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Tubulin polymerizing activity of dictyostatin-1, a polyketide of marine sponge origin

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Abstract

Dictyostatin-1 had previously been isolated from a marine sponge of the genus *Spongia* sp. and described as a cytotoxic agent to murine and human cancer cells, but its mechanism of activity was unknown. In a routine screening assay used to detect cytotoxic compounds of marine origin, dictyostatin-1 was identified as a highly active component in an extract from a Lithistida sponge and exploration into its pharmacology was pursued. Initial studies demonstrated that dictyostatin-1 arrested cells in the G_2/M phase of the cell cycle. Staining of these cells with antitubulin revealed cells having multiple aster formations and microtubule matrix bundling patterns similar to that seen in cells exposed to paclitaxel. Dictyostatin-1 was able to induce the polymerization of purified bovine brain tubulin *in vitro* and the polymerized tubulin remained stable at cold temperatures. Dictyostatin-1 also proved to be highly potent in two paclitaxel-resistant human cancer cell lines expressing active P-glycoprotein. Together, these results indicate that dictyostatin-1 is a potent inducer of tubulin polymerization and retains activity in cells expressing the P-glycoprotein efflux pump.

Keywords: Dictyostatin-1; Induction of tubulin polymerization; Cytotoxic; Antimitotic; P-glycoprotein efflux pump; G_2/M block

1. Introduction

Normal cell division, intracellular transport, cellular motility, cell signaling, and maintenance of cell shape are all dependent on the highly regulated dynamic instability process of the tubulin/microtubule system [1]. The microtubule complex has thus proven to be an important target in the development of anticancer therapeutic agents, such as the vinca alkaloids and paclitaxel. Paclitaxel (Taxol®), a natural product isolated from the Pacific yew tree, *Taxus brevifolia*, is a microtubule interactive agent which has demonstrated significant clinical success in the treatment of breast, lung, and ovarian cancers [2,3]. The mechanism of action of paclitaxel has been well documented and includes the premature polymerization of tubulin resulting in hyperstable microtubule formation, inhibition of cellular proliferation at the G_2/M phase of the cell cycle, mitotic spindle disorganization, and cell death [4].

Despite the clinical successes of paclitaxel and its semisynthetic derivative docetaxel (Taxotere®), the use of these compounds have been limited by poor solubility, undesirable side effects, low oral bioavailability, and limited effectiveness towards P-glycoprotein (P-gp) expressing multidrug-resistant cancers. These incongruities have emphasized the necessity for more efficacious and novel microtubule interactive agents.

Marine organisms are rapidly proving to be a rich source of bioactive secondary metabolites with potential anticancer properties. Recently, eleutherobin [5] from the soft coral *Eleutherobia* sp.; sarcodictyins [6,7] from the stoloniferan coral *Sarcodictyon roseum*; laulimalide [8] from the sponge *Cacospongia mycofijiensis*; and discodermolide [9,10] from the sponge *Discodermia dissoluta*, have all been reported to have tubulin hyperstabilizing properties similar to that of

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Abbreviations: G-PEM, GTP/PIPES/EGTA/magnesium chloride buffer; MAP, microtubule-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P-gp, P-glycoprotein; TCM, tissue culture medium; MPLC, medium pressure liquid chromatography; NMR, nuclear magnetic resonance.

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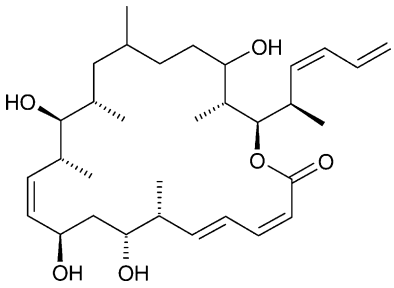

Fig. 1. Structure of dictyostatin-1.

paclitaxel. Although these compounds share little obvious chemical similarities, their shared mechanism of action, as well as their mutually exclusive binding properties with paclitaxel, has led several researchers to establish models of a tubulin-interacting pharmacophore [11–13].

In 1994 a novel macrocyclic lactone, dictyostatin-1 (Fig. 1), derived from a Republic of Maldives marine sponge in the genus *Spongia* sp., was reported to strongly inhibit the growth of a selection of cell lines in the US National Cancer Institute human cancer cell line panel [32], as well as murine P388 lymphocytic leukemia cells [14]. Despite the favorable activity of dictyostatin-1 against cultured human cancer cells, no further research has been reported on this compound. Recently, Shin *et al.* [15] reported on the synthesis of discodermolide analogs with structures similar to that of dictyostatin-1, but the mechanism of action of dictyostatin-1 was not investigated. As part of our on-going search for novel antitumor agents, dictyostatin-1 emerged as a potent cytotoxic agent derived from a Lithistida sponge of the family Corallistidae collected off the North Jamaican Coast. We report here the mechanism of cytotoxic activity to be similar to that of paclitaxel.

2. Materials and methods

2.1. Collection of sponge source material

A sample of Corallistidae (Phylum: Porifera, Class: Demospongiae, Order: Lithistida, Family: Corallistidae) was collected using the Johnson Sea Link manned submersible at a depth of 442 m off the North Jamaican Coast (latitude 18°28.64′N, longitude 78°10.00′W). The sponge morphology is that of a stalked cup with a smooth surface and a warty-lumpy underside, stony in consistency, and white in color. A reference sample preserved in ethanol has been deposited in the Harbor Branch Oceanographic Museum (catalog number 003:00959, DBMR number 23-VIII-93-5-008) and is available for taxonomic evaluation.

2.2. Isolation of dictyostatin-1

Two hundred grams of the frozen Corallistidae sponge was extracted exhaustively with ethanol. After concentration by distillation under reduced pressure, the residue was partitioned between *n*-butanol and water. After solvent removal, the *n*-butanol partition was chromatographed by vacuum column chromatography on a silica gel 60H (EM Science) stationary phase using a step gradient of ethyl acetate in heptane as eluent. The fractions which were eluted with heptane/ethyl acetate (25:75 v/v) and pure ethyl acetate contained dictyostatin-1. Dictyostatin-1 was further purified by MPLC using a 1 cm × 25 cm Altex column custom packed at HBOI using a bulk Vydac Protein and Peptide C18 stationary phase (20–30 μm particle size). The column was eluted with a step gradient of acetonitrile in water as follows: fractions 2–24 eluted with water/acetonitrile (8:2 v/v); fractions 25–50 eluted with water/acetonitrile (6:4 v/v); fractions 51–75 eluted with water/acetonitrile (4:6 v/v); fractions 76–80 eluted with 100% acetonitrile. Flow rate was maintained at 5 mL/min. Fractions were collected dropwise with a total of 350 drops per fraction (volume ∼10 mL). Fractions 57 and 58 were essentially pure dictyostatin-1 (5.7 mg, 0.0028% of wet weight). The planar structure of dictyostatin-1 was defined by one- and two-dimensional NMR spectroscopy and confirmed by comparison of the data with that published for dictyostatin-1 [14].

2.3. Cell culture

A549 human lung adenocarcinoma, MCF-7 and NCI/ADR-RES (formerly MCF-7/ADR) human breast cell lines were obtained from the American Type Culture Collection. The MES-SA and MES-SA/DX5 human uterine cell lines were purchased from the DCTDC Tumor Repository, NCI-Frederick Cancer Research and Development Center. All cell lines were maintained in RPMI-1640 tissue culture medium (TCM) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 60 μg/mL L-glutamine, 18 mM HEPES, 0.05 mg/mL gentamicin, and 10% fetal bovine serum (Life Technologies). The cell lines were cultured in plastic tissue culture flasks and kept in an incubator at 37° in humidified air containing 5% CO_2. All cell lines were subcultured 1:20 every third or fourth day using a 0.5% trypsin, 0.53 mM EDTA solution (Life Technologies) and used within 20 passages of the initial stock culture.

2.4. 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 cells to assess the antiproliferative effects of dictyostatin-1 according to the methods of Alley *et al.* [16]. Briefly, cells were seeded into 96-well tissue culture plates (Nunc) at a density of 6×10^3 cells/well in TCM and allowed to adhere overnight. Cells were incubated with serial dilutions of dictyostatin-1 or paclitaxel in TCM. Positive drug controls were included to monitor the drug sensitivity of each of the cell lines. These included varying dilutions of 5-fluorouracil and doxorubicin (Sigma Chemical Co). After 72-hr expo-

sure, 75 μL of warm PBS containing 5 mg/mL MTT was added to each well; cultures were returned to the incubator, and left undisturbed for 3 hr. To quantitate formation of the resulting reduced formazan spectrophotometrically, culture fluids were removed by aspiration, and 200 μL of acidified isopropanol (2 mL concentrated HCl/L isopropanol) added per well. The absorbance of the resulting solution was measured at 570 nm with a plate reader (TECAN U.S.) and 650 nm reference filter. A linear relationship between cell number 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). A minimum of seven concentrations, each comprising of 4–5 wells, was used in the derivation of the IC_{50}.

2.5. Flow cytometry

A549 cells were transferred into each well of a 6-well culture plate at a density of 5×10^5 cells/well and allowed to adhere overnight at 37°, 5% CO_2. The culture medium was removed from each well and replaced with fresh medium containing dictyostatin-1 or paclitaxel, and incubated for an additional 24 hr. The medium was transferred to a centrifuge tube, the cells trypsinized, pooled with their medium, and centrifuged. All centrifugations for this assay were at 800 *g*, 4° for 5 min. The supernatant was removed, the cells re-suspended in cold 80% ethanol in PBS, and fixed for 1 hr at −20°. The fixed cells were centrifuged and washed twice in cold PBS, re-suspended in 1 mL of propidium iodide staining solution (PBS containing 0.02 mg/mL propidium iodide, 0.1 mg/mL ribonuclease A; Sigma) and incubated in a 37° water bath for 30 min. The cell suspensions were strained through 70 μm filters and analyzed on a Coulter EPICS ELITE flow cytometer (Beckman Coulter Corp.) 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 compound.

2.6. Mircotubule staining in cultured cells

Sterile glass cover slips were placed in each well of a 6-well tissue culture plate and seeded with 7×10^4 A549 cells/well. The cells were allowed to adhere overnight at 37°, 5% CO_2. The culture medium was removed and replaced with fresh medium containing 10, 100, or 1000 nM dictyostatin-1 or paclitaxel and incubated for an additional 24 hr. Cover slips 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 cover slips were rinsed twice in PBS and incubated with 1 μg/mL murine monoclonal anti-α-tubulin (Sigma) in PBS at room temperature for 45 min with intermittent shaking. The primary antibody was removed and replaced with 1 μg/mL FITC-conjugated goat anti-mouse IgG (F_{ab} specific; Sigma). The cells were incubated in the dark at room temperature for 45 min with intermittent shaking. The cover slips were rinsed twice in PBS and incubated with 1 mL of propidium iodide staining solution and incubated in a 37° incubator for 30 min. The cover slips were then gently washed three times in distilled water, air-dried, and mounted on slides with SlowFade™ (Molecular Probes). Cells were observed under epifluorescence and confocal microscopy (Olympus America Inc.).

2.7. Tubulin polymerization

The polymerization of purified bovine brain tubulin (Cytoskeleton Inc.) was measured using a modification of the conditions established by Asnes and Wilson [17]. The polymerization was monitored by changes in the optical density of tubulin solutions at 350 nm in a spectrophotometer equipped with an electronic thermostatted cell holder (Hitachi Instruments, Inc.). Stock solutions of tubulin were diluted on ice in cold G-PEM buffer (1 mM GTP, 80 mM PIPES, 1 mM EGTA, 0.5 mM magnesium chloride; pH 6.8) to a final concentration of 1 mg/mL. The instrument was zeroed with this solution at 4°. Test compounds, or an equivalent volume of ethanol, were then quickly mixed into the tubulin solution to a final concentration of 10 μM 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°/min to 35°, reduced back to 4° at a rate of 2°/min, and held at 4° for an additional 15 min. Purified tubulin without microtubule-associated proteins (MAPs) or drugs did not assemble under these test conditions.

3. Results

3.1. Chemistry

Bioassay-guided fractionation of a crude ethanol extract of a deep water sponge of the family Corallistidae collected in Jamaica, led to the isolation and purification of a potent cytotoxic agent. One- and two-dimensional NMR studies, as well as comparison to published data, identified the active component as the known metabolite dictyostatin-1 (Fig. 1). The full stereochemical assignment of dictyostatin-1 has not yet been achieved and work is on going to provide crystals suitable for X-ray crystallographic analysis, as well as to conduct molecular modeling studies coupled to high field NMR experiments in which crucial overlapping resonances can be resolved.

3.2. Cytotoxicity of dictyostatin-1

Dictyostatin-1 and paclitaxel had comparable cytotoxic IC_{50} values towards A549, MCF-7, and MES-SA cells,

Table 1
Cytotoxicity of dictyostatin-1 and paclitaxel in cultured human cancer cells as determined by MTT metabolism following 72-hr exposure to the test agent

Cell line	IC_{50} (nM)	
	Dictyostatin-1	Paclitaxel
A549	0.95 ± 0.25*	5.13 ± 2.9
MCF-7	1.5 ± 0.9	2.5 ± 0.7
NCI/ADR-RES	20 ± 4.2 (13×)**	3331 ± 652 (1332×)
MES-SA	4.1 ± 1.4	3.3 ± 0.6
MES-SA/DX5	11 ± 2.4 (3×)	1654 ± 230 (501×)

* Values are means ± SD from a minimum of four separate experiments.
** Values in brackets are fold resistance, indicating the reduced potency of the compound in the resistant cell lines.

ranging from 1 to 5 nM after 72-hr exposure (Table 1). Two additional cell lines, NCI/ADR-RES and MES-SA/DX5, were also chosen for this assay as they had previously been characterized with a multidrug-resistant phenotype [18–20]. These cell lines exhibited a strong resistance of 1332- and 500-fold towards paclitaxel, respectively. In contrast, dictyostatin-1 had only a small reduction in potency and the IC_{50} remained at 20 nM or less. This represents a 13-fold resistance in the NCI/ADR-RES cell line and only a 3-fold reduction in cytotoxicity in the MES-SA/DX5 cells.

3.3. Perturbation of cell cycle by dictyostatin-1

Cell cycle analysis by flow cytometry revealed that dictyostatin-1 induced a strong G_2/M block and accumulation of A549 cells in the S phase at concentrations as low as 10 nM (Fig. 2). A large increase of sub-G_1 population was also observed, indicative of cells undergoing active apoptosis. Paclitaxel induced similar changes at 10 nM, but these were not as extensive as those produced by dictyostatin-1. At concentrations of 100 nM or higher the effects of dictyostatin-1 and paclitaxel were indistinguishable from each other as both produced a nearly complete arrest and accumulation of cells in the G_2/M phase.

3.4. Effects on microtubule and nucleus morphology in A549 cells

Dictyostatin-1 promoted the rearrangement of microtubules into bundle formations (Fig. 3) similar to those induced by paclitaxel, although dictyostatin-1 had much more prominent effects at lower concentrations. The percentage of the microtubule matrix condensed into bundles increased with higher concentrations of dictyostatin-1, although there appeared to be little additional bundling at concentrations greater than 100 nM. These bundles were predominantly straight, needle-like structures radiating from near the nucleus towards the periphery of the cell, although at higher concentrations the bundles frequently lay along side the nucleus.

Cells undergoing mitosis frequently exhibited multiple centriolar structures (Fig. 3B) following exposure to dictyostatin-1 at all concentrations tested. The number of centrioles, or asters, seen per mitotic cell generally varied from one to eight although this was frequently difficult to quantify if the centrioles were situated in close proximity

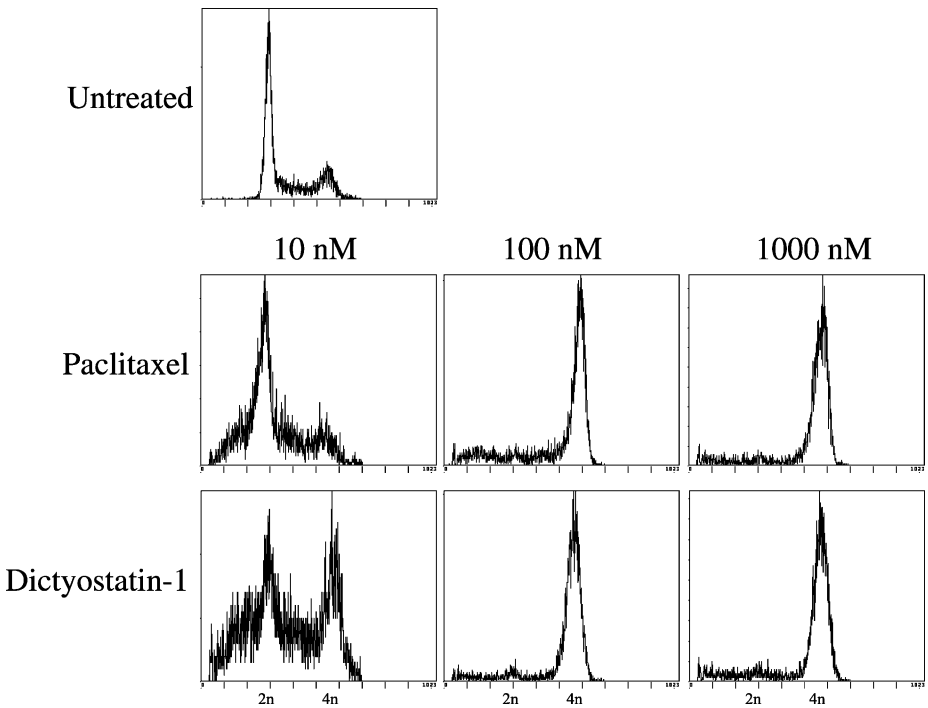

Fig. 2. Cell cycle analysis by flow cytometry of A549 cells incubated for 24 hr with 0.05% ethanol (vehicle control), paclitaxel, or dictyostatin-1. Histograms represent samples of approximately 1×10^4 cells per test and are plotted as cell number (y-axis) vs. fluorescence intensity (x-axis).

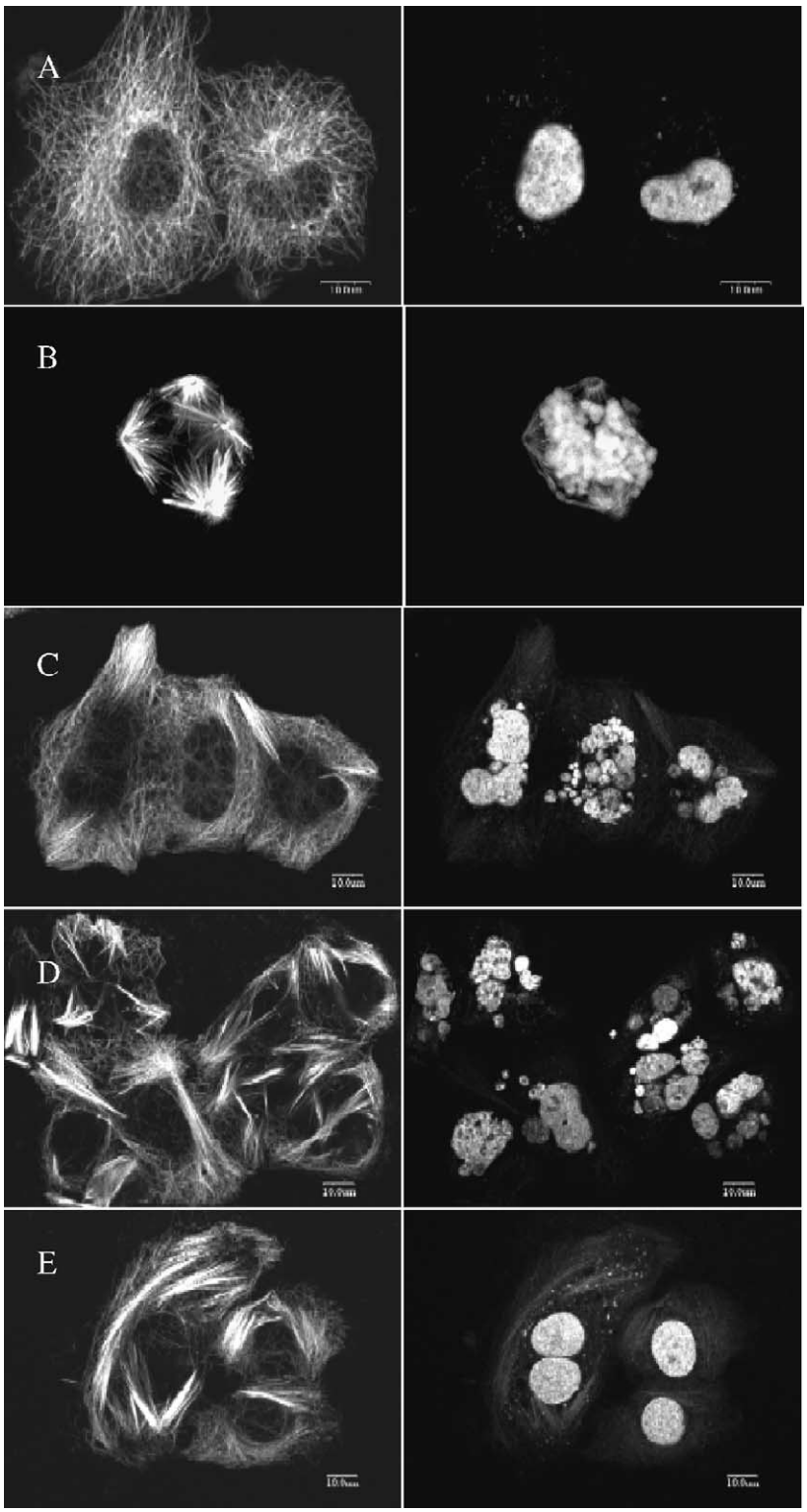

Fig. 3. Immunofluorescence images of A549 cells stained with anti-α-tubulin and propidium iodide and observed by confocal microscopy. Cells were exposed to (A) 0.05% ethanol (vehicle control), or dictyostatin-1 at concentrations of (B) and (C) 10 nM, (D) 100 nM, or (E) 1000 nM. The left and right panels are from a single image but represent the different emission wavelengths from the two different fluorophores used to stain the tubulin or DNA.

to each other. Cells in the mitotic phase of the cell cycle were rounded and had condensed chromosomes which were not arranged along any apparent equatorial plane of division.

Cultures exposed to 10 or 100 nM dictyostatin-1 had a high incidence of cells with nuclear degradation characteristic of active apoptosis. There was little evidence of apoptosis in cells incubated with 1000 nM dictyostatin-1 as

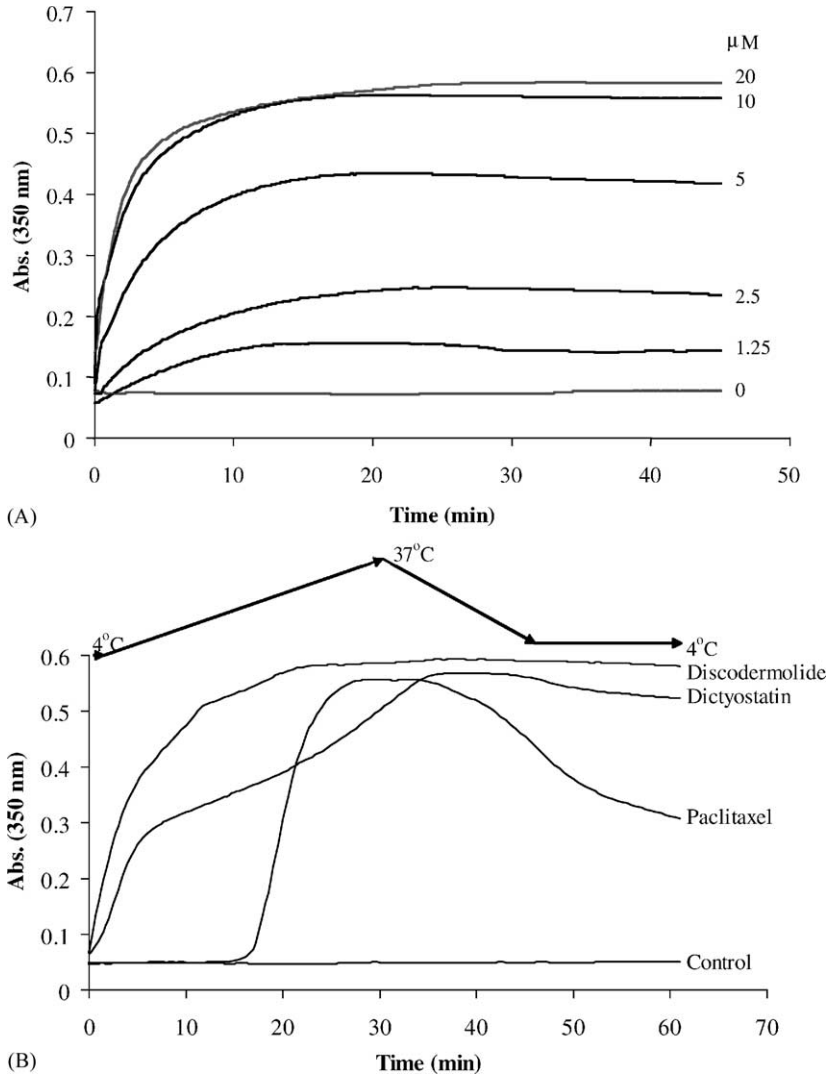

Fig. 4. Polymerization of purified bovine brain tubulin in G-PEM buffer. (A) Concentration-dependent polymerization of tubulin (1 mg/mL) by dictyostatin-1 at 35°. (B) Temperature-dependent tubulin polymerization characteristics of 10 μM dictyostatin-1, discodermolide, or paclitaxel (tubulin concentration, 1 mg/mL). 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.

the nuclei appeared similar in shape to the negative control cultures (Fig. 3E).

3.5. Induction of tubulin polymerization

Dictyostatin-1 induced a rapid polymerization of purified bovine brain tubulin at 35° in a concentration-dependent fashion and in the absence of MAPs or glycerol (Fig. 4A). Maximum tubulin polymerization was attained by 10 μM, which is approximately equimolar to the tubulin concentration. In temperature-controlled experiments, 10 μM dictyostatin-1 promoted microtubule assembly at 4° (Fig. 4B), although this occurred slower than discodermolide-induced polymerization or in experiments which commenced at 35°. In comparison, paclitaxel did not induce tubulin polymerization at temperatures below approximately 17°. Both dictyostatin-1 and paclitaxel induced similar maximum turbidity effects at 10 μM, but the optical density attained by both of these agents was less than that produced in the presence of discodermolide. Tubulin polymers produced in the presence of dictyostatin-1 or discodermolide were much more stable at cold temperatures than those induced by paclitaxel.

4. Discussion

In a routine screen for cytotoxic agents to human cancer cell lines, extracts from a Lithistida marine sponge of the family Corallistidae were found to be highly potent. Bioassay-guided purification led us to the isolation of dictyostatin-1, a macrolide polyketide bearing some similarity in structure to the microtubule stabilizing agent discodermolide. This is the first reported isolation of dictyostatin-1 from a Lithistida sponge and represents a far greater yield of compound than had been reported

previously when it was isolated from a *Spongia* sp. [14]. Dictyostatin-1 had been previously reported as highly cytotoxic to the murine P388 lymphocytic leukemia cell line, as well as in the US National Cancer Institute 60 human tumor cell line screen, but its mechanism of action was unknown. We found similar cytotoxic activity in A549 human lung carcinoma, MCF-7 human breast adenocarcinoma, and MES-SA human uterine sarcoma cell lines.

The cell cycle effects of dictyostatin-1 were investigated as an initial assay to elucidate its potential mechanism of cytotoxicity. These effects were also compared to those of paclitaxel, a known mitotic spindle inhibitor. The accumulation of cells in the G_2/M phase of the cell cycle at all dictyostatin-1 concentrations tested suggested its antiproliferative effects were targeted towards the mitotic apparatus. Aneuploidy and degradation of cellular DNA, such as occurring during active apoptosis, were also observed by flow cytometry: following the treatment of A549 cells with 10 nM dictyostatin-1 there was an increase in the cell population exhibiting fluorescence below the intensity of those bearing a normal complement of DNA. In contrast, high concentrations of dictyostatin-1 did not elicit any apparent apoptotic or aneuploid response and cells accumulated in the G_2/M phase. These effects were highly comparable to those of paclitaxel, as well as to those previously reported for discodermolide [21,22].

Confocal microscopy observation of dictyostatin-1-treated cells stained for α-tubulin confirmed the findings of the cell cycle analysis. Severe microtubule rearrangement indicated that dictyostatin-1 targeted this cytoskeletal structure with morphological effects similar to those induced by paclitaxel, as well as to those we previously reported for discodermolide [23]. Co-staining of these cells with propidium iodide revealed that low concentrations of dictyostatin-1 induced apoptosis, as indicated by the presence of micronuclei bodies, whereas higher concentrations had little effect on nuclear morphology with the exception of an increased incidence of polynucleation. This concentration-dependent mechanism of cytotoxicity has also been described for paclitaxel [24–26] and is presumably due to a minimal fluidity of the microtubule structure necessary for active apoptosis. A greater hyperstabilization of the cytoskeletal architecture at higher concentrations of dictyostatin-1 would disallow even this terminal function.

Some agents which cause the disassembly of microtubules in cultured cells, such as vinca alkaloids and dolastatin, have been shown to increase the turbidity of tubulin solutions through the formation of spiral filaments and ring structures [27,28]. Induction of spiral filament formation by vinca alkaloids only occurs at high concentrations, whereas dolastatin-10 increases optical turbidity of tubulin at 10 μM. Chen and Horwitz [29] recently reported that of the tubulin-interacting compounds only those which hyperstabilize the microtubule structure induce aneuploidy in the absence of mitotic block, whereas microtubule destabilizing compounds, such as vinblastine, do not initiate an aneuploid state among A549 cells. Together, our observations of aneuploidy and microtubule bundling patterns in cells exposed to low concentrations of dictyostatin-1 strongly suggest that the increase in optical density of purified tubulin solution in the presence of dictyostatin-1 is due to the formation of microtubule structures rather than the oligopolymeric aggregates induced by microtubule destabilizing agents. Polymerization studies also verified that the microtubule effects of dictyostatin-1 could occur through a direct interaction with tubulin in the absence of MAPs or other cellular mechanisms regulating the tubulin/microtubule system. The optimum stimulation of tubulin polymerization occurred at a dictyostatin-1 concentration which was equivalent to that of tubulin. Similar findings were previously reported for paclitaxel [4] and discodermolide [30].

Despite the equivalent cytotoxic IC_{50} values of dictyostatin-1 and paclitaxel in the parental cell lines, temperature-controlled experiments revealed that dictyostatin-1 initiated turbidity effects in the cold whereas paclitaxel was ineffective at temperatures below 17°. As had been previously reported for discodermolide [23], the assembly of the microtubule structure from purified bovine brain tubulin in the presence of dictyostatin-1 appears to be independent of temperature within the temperature range tested, although the increase in optical viscosity in colder temperatures was not as rapid as that induced by discodermolide. This observation suggests the hypotheses that dictyostatin-1, as well as the other marine-derived microtubule hyperstabilizing compounds, may act as antimitotic agents in their colder, indigenous marine environment, or that they may prevent the destabilization of the sponge cell microtubule complex during episodes of cold-water currents.

Of particular interest has been the search for potential therapeutics which are effective against cancers that are otherwise refractory towards the effects of paclitaxel. As the expression of the P-gp efflux pump has been strongly correlated with resistance towards paclitaxel [31], we included two resistant cell lines (NCI/ADR-RES (formerly MCF-7/ADR) and MES-SA/DX5) in our screening panel which were known to express P-gp. The resistance of these cells to paclitaxel was confirmed whereas dictyostatin-1 remained highly cytotoxic with only a small increase in the IC_{50}. These results suggest that dictyostatin-1 is either a poor substrate for the efflux activity of P-gp, or its affinity to tubulin is far greater than its affinity to P-gp. The effect of dictyostatin-1 on cells with altered forms of tubulin is currently being explored to determine the efficacy of this compound towards other known mechanisms of paclitaxel resistance.

Despite the similarities in the effects of dictyostatin-1 and paclitaxel, it is as yet unknown whether these compounds are ligands to the same binding site on tubulin, or even if they bind to the same tubulin isoform. Shin *et al.*

[15] synthesized and measured the antiproliferative effects of cyclic discodermolide analogs resembling the structure of dictyostatin-1. Although the latter compound was not included in their testing, the structural similarity between these two marine-derived agents leads to a strong probability that they share a common binding site on tubulin. This research illustrates the growing importance of marine-derived natural products as a novel source of potential therapeutic agents aimed at cancer treatment.

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