Cancer Chemother Pharmacol (2001) 48: 29–36
DOI 10.1007/s002800100287

ORIGINAL ARTICLE

Richard A. Isbrucker · Sarath P. Gunasekera
Ross E. Longley

Structure-activity relationship studies of discodermolide and its semisynthetic acetylated analogs on microtubule function and cytotoxicity

Received: 20 September 2000 / Accepted: 21 January 2001 / Published online: 20 April 2001

Abstract *Purpose*: Discodermolide, a natural product from the marine sponge *Discodermia dissoluta*, has been previously described as an antimitotic agent with microtubule hyperstabilizing properties similar to those of paclitaxel (Taxol). The clinical success of paclitaxel has led to a growing interest in novel antimitotic compounds and the elucidation of their structure-activity characteristics. Analogs of discodermolide were prepared by acetylation of the hydroxyl groups at carbons 3, 7, 11 and/or 17 and tested for biological activity in human tumor cells to determine the structural requirements for tubulin interaction and cytotoxic effects. *Methods*: A549 human lung adenocarcinoma cells were incubated with discodermolide, or its acetylated analogs, and examined for their effects on microtubule architecture, cytotoxicity, and perturbations of the cell cycle. To confirm their direct interaction with tubulin, analogs were assayed for their ability to induce the polymerization of purified bovine brain tubulin. *Results*: Acetylation of discodermolide at the C-7 hydroxyl group potentiated the cytotoxicity of the molecule to A549 cells, whereas acetylation at the C-3 hydroxyl group had little effect on the cytotoxicity of the parent or C-7-acetylated compounds. The acetylation of the hydroxyl groups at the C-11 and C-17 positions severely abrogated the cytotoxicity of the molecule. Cell cycle analysis by flow cytometry revealed that the more cytotoxic analogs caused the accumulation of cells in the G_2/M phase, a mechanism previously reported for discodermolide. All discodermolide analogs with IC_{50} values below 1000 n*M* exhibited microtubule effects to varying degrees in cultured A549 cells, yet only the most cytotoxic promoted the polymerization of purified tubulin. *Conclusions*: Although the parent compound was more effective at polymerizing purified tubulin, acetylation of the C-3 or C-3 and C-7 hydroxyl groups improved its cytotoxicity in whole cells suggesting that acetylation either enhances accumulation of the molecules within cells or imparts a secondary cytotoxic quality not present in the discodermolide molecule. The study reported here is the first to provide information on the structure-activity relationships of discodermolide using human tumor cells and analogs produced by semisynthetic modification of natural discodermolide.

Keywords Discodermolide · Paclitaxel · Tubulin · Structure-activity relationship

This research was supported by a grant from the National Institutes of Health (grant ROICA74227-02) to R.E.L.

R. A. Isbrucker · S. P. Gunasekera · R. E. Longley (✉)
Division of Biomedical Marine Research,
Harbor Branch Oceanographic Institution,
5600 US1 North, Fort Pierce, FL 34946, USA
E-mail: longley@hboi.edu
Tel.: +1-561-4652400
Fax: +1-561-4651523

Introduction

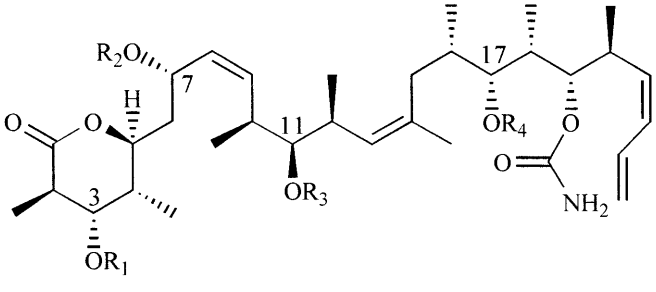
Marine organisms are proving to be a novel and rich source of bioactive compounds [7]. Sedentary marine species, such as soft corals or sponges, have evolved potent chemical defenses to act as antifouling agents and to ward off competitors and predators. Investigations into these marine natural products have proven that many are not general cytotoxic agents but rather are targeted towards specific cellular or biochemical events and therefore hold a strong potential as antimicrobial, anticancer or antiinflammatory agents. Recently, several marine-derived compounds have been described to be specific inhibitors [3, 5, 14, 27] or promoters [17, 22, 26] of tubulin polymerization. Discodermolide, a polyhydroxylactone from the sponge *Discodermia dissoluta*, was initially characterized as a potential immunosuppressive agent [9, 18, 19]. Later, the compound was reported to be a potent antimitotic agent [20] which promotes the hyperstabilization of the microtubule complex [26] – both known mechanisms of the anticancer agent paclitaxel [25].

The success of paclitaxel in clinical trials and in recent oncological use has clearly demonstrated the viability of

targeting microtubule function as a pharmaceutical means of solid tumor cancer treatment. Despite its success as an anticancer agent, the use of paclitaxel has been limited by undesirable side effects, poor solubility, and its ineffectiveness towards P-glycoprotein-expressing multidrug resistance cancers [6, 24], thereby necessitating the need for more efficacious and novel microtubule interactive agents.

Binding of discodermolide and paclitaxel to tubulin dimers in vitro is mutually exclusive, with discodermolide showing a greater affinity for tubulin and displacing radiolabelled paclitaxel from tubulin [12, 15]. Although these two compounds have similar cytotoxicities in some human cancer cell lines [15, 26] and share mechanisms of antimitotic activity, they do not share any readily recognizable chemical homology and diverge in some of their biological effects. Initial studies have shown that paclitaxel-resistant cells remain highly susceptible to the actions of discodermolide [15], and, unlike paclitaxel [21], discodermolide does not induce nitric oxide production (Zhang and Longley, personal communication). These early results suggest additional mechanisms of action of paclitaxel that are not associated with discodermolide.

With the aim of further investigating the mechanism of action of discodermolide in vitro, we conducted the first study of the structure-activity relationships of discodermolide and eight acetylated analogs using human tumor cells. Discodermolide analogs were prepared by the acetylation of the hydroxyl groups at carbons 3, 7, 11, and 17 in the parent compound (Fig. 1). This led to the production of discodermolide-3,7,11,17-tetraacetate (D-3,7,11,17-OAc), -3,7,17-triacetate (D-3,7,17-OAc), -3,7,11-triacetate (D-3,7,11-OAc), -3,7-diacetate (D-3,7-OAc), -3,11-diacetate (D-3,11-OAc), -3,17-diacetate (D-3,17-OAc), -3-acetate (D-3-OAc), and -7-acetate (D-7-OAc). We compared the effects of these discodermolide derivatives with those of paclitaxel in terms of (1) their cytotoxicity to A549 cells, (2) their ability to arrest the proliferation of A549 cells at the G_2/M phase of the cell cycle, (3) their effects on microtubule architecture in A549 cells, and (4) their hyperstabilization of purified bovine brain tubulin.



	R_1	R_2	R_3	R_4
Discodermolide	H	H	H	H
D-3-OAc	$COCH_3$	H	H	H
D-7-OAc	H	$COCH_3$	H	H
D-3,7-OAc	$COCH_3$	$COCH_3$	H	H
D-3,11-OAc	$COCH_3$	H	$COCH_3$	H
D-3,17-OAc	$COCH_3$	H	H	$COCH_3$
D-3,7,11-OAc	$COCH_3$	$COCH_3$	$COCH_3$	H
D-3,7,17-OAc	$COCH_3$	$COCH_3$	H	$COCH_3$
D-3,7,11,17-OAc	$COCH_3$	$COCH_3$	$COCH_3$	$COCH_3$

Fig. 1 Chemical structures of discodermolide and analogs

Materials and methods

Isolation of discodermolide

The lithistid sponge *Discodermia* sp. was collected by a manned submersible from a deep island slope of the tropical western Atlantic Ocean and stored at –20°C until extraction. The wet sponge was soaked in ethanol and the concentrated ethanol extract partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction was chromatographed over silica gel with a methanol/dichloromethane ($MeOH/CH_2Cl_2$) gradient and fractions monitored by thin layer chromatography (TLC) for discodermolide. The fractions that showed the presence of discodermolide were combined for further purification. These combined fractions were further purified by HPLC (SiO_2, 5 μm, 250×10 mm) with 5.5% $MeOH/CH_2Cl_2$ and gave discodermolide as a white solid. Crystallization of the white solid from 5% $MeOH/CH_2Cl_2$ produced colorless crystals of pure discodermolide (yield 0.001% of wet weight). The structure was confirmed by TLC, HPLC, and NMR comparison with that of an authentic discodermolide sample.

Preparation and purification of discodermolide acetates

Discodermolide (16.0 mg) was dissolved in dry pyridine (2.5 ml), treated with acetic anhydride (20 μl) in a Reacti-vial and stirred with the temperature maintained at 15–18°C. The reaction mixture was monitored by TLC to yield the greatest number of analogs and the reaction was halted by adding ice. The solvents were evaporated under a stream of nitrogen and the residue was subjected to HPLC on a SiO_2 gel (5 μm, Phenomenex Semi-prep Lichrosorb) column using a mixture of 6% MeOH in CH_2Cl_2 to yield a mixture of less-polar acetates, a mixture of polar acetates, and traces of unreacted discodermolide. The less-polar acetate mixture was re-chromatographed using the same HPLC system with 3% MeOH in CH_2Cl_2 to give pure D-3,7,11,17-OAc, D-3,7,17-OAc, D-3,7,11-OAc, D-3,7-OAc, D-3,11-OAc, and D-3,17-OAc. Similarly, re-chromatography of the polar fraction under the same conditions with 5% MeOH in CH_2Cl_2 gave pure D-3-OAc and D-7-OAc. The acetates were identified by mass spectral data and their structures were confirmed by extensive one- and two-dimensional NMR studies. These results have been presented elsewhere [10].

Cell culture

A549 cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in RPMI-1640 tissue culture medium (TCM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 60 μg/ml L-glutamine, 18 m*M* HEPES, 0.05 mg/ml gentamicin, and 10% fetal bovine serum (Life Technologies, Gaithersburg, Md.). The cell line was cultured in plastic tissue culture flasks and kept in an incubator at 37°C in humidified air containing 5% CO_2. A549 cells were subcultured 1:20 every 3rd or 4th day using a 0.5% trypsin, 0.53 m*M* EDTA solution (Life Technologies) and used within 20 passages of the initial stock culture.

Cytotoxicity assay

The mitochondrial metabolism of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) to its insoluble, blue, formazan was used for enumerating A549 cells to assess the antiproliferative effects of discodermolide and its analogs [1]. Briefly, A549 cells were seeded into 96-well tissue culture plates (Nunc, Denmark) at a density of 6×10^3 cells/well in TCM and allowed to adhere overnight. Cells were incubated with serial dilutions of discodermolide, or its analogs, diluted in TCM. Positive drug controls were included to monitor drug sensitivity of each of the cell lines. These included various dilutions of 5-fluorouracil and doxorubicin (Sigma Chemical Co., St Louis, Mo.). After 72 h exposure, 75 µl of warm phosphate-buffered saline (PBS) containing 5 mg/ml MTT was added to each well; cultures were returned to the incubator, and left undisturbed for 3 h. To quantitate the formation of the resulting reduced formazan spectrophotometrically, culture fluids were removed by aspiration, and 200 µl of acidified isopropanol (2 ml concentrated HCl/liter isopropanol) added per well. The absorbance of the resulting solution was measured at 570 nm with a plate reader (TECAN Spectra II Plate Reader, TECAN U.S., Research Triangle Park, N.C.) and a 650 nm reference filter. A linear relationship between cell numbers and formazan production has been routinely observed over the range of cell densities used in these experiments. The concentration of the agent causing 50% cytotoxicity (IC_{50}) and the 95% confidence intervals were calculated by nonlinear regression of log-transformed data (Prism, version 3.00; GraphPad Software, San Diego, Calif.). Four to six wells of each of a minimum of seven concentrations were used in the derivation of the IC_{50}.

Flow cytometry

A549 cells were transferred into each well of a six-well culture plate at a density of 5×10^5 cells/well and allowed to adhere overnight at 37°C in an atmosphere containing 5% CO_2. The TCM was removed from each well and replaced with fresh TCM containing discodermolide or its analogs and incubated for an additional 24 h. The TCM was transferred to a centrifuge tube, the cells trypsinized and pooled with their TCM and centrifuged. All centrifugations for this assay were at 800 *g* at 4°C for 5 min. The supernatant was removed, the cells resuspended in cold 80% ethanol in PBS, and fixed for 1 h at –20°C. The fixed cells were centrifuged and washed twice in cold PBS, resuspended in 1 ml propidium iodide staining solution (PBS containing 0.02 mg/ml propidium iodide, 0.1 mg/ml ribonuclease A; Sigma) and incubated in a 37°C water bath for 30 min. The cell suspensions were strained through 70 µm filters (Becton Dickinson; Franklin Lakes, N.J.) and analyzed on a Coulter EPICS ELITE flow cytometer (Beckman Coulter Corporation, Miami, Fl.) with excitation at 488 nm and emission at 690 nm. Approximately 1×10^4 cells were analyzed and used to construct DNA histograms for each analog of discodermolide.

Microtubule staining in cultured cells

Sterile glass coverslips were placed in each well of a six-well tissue culture plate and seeded with 7×10^4 A549 cells/well. The cells were allowed to adhere overnight at 37°C in an atmosphere containing 5% CO_2. The TCM was removed and replaced with fresh TCM containing 10–1000 n*M* discodermolide or its analogs and incubated for an additional 24 h. Coverslips containing the adhered cells were fixed in 3.7% formalin in PBS for 10 min at room temperature and then permeabilized in 2% Triton X-100 (Sigma) in PBS for 5 min. The coverslips were rinsed twice in PBS and incubated with murine monoclonal anti-α-tubulin (Sigma) (1 µg/ml in PBS) at room temperature for 45 min with intermittent shaking. The primary antibody was removed and replaced with FITC-conjugated goat anti-mouse IgG (F_{ab} specific; 1 µg/ml in PBS; Sigma). The cells were incubated in the dark at room temperature for 45 min with intermittent shaking. The coverslips were washed three times in distilled water, air-dried and mounted on slides with SlowFade (Molecular Probes, Eugene, Ore.). Cells were observed under an epifluorescence microscope (Olympus America, Melville, N.Y.).

Tubulin polymerization

The polymerization of purified bovine brain tubulin (Cytoskeleton, Denver, Colo.) was measured using a modification of the conditions established by Asnes and Wilson [2]. The polymerization was monitored by changes in the optical density of tubulin solutions at 350 nm in a Hitachi U-3010 spectrophotometer equipped with an electronic thermostatted cell holder. Stock solutions of tubulin were diluted on ice in cold G-PEM buffer (1 m*M* GTP, 80 m*M* PIPES, 1 m*M* EGTA, 0.5 m*M* magnesium chloride; pH 6.8) to a final concentration of 1 mg/ml. The instrument was zeroed with this solution at 4°C. Test compounds, or an equivalent volume of ethanol, were then quickly mixed into the tubulin solution to a final concentration of 10 µ*M* and the absorbance continually monitored over a 61-min period. Within this time the temperature of the thermoelectric cell holder was increased at a rate of 1°C/min to 35°C, reduced back to 4°C at a rate of 2°C/min, and held at 4°C for an additional 15 min. Purified tubulin in the absence of microtubule-associated proteins (MAPs) or drugs did not assemble under these test conditions.

Results

Cytotoxicity of discodermolide analogs

Cytotoxicity studies of discodermolide and its analogs were conducted using the A549 human lung adenocarcinoma cell line to elucidate initial structure-activity relationships of the pharmacophore (Table 1). Analogs acetylated only at the hydroxyls of C-3 or C-3 and C-7 showed equal or greater cytotoxicity towards A549 cells compared to the parent compound. The inclusion of acetoxy groups at either C-11 or C-17 greatly decreased the cytotoxicity of the molecule despite the presence of acetoxy moieties at positions C-3 and/or C-7. The relative order of cytotoxicities of the compounds towards A549 human lung cancer cells was D-7-OAc = D-3,7-OAc > D-3-OAc = paclitaxel = discodermolide > D-3,11-OAc > D-3,7,11-OAc = D-3,17-OAc > > D-3,7,17-OAc = D-3,7,11,17-OAc.

Table 1 Cytotoxicity of paclitaxel, discodermolide and discodermolide analogs in A549 human lung adenocarcinoma cells as determined by MTT metabolism following 72 h exposure to the test agent. Values are the IC_{50} (n*M*) and the 95% confidence interval derived from six to eight concentrations. Each value is representative of a single experiment which was repeated a minimum of two times

Test agent	IC_{50}
Paclitaxel	4.1 (3.4–4.9)
Discodermolide	3.5 (1.6–7.2)
D-3-OAc	3.8 (1.2–12.6)
D-7-OAc	0.8 (0.3–1.9)
D-3,7-OAc	0.8 (0.4–1.5)
D-3,11-OAc	164 (80–334)
D-3,17-OAc	524 (410–640)
D-3,7,11-OAc	545 (410–726)
D-3,7,17-OAc	> 1000
D-3,7,11,17-OAc	> 1000

Perturbation of cell cycle by discodermolide analogs

Previous results have shown that discodermolide blocks the proliferation of cultured cells at the G_2/M phase of the cell cycle [20]. Discodermolide completely blocked the proliferation of A549 cells at 100 n*M* (Fig. 2). When discodermolide analogs were tested for this effect only those compounds acetylated at C-3 and/or C-7 arrested cells at the G_2/M phase at this same concentration (Fig. 2). The remaining analogs had no effect on the distribution of cells in the cell cycle at 100 n*M* (results not shown), but it is important to note that these analogs all had IC_{50} values towards A549 cells that were greater than 100 n*M* (Table 1).

Effects on microtubule morphology in A549 cells

The effects of discodermolide analogs on microtubule architecture in A549 cells were investigated in order to determine if this was a mechanism of their activity. Figure 3 shows the morphological changes induced by 100 n*M* paclitaxel, discodermolide, or the discodermolide analogs on the microtubule matrix of A549 cells. Paclitaxel, discodermolide, D-3-OAc, D-7-OAc and D-3,7-OAc induced microtubule bundling in A549 cells at 100 n*M*, and these effects were easily detected at concentrations as low as 10 n*M* (not shown). Discodermolide and D-3-OAc induced comparable morphological changes when tested at equivalent concentrations whereas D-7-OAc and D-3,7-OAc induced a more pronounced bundling morphology in cells when tested at 10 or 100 nM. D-3,11-OAc, D-3,17-OAc, and D-3,7,11-OAc did not induce bundling at 100 n*M* although some evidence of altered cytoskeletal arrangement was observed, such as the presence of cells with multiple aster formations (Fig. 3G, H, I). Also, the nuclear degradation characteristic of apoptosis was observed in these three cultures. Cells exposed to 1000 n*M* D-3,17-OAc showed a clear indication of microtubule bundling, although this was not extensive (not shown). D-3,7,17-OAc and D-3,7,11,17-OAc had no observable effect on the microtubule matrix at 100 nM, but at 1000 nM the D-3,7,17-OAc did induce multiple aster formations in some cells, whereas D-3,7,11,17-OAc had no observable effect.

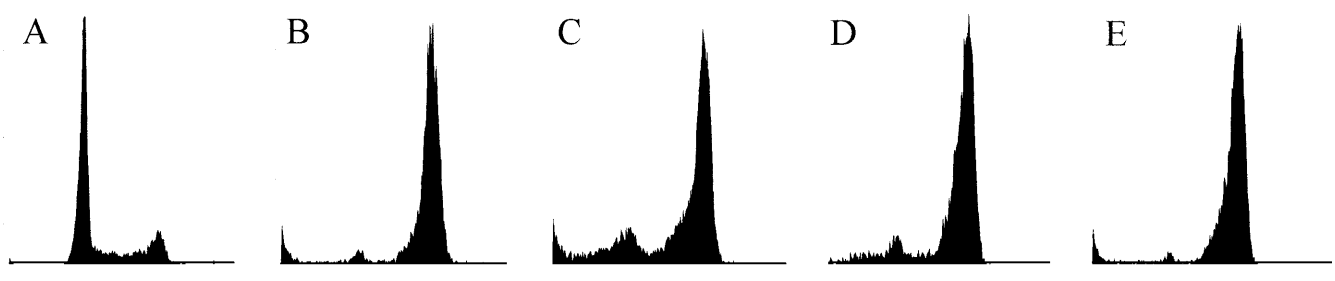
Induction of purified bovine brain tubulin polymerization

Monitoring the polymerization characteristics of purified bovine brain tubulin in the presence of paclitaxel, discodermolide or discodermolide analogs allowed the effect of these compounds on microtubules to be determined in the absence of cellular barriers or microtubule control mechanisms. All compounds were tested at 10 μ*M* with 1 mg/ml purified bovine brain tubulin. This high concentration of test agent was based on the observation that the maximum assembly of tubulin by paclitaxel occurs at approximately equimolar concentrations of drug and tubulin [16]. Under these test conditions only discodermolide, D-3-OAc, D-7-OAc, D-3,7-OAc, and paclitaxel were able to induce tubulin assembly (Fig. 4). The remaining analogs did not induce the polymerization of tubulin. The subsequent addition of discodermolide to these otherwise negative solutions resulted in the rapid assembly of tubulin thereby ensuring that the assay conditions remained viable (not shown).

Discodermolide-induced polymerization of tubulin in vitro has previously been shown to occur rapidly at temperatures as low as 4°C [26]. We confirmed this finding and demonstrated that a rapidly induced polymerization could occur in the absence of microtubule-associated proteins or glycerol (Fig. 4). On the other hand, temperature-controlled experiments show that paclitaxel-induced polymerization of tubulin does not readily occur below 17°C. D-3-OAc, D-7-OAc, and D-3,7-OAc induced little or no tubulin polymerization at 4°C but exhibited strong turbidity effects at temperatures above 6°C. As a group, these three analogs appeared to have similar, or greater, activity in this assay than did paclitaxel. Although all samples were tested at equimolar concentrations and volume of tubulin, discodermolide-induced polymerization consistently yielded a greater change in absorbance than did paclitaxel or discodermolide analog.

To study the stabilizing activity of these compounds, the cuvette temperature was returned to 4°C as the turbidity was continually monitored (Fig. 4). The subsequent stability of the tubulin complexes appeared to

Fig. 2A–E Cell cycle analysis by flow cytometry of A549 cells exposed to 100 n*M* discodermolide, its analogs, or paclitaxel, for 24 h. Histograms represent samples of approximately 1×10^4 cells per compound tested and are plotted as cell number (*y*-axis) vs fluorescence intensity (*x*-axis). **A** 0.05% ethanol (vehicle control); **B** discodermolide; **C** D-3-OAc; **D** D-7-OAc; **E** D-3,7-OAc



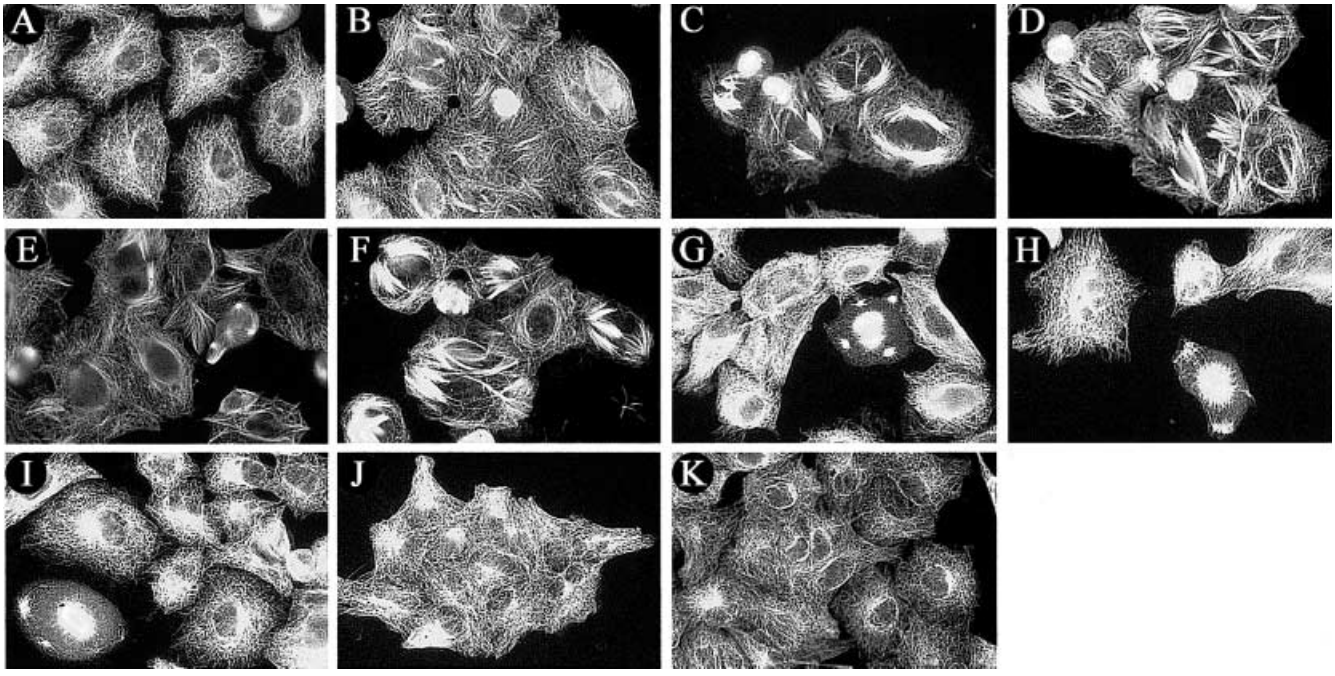


Fig. 3A–K Immunofluorescence images of A549 cells stained with anti-α-tubulin. Cells were exposed to 100 n*M* compounds for 24 h prior to fixing and staining. **A** 0.05% ethanol (vehicle control); **B** paclitaxel; **C** discodermolide; **D** D-3-OAc; **E** D-7-OAc; **F** D-3,7-OAc; **G** D-3,11-OAc; **H** D-3,17-OAc; **I** D-3,7,11-OAc; **J** D-3,7,17-OAc; **K** D-3,7,11,17-OAc

coincide with the temperature at which polymerization was induced. Paclitaxel and D-3,7-OAc-stabilized microtubules showed a rapid decrease in turbidity at 4°C, whereas complexes formed in the presence of D-3-OAc or D-7-OAc were more stable. Discodermolide-stabilized structures were not affected by changes in temperature.

Discussion

The cytotoxicity study reported here has confirmed and extended the previous work indicating the sensitivity of cultured mammalian cells to discodermolide [4, 20, 26]. From the order of potency of discodermolide analogs in A549 cells it is apparent that the addition of acetyl moieties at either C-11 or C-17 severely abrogates the cytotoxicity of the pharmacophore, whereas alterations closer to the lactone ring augment this activity. IC_{50} data of these same discodermolide analogs towards the murine P388 macrophage cell line produced a relative order of potency comparable to that in A549 human tumor cells [10]. Also, our results coincide with the T-cell proliferation-inhibiting effects of synthetic discodermolide analogs as reported by Hung et al. [13]. These authors reported that D-17-OAc is approximately ten times less active than discodermolide and that 17-*epi*-discodermolide was completely inactive in their assay.

However, the results presented here also indicate that the cytotoxic potential of discodermolide and its analogs

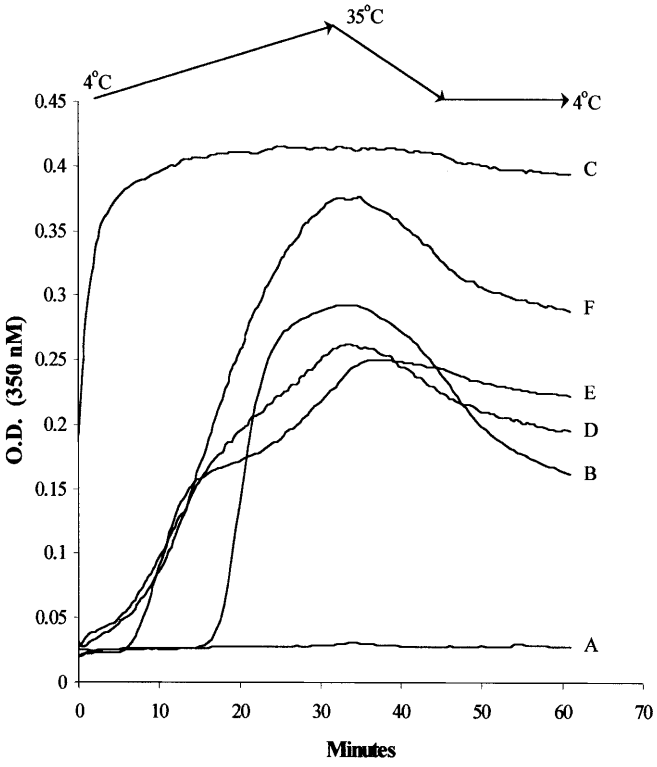


Fig. 4 Polymerization of 1 mg/ml purified bovine brain tubulin in G-PEM buffer induced by 10 μ*M* of paclitaxel (B), discodermolide (*C*), D-3-OAc (*D*), D-7-OAc (*E*), or D-3,7-OAc (*F*). Vehicle control (*A*) and the remaining discodermolide analogs all had similar baseline results. The arrows above the graph indicate the temperature of the cuvette holder throughout the assay. Each curve represents a single assay, which was repeated at least twice for verification

do not necessarily reflect their tubulin-polymerizing characteristics. Although D-3-OAc, D-7-OAc and D-3,7-OAc are equally or more cytotoxic to A549 cells than discodermolide, none of these analogs is able to induce the polymerization of purified tubulin at low temperatures (4°C) and the maximal polymer absorbance value at 35°C was consistently less than that routinely observed with discodermolide. This latter observation could indicate either the incomplete association of tubulin monomers into the microtubule structures, or that the optical characteristics of microtubules formed is different between discodermolide and its acetylated analogs. These possibilities are currently being investigated. Once the microtubule structures were assembled in the presence of the test agents, they showed a dissociation upon a reduction of temperature to 4°C. Even at temperatures below those required for paclitaxel- or analog-induced assembly, the microtubule dissociation rate was consistently slower than the rate of assembly. As previously reported [26], the assembly of the microtubule structure from purified bovine brain tubulin in the presence of discodermolide appears to be independent of temperature within the temperature range tested. This observation of discodermolide-induced tubulin polymerization at low temperatures suggests the hypothesis that discodermolide may act as an antimitotic agent in its colder, indigenous, marine environment.

Discodermolide has been previously demonstrated to arrest cells in the G_2/M phase of the cell cycle [20]. Analysis of cell cycle effects of the discodermolide analogs on A549 cells revealed antimitotic effects similar to those of discodermolide and of analogs acetylated at positions C-3 and/or C-7, but not of any bearing C-11 or C-17 acetylations. It should be noted that these samples were tested only at a concentration of 100 n*M*, and that all analogs containing the C-11 or C-17 acetoxy groups had IC_{50} values greater than this concentration.

Morphological changes to the microtubule matrix observed in A549 cells exposed to the analogs acetylated at positions C-3 and/or C-7 demonstrate the ability of these analogs to induce similar, or more extensive, in situ bundling of microtubules than does the parent compound, even at concentrations as low as 10 n*M*. A hallmark feature of altered microtubule function is the development of aberrant, or multiple, aster body formations in cells entering the early stages of mitosis [15]. We have found that these structures provide a highly sensitive indicator for the presence of microtubule-altering compounds as they occur at concentrations below which microtubule bundling is difficult to observe by epifluorescence microscopy. Using this visual indicator of activity it became apparent that the analogs acetylated at positions C-11 and/or C-17 retained some low, but measurable, tubulin-interacting properties despite their low cytotoxicity or apparent lack of effect on the distribution of cells within the cell cycle at the concentrations tested. Only the tetra-acetylated analog was inactive in all assays conducted here, even in cells exposed to concentrations as high as 1000 n*M*. From these results it is apparent that the primary mechanism of cytotoxic action of D-3-OAc, D-7-OAc and D-3,7-OAc is through the hyperstabilization of the microtubule complex and the subsequent inhibition of normal mitotic spindle function.

The differences in the analog-induced polymerizing characteristics in whole cell microtubules versus those formed by purified tubulin suggest that the analogs interact with the tubulin proteins in a fashion different from that of discodermolide. D-3-OAc, D-7-OAc and D-3,7-OAc may require the presence of other factors, or non-tubulin proteins, in order to function fully as microtubule-hyperstabilizing agents in whole cells.

It is also plausible that these analogs are deacetylated in cultured cells to produce discodermolide and that acetyl groups at positions C-11 or C-17 may not be susceptible to the actions of the deacetylases. Therefore, the inability to correlate cytotoxicity with tubulin-hyperstabilizing qualities must be due to the complex regulatory mechanisms of microtubule polymerization processes within a cell and its high sensitivity to endogenous or exogenous factors which were not reflected in our purified tubulin-polymerizing assay. The increased cytotoxic potency of D-7-OAc and D-3,7-OAc could also indicate that these compounds are either more cell-membrane permeable, or that the acetylation at the C-7 hydroxyl group imparts additional cytotoxic qualities to the compounds – factors that are important in determining the potential effectiveness of novel compounds as anticancer agents.

The inability of the remaining five analogs to induce the polymerization of purified tubulin at a concentration of 10 μ*M* clearly demonstrates that acetylation of the hydroxyl groups at the C-11 and/or C-17 positions of the discodermolide molecule dramatically reduces the affinity of the compound for tubulin and that their decreased effectiveness against whole cells is not necessarily due to the presence of any cellular barriers. In contrast to this, acetylation of the hydroxyl groups at the C-3 and C-7 positions, which lie closer to the substituted lactone functionality, confers greater cytotoxicity to the molecule but through a process which may not be directly related to its ability to induce the polymerization of tubulin. Nonetheless, our study shows that chemical modifications (protection by acetylation reactions) involving the central region (C-11, C-17) of the discodermolide molecule serve to impair its biological activities, indicating the potential importance of these hydroxyl moieties in the overall antimitotic activity of the compound. These results, coupled with the potentiation of biological activities of the modified C-3 and C-7 sites, serve to provide us with important information regarding the structure-activity relationship of the discodermolide molecule and a particular insight into the determination of the active pharmacophore of the compound.

Three models of a common tubulin-interacting pharmacophore have recently been proposed based,

primarily, on structure-activity relationship studies of taxane and epothilone variants [8, 11, 23]. In contrast to the relatively rigid ring structures of paclitaxel and epothilone with few low-energy conformations, the linearity of the discodermolide molecule, with only two isolated double bonds in the middle of the molecule to contribute to its relative rigidity, makes the molecule relatively flexible and readily adaptable to these proposed low-energy conformations. Since acetylation of hydroxyl groups would have only a small impact on the final flexibility of the discodermolide analogs, the data presented here provide support particularly to the identification of functional groups involved in the active pharmacophore. Further studies examining discodermolide analogs of varied structural flexibility are currently being conducted.

Our results provide no indication as to the role of the terminal diene structure in the activity of discodermolide, although other authors have reported a significant decrease in potency with the addition of an extended pivaloyl group at C-24 [13]. Alteration of functional groups associated with, or close to, the lactone structure does not appear to greatly reduce discodermolide activity. Thiophenylation at C-1 has no effect on the potency of the pharmacophore [13], thereby eliminating a direct role for that carbonyl oxygen in either the bonding activity or stereochemistry of discodermolide. Although acetylation at the C-3 or C-7 position changes the potential reactivity of these functional groups, their stereo placement is not affected during synthesis and their overall influence on the conformation of the discodermolide pharmacophore is unlikely to be strong.

The results presented here clearly illustrate the involvement of the C-11 and C-17 hydroxyl groups in the biological activity of discodermolide, in agreement with the previous findings of Hung et al. [13]. Interestingly, these authors reported that acetylation at C-17 reduces the cytotoxic effects of discodermolide tenfold, whereas epimerization of the C-17 hydroxyl group renders the final molecule inactive. Together, these results indicate the role of the C-17 hydroxyl group in maintaining the favorable stereochemistry of the pharmacophore, possibly through a hydrogen bonding with the neighboring carbamate group.

These results will, hopefully, allow us to design simple analogs with potent biological activities similar to that of discodermolide, and have provided important clues into the structure-activity relationship between the discodermolide molecule and its interaction with tubulin.

Acknowledgements The authors wish to acknowledge Ms. Patricia Linley for providing technical assistance in performing the A549 cytotoxicity assays. This is Harbor Branch Oceanographic Institution contribution No. 1396.

References

1. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 48:589
2. Asnes CF, Wilson L (1979) Isolation of bovine brain microtubule protein without glycerol: polymerization kinetics change during purification cycles. Anal Biochem 98:64
3. Bai RL, Paull KD, Herald CL, Malspeis L, Pettit GR, Hamel E (1991) Halichondrin B and homohalichondrin B, marine natural products binding in the vinca domain of tubulin. Discovery of tubulin-based mechanism of action by analysis of differential cytotoxicity data. J Biol Chem 266:15882
4. Balachandran R, ter Haar E, Welsh MJ, Grant SG, Day BW (1998) The potent microtubule-stabilizing agent (+)-discodermolide induces apoptosis in human breast carcinoma cells – preliminary comparisons to paclitaxel. Anticancer Drugs 9:67
5. Blokhin AV, Yoo HD, Geralds RS, Nagle DG, Gerwick WH, Hamel E (1995) Characterization of the interaction of the marine cyanobacterial natural product curacin A with the colchicine site of tubulin and initial structure-activity studies with analogues. Mol Pharmacol 48:523
6. Casazza AM, Fairchild CR (1996) Paclitaxel (Taxol) mechanisms of resistance. Cancer Treat Res 87:149
7. Fenical W (1997) New pharmaceuticals from marine organisms. Trends Biotechnol 15:339
8. Giannakakou P, Gussio R, Nogales E, Downing KH, Zaharevitz D, Bolluck B, Poy G, Sackett, Nicolaou KC, Fojo T (2000) A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells. Proc Natl Acad Sci U S A 97:2904
9. Gunasekera SP, Gunasekera M, Longley RE, Schulte GK (1991) Discodermolide: a new bioactive polyhydroxylated lactone from the marine sponge, *Discodermia dissoluta*. J Org Chem 55:4912 (correction J Org Chem 56:1346, 1991)
10. Gunasekera SP, Longley RE, Isbrucker RA (2001) Acetylated analogues of the microtubule stabilizing agent discodermolide: preparation and biological activity. J Nat Prod 64:171
11. He L, Jagtap PG, Kingston DGI, Shen HJ, Orr GA, Horwitz SB (2000) A common pharmacophore for Taxol and the epothilones based on the biological activity of a taxane molecule lacking a C-13 side chain. Biochemistry 30:3972
12. Hung DT, Chen J, Schreiber SL (1996) (+)-Discodermolide binds to microtubules in stoichiometric ratio to tubulin dimers, blocks Taxol binding and results in mitotic arrest. Chem Biol 3:287
13. Hung DT, Nerenberg JB, Schreiber SL (1996) Synthesis of discodermolides useful for investigating microtubule binding and stabilization. J Am Chem Soc 118:11054
14. Koiso Y, Morita K, Kobayashi M, Wang W, Ohyabu N, Iwasaki W (1996) Effects of arenastatin A and its synthetic analogs on microtubule assembly. Chem Biol Interact 102:183
15. Kowalski RJ, Giannakakou P, Gunasekera SP, Longley RE, Day BW, Hamel E (1997) The microtubule-stabilizing agent discodermolide competitively inhibits the binding of paclitaxel (Taxol) to tubulin polymers, enhances tubulin nucleation reactions more potently than paclitaxel, and inhibits the growth of paclitaxel-resistant cells. Mol Pharmacol 52:613
16. Kumar N (1981) Taxol-induced polymerization of purified tubulin. J Biol Chem 256:10435
17. Long BH, Carboni JM, Wasserman AJ, Cornell LA, Casazza AM, Jensen PR, Lindel T, Fenical W, Fairchild CR (1998) Eleuthrobin, a novel cytotoxic agent that induces tubulin polymerization, is similar to paclitaxel (Taxol). Cancer Res 58:1111
18. Longley RE, Caddingan D, Harmody D, Gunasekera M, Gunasekera SP (1991) Discodermolide – a new, marine-derived immunosuppressive compound. I. In vitro studies. Transplantation 52:650
19. Longley RE, Caddingan D, Harmody D, Gunasekera M, Gunasekera SP (1991) Discodermolide – a new, marine-derived immunosuppressive compound. II. In vivo studies. Transplantation 52:656

20. Longley RE, Gunasekera SP, Faherty D, Mclane J, Dumont F (1993) Immunosuppression by discodermolide. Ann N Y Acad Sci 696:94
21. Manthey CL, Perera PY, Salkowski CA, Vogel SN (1994) Taxol provides a second signal for murine macrophage tumoricidal activity. J Immunol 152:825
22. Mooberry SL, Tien G, Hernandez AH, Plubrukarn A, Davidson BS (1999) Laulimalide and isolaulimalide, new paclitaxel-like microtubule-stabilizing agents. Cancer Res 59:653
23. Ojima I, Chakravarty S, Inoue T, Lin S, He L, Horwitz SB, Kuduk SD, Danishefsky SJ (1999) A common pharmacophore for cytotoxic natural products that stabilize microtubules. Proc Natl Acad Sci U S A 96:4256
24. Sackett D, Fojo T (1997) Taxanes. Cancer Chemother Biol Response Modif 17:59
25. Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci U S A 77:1561
26. ter Haar E, Kowalski RJ, Hamel E, Lin CM, Longley RE, Gunasekera SP, Rosenkranz HS, Day BW (1996) Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than Taxol. Biochemistry 35:243
27. Turner T, Jackson WH, Pettit GR, Wells A, Kraft AS (1998) Treatment of human prostate cancer cells with dolastatin 10, a peptide isolated from a marine shell-less mollusc. Prostate 34:175