 were employed to determine if vitilevuamide occupied any of these known sites. Vinblastine binding to tubulin was inhibited by vitilevuamide. Lineweaver–Burk analysis yielded lines that intercepted at the negative abscissa, indicating that this inhibition was non-competitive (Fig. 4A). Dixon analysis [33] of the data presented in Fig. 4A yielded an apparent K_i value for

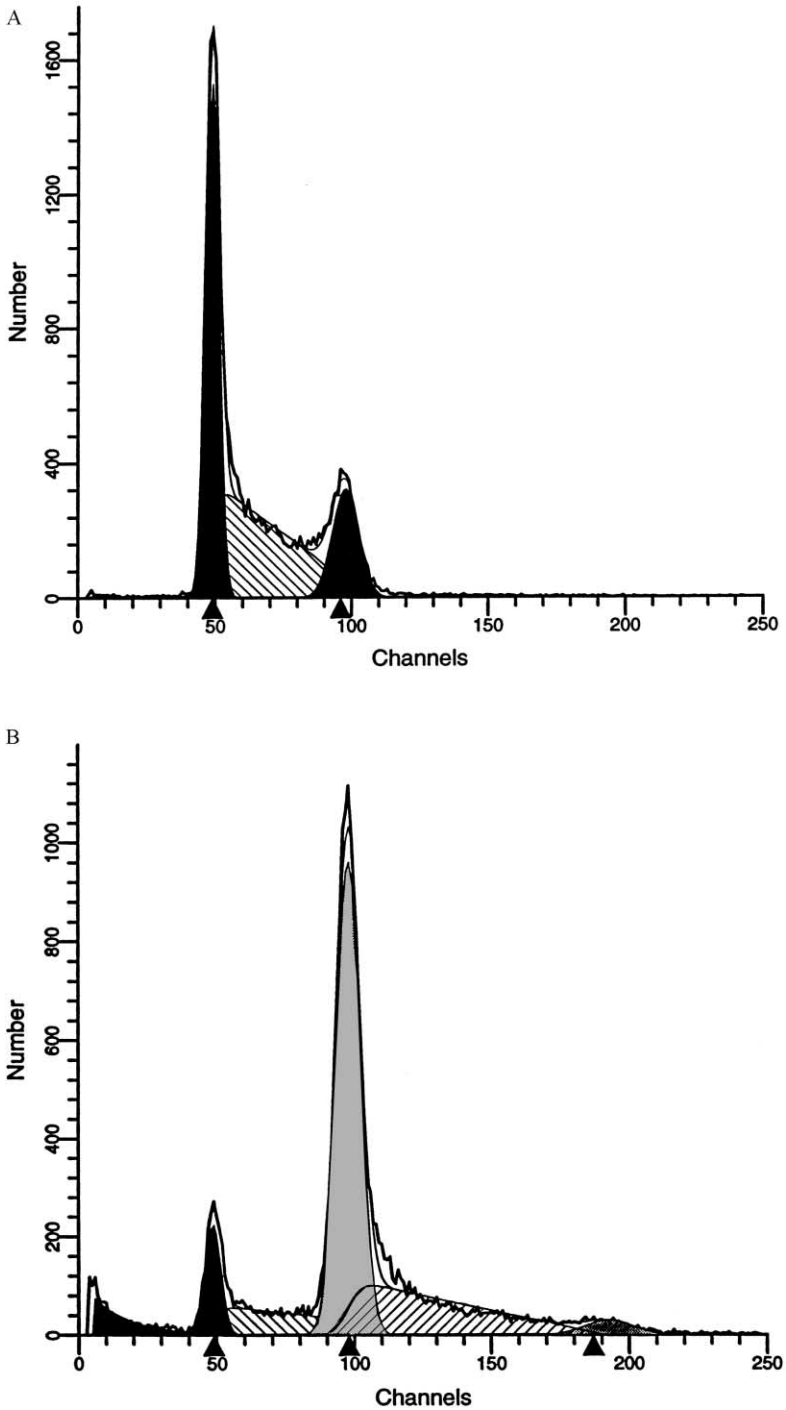

Fig. 3. Effect of vitilevuamide on cell cycle progression. Chinese hamster ovary cells were plated in growth medium in 25 mL flasks at a density of 2–3 million cells per flask. The cultures were then treated with vehicle (A) or 15 μM vitilevuamide (B) for 16 hr, and cells were processed for propidium iodide staining and FACS analysis. Cell distribution in G_0/G_1, S, and G_2/M was determined using the Varity Modfit program. The areas under the left solid peak, the middle stripped peak, and the right solid peak represent the fraction of cells in G_0/G_1, S, and G_2/M, respectively. Cells were treated in triplicate and pooled prior to FACS analysis.

inhibition of [^{3}H]vinblastine binding of 1.8 μM. Vitilevuamide did not inhibit the binding of dolastatin 10 to tubulin at low concentrations. Inhibition of binding was seen at higher concentrations. However, no clear concentration-dependent relationship could be determined (Fig. 4B). Since a binding relationship could not be determined between vitilevuamide and dolastatin 10, an apparent K_i value was not calculated. It was noted that higher concentrations of vitilevuamide and dolastatin promote tubulin aggregation. It is probable that tubulin aggregation resulted in the decreased binding of dolastatin 10 seen at higher concentrations of vitilevuamide. A similar observation with dolastatin has been reported in the literature [28].

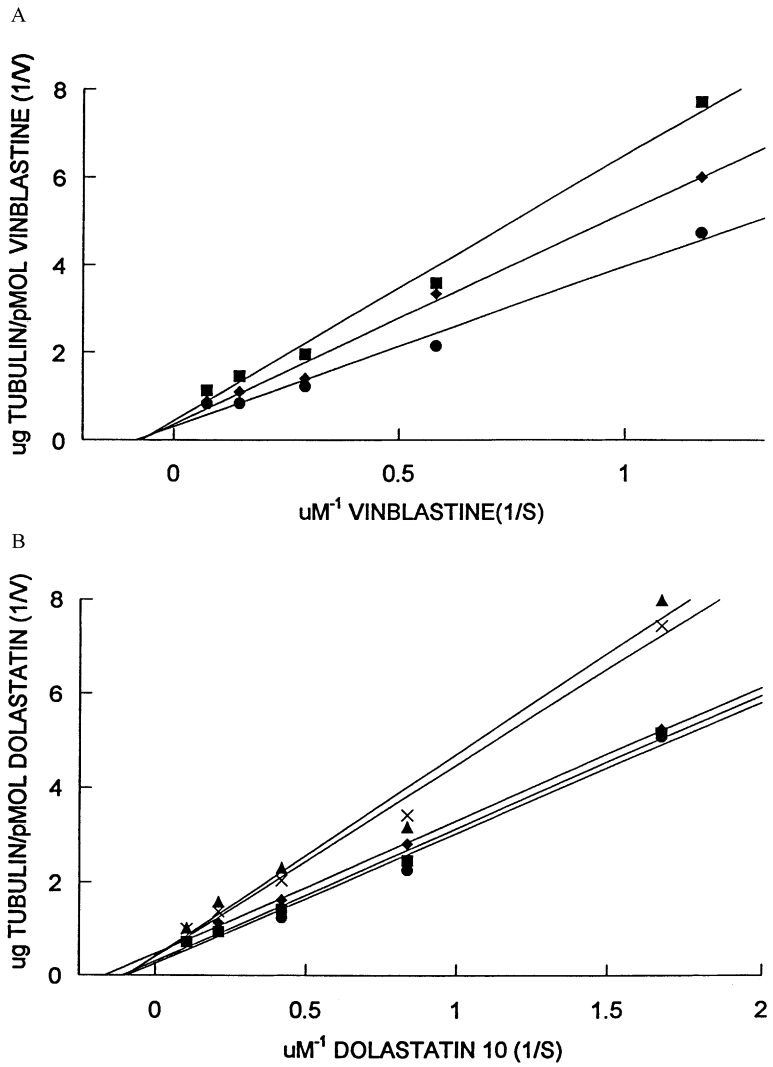

Fig. 4. Effect of vitilevuamide on vinblastine or dolastatin 10 binding to tubulin. Each 0.5 mL reaction contained 0.1 M MES, 0.5 mM $MgCl_2$, 1.0 mg/mL of tubulin, 2% (v/v) DMSO, the indicated concentration of [^{3}H]vinblastine (A) or [^{3}H]dolastatin (B), and the indicated concentration of vitilevuamide summarized below. Tubulin was added last in every experiment and allowed to incubate at 37° for 45 min. Reaction mixtures were then added to 1 mL microspin columns of bio-gel P-30 and processed as described previously [28,29]. The following concentrations of vitilevuamide in panel A were used: (●) none; (◆) 1.56 μM; and (■) 3.4 μM. The following concentrations of vitilevuamide in panel B were used: (●) none; (■) 3.1 μM; (◆) 6.2 μM; (×) 7.8 μM; and (▲) 15.8 μM. All lines were drawn by linear regression, and R^2 values for all fits were above 0.96. Each regression line represents data from a minimum of three individual experiments.

Since all known agents that bind in the vinca domain also prevent GTP exchange on tubulin [32], the effects of vitilevuamide on the binding of radiolabeled GTP to tubulin were studied in comparison to vinblastine and hemiasterlin.

Vinblastine is the least active in this respect; maytansine, spongistatin 1, dolastatin 10, and phomopsin A are some of the most active [32]. Fig. 5 illustrates the percentage of GTP bound per microgram of tubulin in the presence of vitilevuamide, vinblastine, and hemiasterlin. Vitilevuamide inhibited GTP binding only weakly, even at concentrations as high as 80 μM. This was similar to vinblastine, but not as active as hemiasterlin.

Vitilevuamide did not inhibit the binding of colchicine to tubulin. Many tubulin active drugs, especially those that bind to the vinca domain, actually strengthen the binding of colchicine to tubulin and can prevent the natural decay of the colchicine–tubulin complex [30–32]. Vitilevuamide prevented the loss of colchicine binding activity when tubulin was preincubated with the drug for 3 hr prior to colchicine, as did vinblastine and hemiasterlin (Table 1). Without a preincubation, we did not observe the enhancement of colchicine binding described by others [30–32].

3.5. ILS of mice with lymphocytic leukemia

Mice injected with P388 lymphocytic leukemia cells that were treated with vitilevuamide lived longer than mice receiving no treatment. Vitilevuamide was able to produce a highly significant 70% ILS in mice treated with 30 μg/kg per dose (Table 2). At doses of 12 and 6 μg/kg per dose,

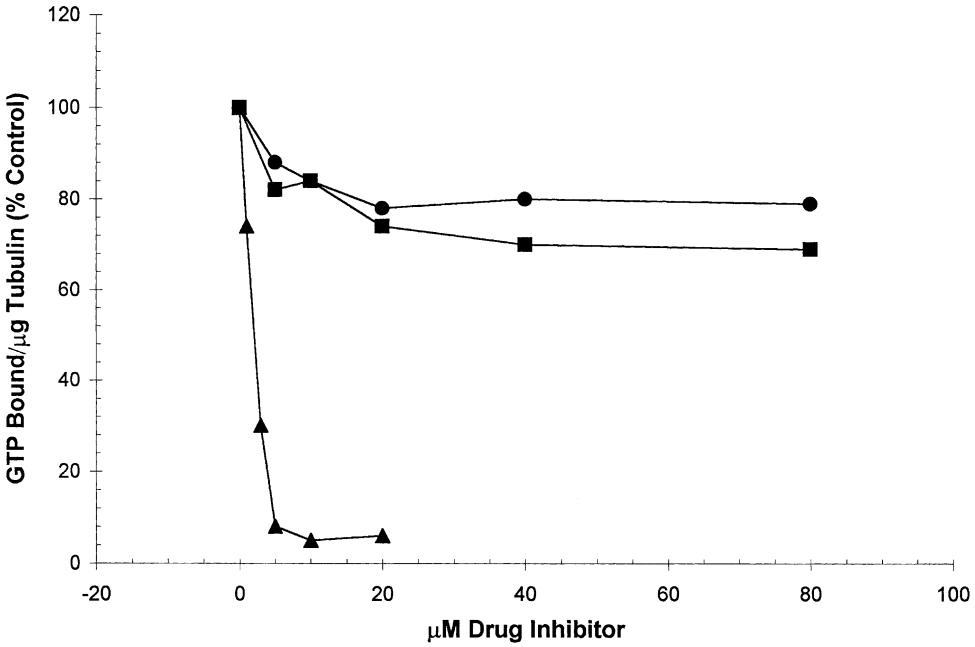

Fig. 5. Inhibitory effects of vitilevuamide, hemiasterlin, and vinblastine on the binding of radiolabeled GTP to tubulin. Each 0.5 mL reaction mixture contained 0.5 mg/mL (5 μM) tubulin, 50 μM [8-^{3}H]GTP, 0.1 M MES (1 M stock solution adjusted to pH 6.9 with NaOH), 0.5 mM $MgCl_2$, 2% (v/v) DMSO, and the indicated concentration of inhibitor ((●) vinblastine; (■) vitilevuamide; (▲) hemiasterlin). Incubation was for 10 min at 4°. 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 [29,30]. Protein and radioactivity in the filtrates were quantified. Values were then normalized to the control reaction mixtures.

Table 1
Stabilization of colchicine binding activity of tublin by vitilevuamide, vinblastine, and hemiasterilin[a]

Drug	[^{3}H]Colchicine (pmol bound/pmol tublin)	
	Not preincubated	Preincubated
None	0.22	0.09
Hemiasterilin	0.24	0.22
Vinblastine	0.21	0.19
Vitilevuamide	0.20	0.20

[a] Each 0.1 mL reaction mixture contained 0.4 mg/mL of tublin, 0.1 M MES (pH 6.9), 0.1 M EDTA, 1 mM GTP, 0.5 mM $MgCl_2$, 1 mM 2-mercaptoetheanol, 1 mM EGTA, 5% DMSO, 60 μM [^{3}H]colchicine, and the indicated drug at 50 μM. If indicated, the reaction mixtures were preincubated for 3 hr at 37° prior to the addition of the [^{3}H]colchicine. Incubation was for 2 hr at 37°, after the addition of [^{3}H]colchicine. The data presented in the table represent average values obtained in two independent experiments, each of which contained triplicate samples.

Table 2
Antitumor activity of vitilevuamide against murine P388 lymphocytic leukemia

Dose (μg/kg per dose)[a]	ILS_{max} (%)[b]
130	−45 (toxic)
60	−13 (toxic)
30	70
12	20
6	8

[a] P388 lymphocytic leukemia cells (1×10^6 per mouse) were inoculated i.p. on day 0. Drug was injected on days 1, 5, and 9.

[b] ILS_{max}, maximal increase in life-span over control.

vitilevuamide also showed an ILS of 20 and 8%, respectively. At higher doses, vitilevuamide was too toxic and actually decreased the life-span of these mice. These *in vivo* experiments demonstrated that vitilevuamide is active as an antineoplastic agent.

4. Discussion

In the present study, it was shown that vitilevuamide is cytotoxic to several human tumor cell types and CHO cells in culture. The LC_{50} for cell cytotoxicity was in the range of 6–311 nM. Subsequent experiments have shown that this toxicity results from the ability of vitilevuamide to bind and inhibit tubulin polymerization. Using FACS and cell cycle analysis, it was found that vitilevuamide produced a selective accumulation of CHO cells in the G_2/M phase of the cell cycle. In cultures treated for 16 hr with 15 μM vitilevuamide, 51.6% of the cells accumulated in G_2/M, compared with 17.0% in controls. The increase in G_2/M cells was accompanied by a decrease in G_0/G_1 cells. The results of the FACS analysis indicated that vitilevuamide inhibited cell cycle progression at mitosis. Further analysis needs to be performed to determine the mitotic index of cells treated with vitilevuamide.

Based on its ability to disrupt microtubule function in rat glioma cells, we investigated whether vitilevuamide could directly affect microtubule polymerization. In a system using isolated bovine brain microtubule protein consisting of tubulin and microtubule-associated proteins (MAPs), we

found that vitilevuamide inhibited microtubule assembly *in vitro*. The IC_{50} concentration was approximately 2 μM. This value was similar to those reported for dolastatin 10 (1.2 μM), phomopsin A (1.4 μM), and vinblastine (1.5 μM) [8,17,30], even though the literature values were determined in a slightly different glutamate-induced polymerization system. Vitilevuamide was a more potent inhibitor of tubulin polymerization than maytansine (3.4 μM), rhizoxin (6.9 μM), or halichondrin B (7.2 μM).

Vitilevuamide is a new member of the group of drugs that inhibit vinca alkaloid binding to tubulin. With [^{3}H]vinblastine as the substrate, kinetic analysis revealed that this inhibition was non-competitive. It has previously been shown that the natural products dolastatin 10, halichondrin B, phomopsin A, spongistatin 1, and cryptophycin 1 are also non-competitive inhibitors of the vinca alkaloid binding site, while the macrolides maytansine and rhizoxin inhibit in a competitive fashion [8,13,30,34]. Our results suggest that vitilevuamide binds to a site distinct from, but in close proximity to, the vinca site known as the vinca domain. Dixon analysis revealed an apparent K_i of 1.8 μM for vitilevuamide inhibition of [^{3}H]vinblastine binding. This compares well to other K_i values reported for other non-competitive inhibitors of vinblastine binding. Vitilevuamide is a slightly weaker inhibitor than spongistatin 1 (K_i 1.3 μM) [13] and stronger than halichondrin B (K_i 5.0 μM) [8]. The competitive inhibitor maytansine (K_i 0.9 μM) [8] is a stronger inhibitor, while vincristine (K_i 1.8 μM) [13] is about equal.

The effect of vitilevuamide on dolastatin 10, another tubulin-binding marine peptide with anticancer potential, was probed. Our results show that vitilevuamide inhibited dolastatin binding only at concentrations that induced tubulin aggregation. It is possible that an inhibitory binding relationship exists between vitilevuamide and dolastatin 10; however, experimental conditions allowing aggregation prevented its detection. Thus, no clear relationship for inhibition of dolastatin 10 binding to tubulin could be determined. Bai *et al.* have shown that spongistatin 1 non-competitively inhibits the binding of both vinblastine and dolastatin to tubulin [13], while cryptophycin 1 competitively inhibits the binding of dolastatin 10 to tubulin [34]. There exists a similarity in the cyclic ring size of spongistatin 1 and vitilevuamide. It may be possible that vitilevuamide binds in the distinct site where spongistatin 1 binds. However, until we can obtain a radiolabeled form of spongistatin or vitilevuamide, this cannot be determined.

Like phomopsin A, dolastatin 10, and vinblastine, vitilevuamide enhanced colchicine binding to tubulin, although vitilevuamide required a preincubation period in order to exhibit this. It is a property common to drugs that modulate the vinca alkaloid domain of tubulin. All agents that prevent the time-dependent decay of colchicine binding to tubulin (vinblastine, dolastatin 10, phomopsin A, and cryptophycin 1) have also been shown to induce tubulin aggregation. Vitilevuamide falls within this class, producing tubulin aggregation at high concentrations, as seen by light microscopy. The exact nature of these aggregates is unclear, and further investigation is needed to determine if the aggregates are similar to those produced by dolastatin 10 or by cryptophycin 1.

Binding of GTP to tubulin is weakly inhibited by vitilevuamide, vinblastine, and cryptophycin 1 [35], but is strongly inhibited by other natural products such as hemiasterlin, spongistatin 1, phomopsin A, and dolastatin 10. Further studies will determine if preincubation of vitilevuamide with tubulin will enhance its inhibition of GTP binding, as seen with cryptophycin 1 [34]. Our data suggest that vitilevuamide binds tubulin close to the so-called ''peptide site'' within the vinca domain, and that the precise interaction may be unique from other tubulin active compounds.

The potent cytotoxic profile of vitilevuamide made it a viable candidate for use *in vivo*. Results obtained in mice harboring P388 lymphocytic leukemia suggest that vitilevuamide does indeed have anticancer potential. Vitilevuamide was potent in its ability to increase the life-span of leukemic mice, and that warrents further *in vivo* testing.

We conclude that vitilevuamide is a structurally unique cytotoxic marine metabolite that inhibits tubulin polymerization as its mechanism of action. Like many of the complex groups of natural products, vitilevuamide inhibits the binding of vinca alkaloids to tubulin and nucleotide exchange (although weakly). It was shown that vitilevuamide does not inhibit colchicine binding but rather stabilizes it when preincubated with tubulin. Competitive binding assays determined that vitilevuamide interacts with tubulin at a site that is distinct from vinblastine, colchicine, dolastatin 10, or GTP. Unfortunately, vitilevuamide is structurally complex, preventing easy chemical synthesis. Also, the low yield of vitilevuamide obtained from the ascidians in which it is found makes large-scale collection inefficient. However, the isolation of vitilevuamide from two different genera of tunicates suggests the possibility that it is the product of a microbial symbiont. If this is the case, vitilevuamide could be produced in fermentation culture, and may form the basis for a new class of anticancer agents.

Acknowledgments

We would again like to thank R.-D. Haugwitz and D.-J. Newman for providing us with dolastatin 10. We would also like to thank Ernest Hamel for helpful suggestions in interpreting our data.

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