Discodermolide, A Cytotoxic Marine Agent That Stabilizes Microtubules More Potently Than Taxol[†,‡]

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ABSTRACT: Computer-assisted structure analysis indicated (+)-discodermolide, a polyhydroxylated alkatetraene lactone marine natural product, was an antimitotic compound, and we confirmed this prediction. Previous work had shown an accumulation of discodermolide-treated cells in the G_2/M portion of the cell cycle, and we have now found that discodermolide arrests Burkitt lymphoma cells in mitosis. Discodermolide-treated breast carcinoma cells displayed spectacular rearrangement of the microtubule cytoskeleton, including extensive microtubule bundling. Microtubule rearrangement that occurred with 10 nM discodermolide required 1 μM taxol. Discodermolide had equally impressive effects on tubulin assembly *in vitro*. Near-total polymerization occurred at 0 °C with tubulin plus microtubule-associated proteins (MAPs) under conditions in which taxol at an identical concentration was inactive. Without MAPs and/or without GTP, tubulin assembly was also more vigorous with discodermolide than with taxol under every reaction condition examined. Discodermolide-induced polymer differed from taxol-induced polymer in that it was completely stable at 0 °C in the presence of high concentrations of Ca^{2+}. In a quantitative assay designed to select for agents more effective than taxol in inducing assembly, discodermolide had an EC_{50} value of 3.2 μM versus 23 μM for taxol.

Antimitotic agents almost universally interact with the major component of microtubules, the heterodimeric protein tubulin, and the most potent of these compounds have been derived from natural sources. In recent years, a wide array of structurally diverse antimitotic agents have been isolated from marine organisms (the dolastatins, halichondrins, spongistatins, and curacins) (Hamel, 1996). In addition to their intrinsic interest, various antimitotic agents have found roles in treatment of human diseases, particularly cancer. The most recently developed clinically useful agents are the taxoids, taxol and taxotere (Rowinsky et al., 1992). Most antimitotic agents inhibit microtubule assembly, but the taxoids have been unusual in that they both enhance polymer stability and hypernucleate microtubule assembly by permitting it to occur under otherwise unfavorable conditions (Schiff et al., 1979; Grover et al., 1995). In this report we describe our discovery that the lactone-bearing, polyhydroxylated alkatetraene (+)-discodermolide[1] (Figure 1), derived from the Caribbean sponge *Discodermia dissoluta* (Gunasekera et al., 1990), inhibits mitosis, induces spectacular microtubule bundles in breast carcinoma cells, and potently induces formation of stable tubulin polymer *in vitro*.

These findings grew out of studies using computational methods to determine the structural basis for inhibition of tubulin polymerization (Ter Haar et al., 1994) and to find new potent inhibitors useful in the treatment of breast cancers (Ter Haar & Day, 1995). These studies involved searching large structural data bases and using our developed quantitative structure−activity relationships to predict antitubulin activity in previously described compounds not known to have this property. Discodermolide was among the compounds predicted to have such a mechanism of action.

The original isolation of discodermolide was bioassay based, and it was found to have apparent immunosuppressive activity (Longley et al., 1991a,b, 1993). Due both to its biological activity and its interesting structure, several approaches to its preparation have appeared (Clark & Heathcock, 1993; Evans et al., 1993; Golec & Gillespie, 1993; Golec & Jones, 1993; Patterson & Wren, 1993; Yang & Myles, 1994a,b), culminating in communications describing syntheses of its (−)- and (+)-enantiomers (Nerenberg et al., 1993; Hung et al., 1994). Of particular interest to us was the potent antiproliferative activity of discodermolide (reported IC_{50} values as low as 6 nM in various cell lines)

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[‡] Dedicated to the memory of Dr. Matthew Suffness.

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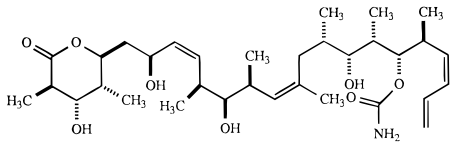
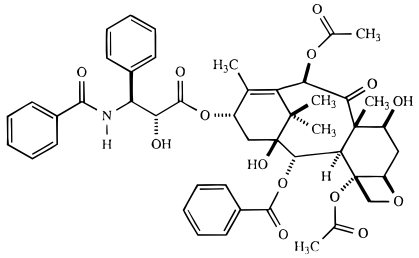


(+)-discodermolide



taxol

FIGURE 1: Structural formulas of (+)-discodermolide and taxol.

with cell cycle arrest at G_2/M (Longley et al., 1991a,b, 1993; Hung et al., 1994).

MATERIALS AND METHODS

Materials. (+)-Discodermolide was isolated from *D. dissoluta* as described previously (Gunasekera et al., 1990). Taxol was generously provided by the Drug Synthesis & Chemistry Branch of the National Cancer Institute. Unless otherwise indicated, both drugs were dissolved in dimethyl sulfoxide, and control reaction mixtures contained solvent equivalent to that in drug-treated cultures or reaction mixtures. Electrophoretically homogeneous tubulin and heat-treated MAPs[2] were prepared from bovine brain (Hamel & Lin, 1984). Tubulin was freed from unbound nucleotide by gel filtration chromatography and reconcentrated as described elsewhere (Grover et al., 1995). All studies with tubulin contained 0.1 M Mes (taken from a 1 M stock solution adjusted to pH 6.9 with NaOH), 4% dimethyl sulfoxide, tubulin at 1 mg/mL (10 μM), and additional components as indicated. Human Burkitt lymphoma CA46 cells were a gift from Dr. P. O'Connor, NCI; MCF-7 human breast carcinoma cells (28th passage) were a gift from Dr. M. Lippman, Georgetown University; and MDA-MB231 human breast carcinoma cells were from the American Type Culture Collection. Culture media and FCS were from Gibco-BRL or Biofluids. Permanox slides were from Nunc, PERMFLUOR mounting fluid from Fisher Scientific, and antibodies and GTP (repurified by triethylammonium bicarbonate gradient anion exchange chromatography) from Sigma Chemical Co.

Inhibition of MCF-7 and MDA-MB231 Cell Growth. Cells were routinely grown in DMEM medium containing phenol red with 5% FCS at 37 °C in a 5% CO_2 humidified atmosphere. The cells were switched to IMEM medium without phenol red containing 10% FCS for seeding/replating 24 h prior to drug treatment. For growth curves of cells continuously exposed to drug, all measurements were made on three independent incubations in blinded triplicate (n = 9). Cells in log phase were seeded in eight-well plates (10^6 cells/well) in 2 mL of IMEM with 10% FCS and allowed to attach for 24 h. The medium was removed, and cells were gently washed with HBSS. IMEM with 5% FCS (2.0 mL) containing varying drug concentrations was added. Every 12 h for 96 h attached cells were trypsinized and pooled with detached cells. Total cells were collected by centrifugation, washed, and reharvested by centrifugation. Viable cell number was determined microscopically on a hemacytometer by trypan blue exclusion. The IC_{50} value was defined as the drug concentration required to inhibit increase in viable cell number by 50%.

Indirect Immunofluorescence. Breast cancer cells (800−2500 cells/40 μL) were plated on sterile, eight-well Permanox cell culture slides in IMEM containing 10% FCS and allowed to attach and grow for 24 h. Medium was removed, and attached cells were treated with varying drug concentrations in IMEM containing 5% FCS at 37 °C. After 48 h, medium was removed and attached cells were fixed by a 10 min treatment with 3% formaldehyde in 10 mM PBS. The cells were further fixed and permeabilized with methanol at −20 °C, air-dried, treated with Triton X-100 in PBS for 20 min, preincubated for 30 min with 2% dry milk in PBS at room temperature, and incubated overnight with 300 μL/well of mouse monoclonal anti-β-tubulin antibody (10−20 μg/mL) at 4 °C. Following a 35 min rinse in PBS containing 2% dry milk, cells were incubated for 4 h at room temperature with 300 μL/well of fluorescein-5-isothiocyanate-conjugated goat anti-mouse IgG_1 (125 μg/mL). Following successive 15 min rinses with PBS containing 2% dry milk and with PBS, slides were wet-mounted in PERMFLUOR.

Inhibition of Burkitt Lymphoma Cell Growth. Cells were grown in 5 mL suspension cultures at 37 °C in a humidified 5% CO_2 atmosphere in RPMI medium supplemented with 0.03% L-glutamine, 16% FCS, 10 μg/mL gentamycin sulfate, 1% dimethyl sulfoxide, and varying drug concentrations. The initial inoculum was 10^5 cells/mL. For determination of IC_{50} values, cells were grown for 24 h and counted in a Coulter counter. The IC_{50} was defined as the drug concentration required to inhibit increase in cell number by 50%. For determination of mitotic index, cells were harvested by centrifugation after 14 h, fixed in methanol, stained with Giemsa, and scored by microscopic examination.

Tubulin Assembly. Polymerization was followed turbidimetrically at 350 nm in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. Baselines were established with the reaction mixtures, containing all components except drug, held at 0 °C by the temperature controller. Drug (or an equivalent amount of dimethyl sulfoxide) was added and mixed into the reaction mixture as quickly as possible, and the reaction was followed as indicated at 0 °C. Temperature was changed as indicated in the individual experiments. Using the electronic temperature controllers, temperatures rose at about 0.5 °C/s and fell at about 0.1 °C/s.

An assembly EC_{50} value for discodermolide with tubulin in 0.4 M monosodium glutamate (taken from a 2 M stock solution adjusted to pH 6.6 with HCl) was determined in three independent experiments as described elsewhere (Lin et al., 1996). In brief, the drug concentration required to cause 50% of the tubulin to enter a polymer harvestable by centrifugation was determined. Incubation of reaction mixtures was at room temperature for 15 min, and centrifugation was for 10 min at room temperature at 14 000 rpm in an Eppendorf centrifuge. All EC_{50} values shown were

[2] Abbreviations: FCS, fetal calf serum; HBSS, Hank's balanced salt solution; MAPs, microtubule-associated proteins; Mes, 4-morpholineethanesulfonate; NCI, National Cancer Institute; PBS, Na^+/K^+ phosphate-buffered normal saline.

obtained in at least three independent experiments. Average values ±SE are given.

Electron Microscopy. Aliquots were taken from spectrophotometrically followed reaction mixtures and applied to 200-mesh carbon-coated, Formavar-treated, copper grids. The sample was followed by several drops of 0.5% uranyl acetate, and excess stain was drained off with filter paper. The grids were examined in a Zeiss model 10CA electron microscope.

RESULTS

Effects of Discodermolide on Cultured Human Cells. We initially studied the effects of discodermolide on the growth of two human breast carcinoma cell lines, estrogen receptor positive MCF-7 cells and estrogen receptor negative MDA-MB231 cells. Both lines were examined for drug effects on growth by quantifying numbers of viable cells and by indirect immunofluorescence for drug effects on the microtubule cytoskeleton. Since qualitatively and quantitatively similar data were obtained with both lines, only studies with the MCF-7 cells are shown. The IC_{50} value we obtained for discodermolide after 48 h of continuous exposure to the drug was 2.4 nM, not much different from the 2.1 nM value obtained for taxol.

The indirect immunofluorescence patterns obtained with discodermolide were quite distinct from those obtained with taxol, as shown in Figure 2. Unlike inhibitors of microtubule assembly, which uniformly cause the disappearance of microtubules [for example, see Bai et al. (1993)], discodermolide treatment, like taxol treatment (De Brabander et al., 1981), caused major rearrangement of cellular microtubules. The untreated MCF-7 cells (Figure 2A) displayed a complex microtubule network, most dense around the nucleus with fine detail best visualized at the cell periphery. Filamentous and bundled microtubules, retracted and concentrated around the nucleus, were noted at discodermolide concentrations as low as 10 nM (Figure 2B). These bundles became increasingly prominent and elongated as the concentration of discodermolide was increased. Figure 2C presents cells treated with 1 μM discodermolide. In comparison, treatment with 10 nM taxol had little to no effect on the overall appearance of the cellular microtubule network (Figure 2D). Even with 10 μM taxol (Figure 2F), the bundled microtubules were less extensive than occurred with 1 μM discodermolide. Bundles were less abundant in the breast carcinoma cells following treatment with 1 μM taxol (Figure 2E) and were not observed at lower taxol concentrations.

Antitubulin agents without exception cause cells to accumulate in mitotic arrest (Hamel, 1996). While earlier flow cytometric studies had indicated a substantial increase in the G_2/M population following discodermolide treatment (Longley et al., 1991a,b, 1993; Hung et al., 1994), few mitotic MCF-7 cells were observed in the immunofluorescence studies. This probably was a consequence of detachment of the more spherical mitotic cells from the slides on which they were growing. We have found that Burkitt lymphoma CA46 cells are particularly useful in the study of compounds that interact with tubulin, for, following treatment with such drugs, very high percentages of CA46 cells arrest in classic "C-mitosis", with condensed chromosomes, no nuclear membrane, and, presumably, no spindle [see, e.g., Beutler et al. (1993)]. Initially, we measured the effect of increasing concentrations of discodermolide on the growth of this cell line by quantifying increase in cell number. An IC_{50} value of 30 nM was obtained (Figure 3), versus 40 nM for taxol (not shown). We then confirmed that this inhibition was associated with an increase in the mitotic index (Figure 3). At 1 μM discodermolide, the highest drug concentration examined, 68% of the cells were in mitotic arrest.

Effects of Discodermolide on Tubulin Assembly and Polymer Stability. The cellular findings described above led us to examine discodermolide for potential in vitro interactions with purified tubulin. In all experiments performed, in which 10 μM discodermolide was compared with 10 μM taxol in inducing polymerization of 10 μM tubulin, substantially more vigorous assembly occurred with discodermolide than with taxol.

Figure 4 presents results of an experiment in which the reaction mixtures contained heat-treated MAPs and 100 μM GTP, the only reaction condition used in our studies in which tubulin polymerization will occur in the absence of drug. Upon addition of 10 μM discodermolide to such a reaction mixture held at 0 °C, rapid polymerization began immediately, and the reaction went to near completion at this temperature (curve 3). With 10 μM taxol (curve 2), significant assembly only occurred when the temperature was increased to 10 °C, and there was a further increase in turbidity when the temperature was raised to 20 °C. Without drug (curve 1), assembly occurred at 37 °C only. When the temperature was returned to 0 °C at the end of the experiment, the control polymer disassembled completely. The residual turbidity probably represents protein denaturation, as no microtubules were found on electron microscopic examination of such samples. There was also modest decay of turbidity in the taxol sample when the temperature was returned to 0 °C, but with discodermolide the turbidity reading was completely stable.

When this experiment was repeated, but with the temperature increased from 0 to 37 °C before the discodermolide-induced reaction at 0 °C was complete, the rate of the drug-induced assembly reaction increased as the temperature rose (Figure 5A). In this experiment we also explored the combined effects of low temperature and Ca^{2+} on the taxol- (Collins & Vallee, 1987) and discodermolide-induced polymers. We found that 0.6 mM $CaCl_2$ caused greater than 90% disassembly at 37 °C of the polymer in the reaction without drug (data not presented), but both 0.6 and 5 mM $CaCl_2$ caused no significant depolymerization of either taxol- or discodermolide-induced polymer at 37 °C. When the reaction temperature was reduced to 0 °C, however, the taxol-induced polymer underwent relatively rapid disassembly that was both more rapid and more extensive with 5 mM $CaCl_2$ as compared with 0.6 mM $CaCl_2$. In contrast, the discodermolide-induced polymer was completely stable at both $CaCl_2$ concentrations (data shown for 5 mM $CaCl_2$ only).

The results of an experiment in which heat-treated MAPs but not GTP was included in the reaction mixture are shown in Figure 5B (no assembly occurred without drug; data not shown). Again, with discodermolide, but not with taxol, an assembly reaction began at 0 °C when the drug was added to the reaction mixture. The reaction at 0 °C without GTP was slower than that with GTP (see Figure 5A). When the temperature was raised to 37 °C, both drugs induced rapid polymerization. While addition of either 0.6 or 5 mM $CaCl_2$ alone did not destabilize the taxol-induced polymer, it

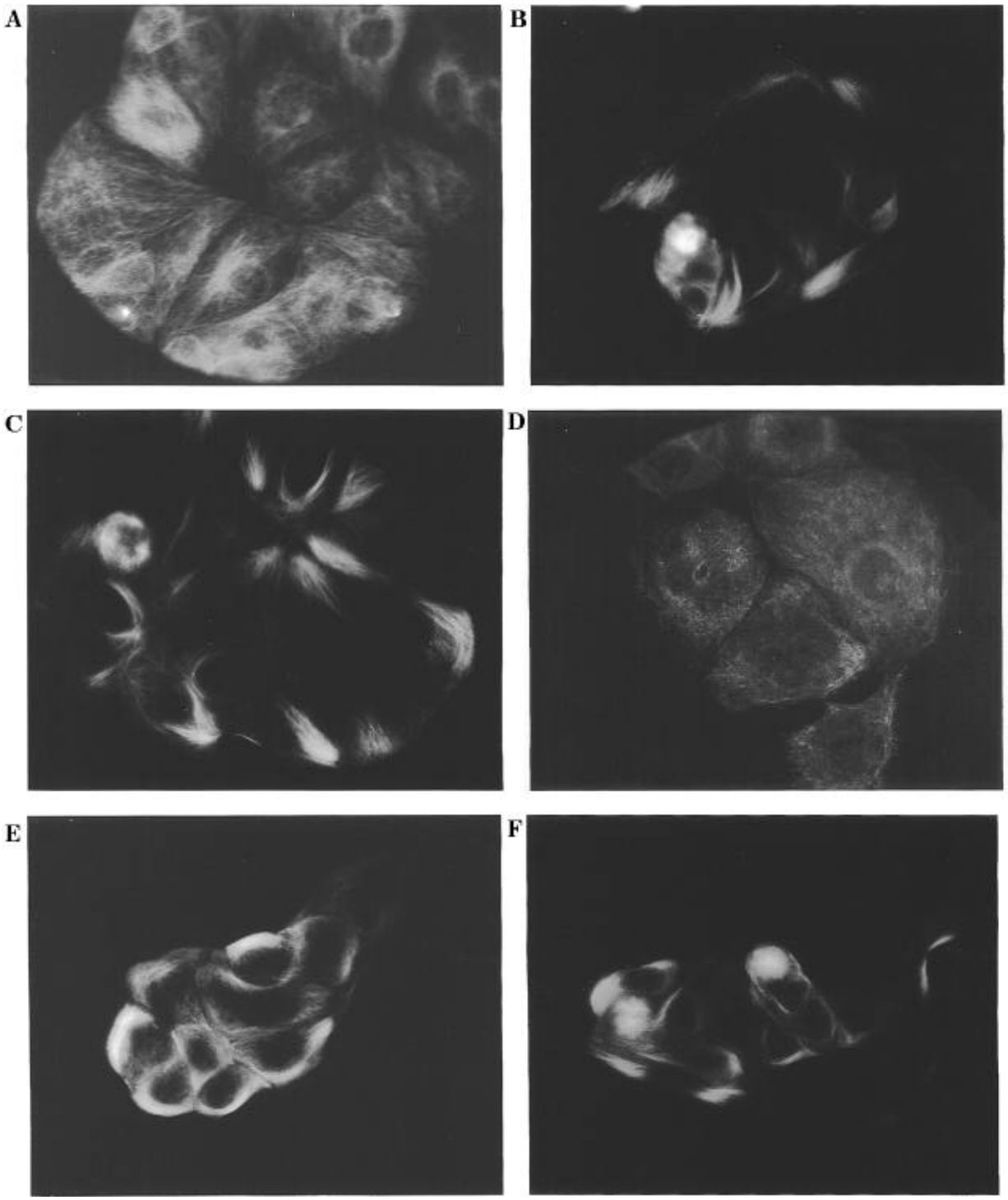


FIGURE 2: Potent perturbation of cellular microtubules by discodermolide as compared with taxol. Photomicrographs of MCF-7 cells examined by indirect immunofluorescence, with the primary antibody directed against β-tubulin. (A) No drug; (B−C) effects of 48 h of continuous exposure to 10 nM and 1 μM discodermolide, respectively; (D−F) effects of 48 h of continuous exposure to 10 nM, 1 μM, and 10 μM taxol, respectively. Magnification: $\times$155.

disassembled once the temperature was reduced to 0 °C. There was little difference in the destabilizing effects of the two $CaCl_2$ concentrations in the absence of GTP. The discodermolide-induced polymer was stable at 0 °C in the absence and in the presence of both concentrations of $CaCl_2$ (data shown for 5 mM $CaCl_2$ only).

Figure 5C presents results of an experiment in which 100 μM GTP but not MAPs was included in the reaction mixture (no reaction occurs without drug). Under this reaction condition, assembly with discodermolide at 0 °C was very slow. When the temperature was raised to 37 °C, vigorous assembly occurred with discodermolide and relatively sluggish assembly occurred with taxol. The combination of 0.6 mM $CaCl_2$ and 0 °C induced depolymerization of the taxol polymer, as did 5 mM $CaCl_2$. Addition of 5 mM $CaCl_2$ also caused an increase in turbidity of unknown origin in the taxol sample at 37 °C, but, as with MAPs only, the destabilizing effects of the two $CaCl_2$ concentrations were similar. With GTP, only the discodermolide polymer was stable to 0 °C in the absence (not shown) and presence of both concentrations of $CaCl_2$. As with taxol, addition of 5 mM $CaCl_2$ caused an immediate increase in turbidity at 37 °C.

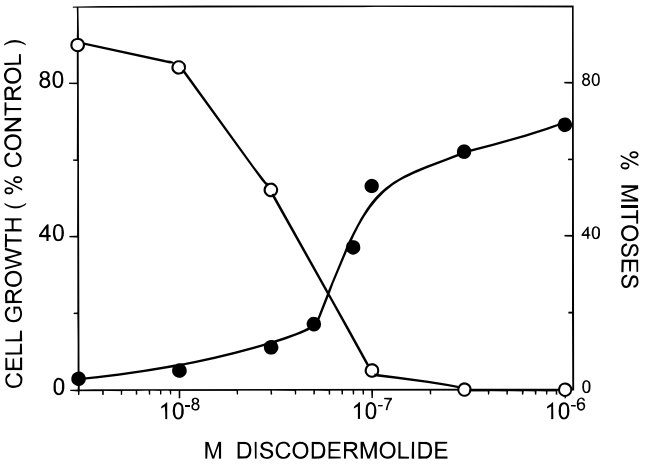


FIGURE 3: Effects of discodermolide on Burkitt lymphoma CA46 cells. Open circles, inhibition of increase in cell number after 24 h; closed circles, increase in mitotic figures after 14 h.

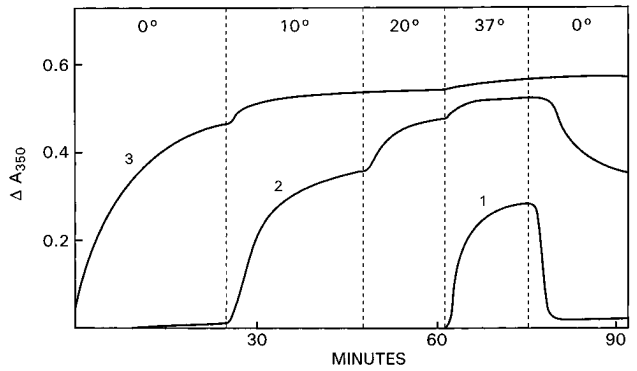


FIGURE 4: Potent stimulation of microtubule assembly by discodermolide as compared with taxol. Each reaction mixture contained 0.1 M Mes, 4% dimethyl sulfoxide, 1 mg/mL (10 μM) tubulin, 100 μM GTP, and 0.5 mg/mL heat-treated MAPs. Baselines were established at 0 °C, and either no drug (curve 1), 10 μM taxol (curve 2), or 10 μM discodermolide (curve 3) was added. The trace for curve 3 indicates the earliest part of the reaction for which data were obtained. The temperature setting on the controller was changed at the indicated times.

The results of an experiment in which neither GTP nor MAPs was included in the reaction mixture are shown in Figure 5D (no assembly occurs without drug). Under this reaction condition, 10 μM taxol is inactive with 10 μM tubulin (Grover et al., 1995), but discodermolide induced assembly at 37 °C. This polymer was stable to cold in the absence (not shown) and presence of Ca^{2+}, with no disassembly occurring even with 5 mM $CaCl_2$. Again, 5 mM $CaCl_2$, but not 0.6 mM $CaCl_2$, caused a rise in turbidity when added to the reaction mixture at 37 °C.

Recent studies with 2-debenzoyl-2-*meta*-azidobenzoyltaxol and other C-2 modified taxol derivatives (Chaudhary et al., 1994; Grover et al., 1995), many of which have enhanced activity relative to taxol in inducing assembly, led us to develop an assay in which such compounds could be compared quantitatively in terms of drug concentration to taxol (Lin et al., 1996; see Materials and Methods). The drug concentration required to induce 50% assembly of 10 μM tubulin at room temperature was measured. This assay was specifically designed to maximize the difference between 2-debenzoyl-2-*meta*-azidobenzoyltaxol ($EC_{50} = 4.7 \pm 0.1$ μM) and taxol ($EC_{50} = 23 \pm 0.2$ μM). Discodermolide was highly active in the assay, yielding an EC_{50} value of 3.2 ± 0.3 μM. Results from experimental sequences with discodermolide and taxol are shown in Figure 6. This difference might well be far greater if the assay incubation was on ice. Note that 50% of the tubulin (i.e., 5 μM) was induced to assemble by an apparently substoichiometric concentration of discodermolide.

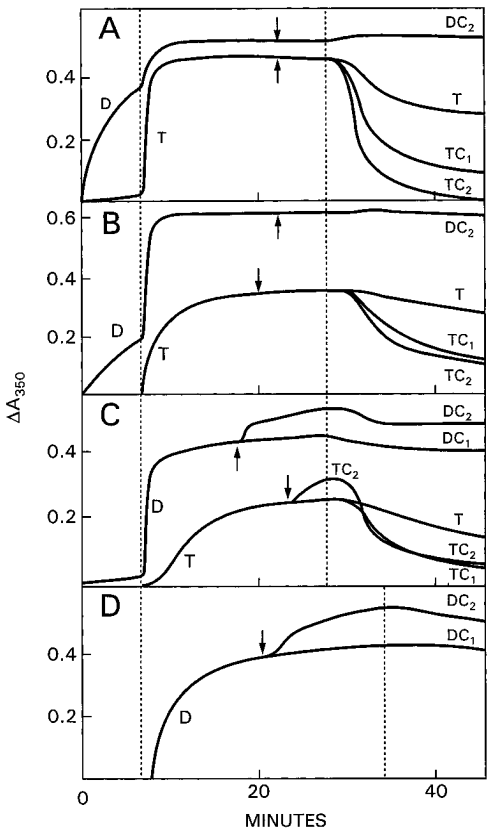


FIGURE 5: Comparison of the effects of MAPs, GTP, and Ca^{2+} on discodermolide- and taxol-induced tubulin polymerization. Reaction mixtures, containing 0.1 M Mes, 10 μM tubulin, and other components as indicated but lacking drug, were held at 0 °C and baselines were established. At time zero, 10 μM taxol (indicated by "T") or 10 μM discodermolide (indicated by "D") was added and rapidly mixed into the reaction mixtures. In panels A−C, turbidity was rising at the earliest time point obtained following addition of discodermolide to the reaction mixtures. Temperature was held at 0 °C for about 4.5 min, at which point the temperature controller was set at 37 °C (indicated by first vertical dashed line). At the arrows, $CaCl_2$ was added to the indicated reaction mixtures (0.6 mM indicated by "C1" and 5 mM indicated by "C2"). At the time indicated by the second vertical dashed line, the temperature controller was set at 0 °C. (A) The reaction mixtures also contained 100 μM GTP and 0.5 mg/mL heat-treated MAPs. (B) The reaction mixtures also contained 0.5 mg/mL heat-treated MAPs (no GTP). (C) The reaction mixtures also contained 100 μM GTP (no MAPs). (D) No further addition (no MAPs, no GTP).

Electron Microscopic Studies. It was important to document that the turbidity profiles obtained with discodermolide, especially at 0 °C, represented authentic assembly reactions, as opposed to, for example, protein or drug precipitation. Aliquots of reaction mixtures taken from samples incubated essentially as described in Figure 4 were therefore examined by electron microscopy. Aliquots were removed from the discodermolide sample after 5 and 20 min at 0 °C, and successively after 20 min each at 10, 20, and 37 °C, from the taxol sample after 20 min successively at 10, 20, and 37 °C, and from the sample without drug after 20 min at 37 °C. Figure 7A shows high and low magnification views of polymer formed after a 5 min incubation at 0 °C with discodermolide. Large numbers of very short polymers,

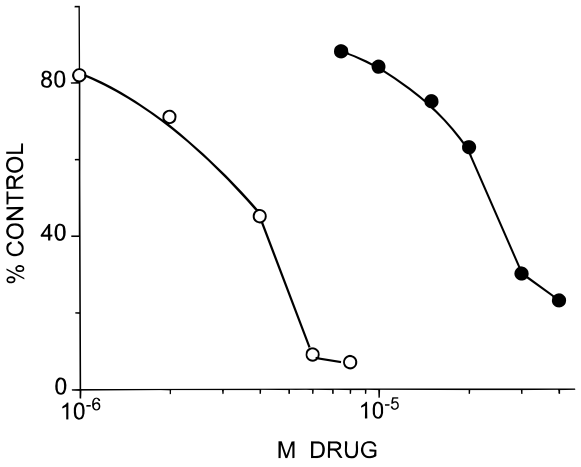


FIGURE 6: Quantitative comparison of discodermolide and taxol in inducing tubulin polymerization in 0.4 M glutamate without GTP at room temperature. Each 100 μL reaction mixture contained 0.4 M monosodium glutamate (pH 6.6), 4% dimethyl sulfoxide, 1 mg/mL (10 μM) tubulin, and the indicated concentrations of discodermolide (open circles) or taxol (closed circles). The reaction mixtures were incubated at room temperature for 15 min and centrifuged in an Eppendorf model 5425C microcentrifuge for 10 min at room temperature. Protein in the supernatants was compared to protein in the supernatants of reaction mixtures without drug. Without drug there was less than a 5−10% reduction in total protein by centrifugation, and no visible pellet formed. The drug-containing supernatants were compared to the drug-free supernatants to determine percent of control values.

largely microtubules but some sheets as well, were present. The samples taken at the end of the 0 °C incubation, as well as after the higher temperature incubations, all showed large numbers of microtubules (with some sheets) that appeared somewhat longer than those present in the initial sample. Figure 7B presents the sample taken at 20 °C. A preliminary determination of the lengths of these polymers was made, with 50 polymers measured at each temperature (the number of polymers was relatively low because only structures with both ends clearly distinguishable were measured, and such microtubules were relatively rare). Average lengths (± SD) were as follows: after 5 min at 0 °C, 0.35 (± 0.16) μm; after 20 min at 0 °C, 0.53 (± 0.25) μm; after a further 20 min at 10 °C, 0.59 (± 0.28) μm; after a further 20 min at 20 °C, 0.58 (± 0.28) μm; and after a further 20 min at 37 °C, 0.58 (± 0.30) μm.

With taxol, the polymer also consisted primarily of microtubules plus some sheets [data not presented; see Grover et al. (1995) for taxol-induced structures under the same reaction conditions used here]. The taxol-induced microtubules were longer than those formed with discodermolide. The polymers induced by taxol at 10 °C were shorter in length (0.70 ± 0.26 μm) than those observed at 20 °C and 37 °C (1.65 ± 0.63 and 1.69 ± 0.57 μm, respectively). Polymer morphology in the control sample consisted almost entirely of microtubules with an average length of 3.26 ± 1.61 μm.

Samples of discodermolide-induced polymer formed in reaction mixtures +MAPs/−GTP, −MAPs/+GTP, and −MAPs/−GTP were also examined by electron microscopy (data not presented). Omission of GTP but not MAPs led to some increase in sheet formation, while omission of MAPs but not GTP led to a large increase in sheets, with few microtubules observed. As seen with 2-debenzoyl-2-*meta*-azidobenzoyltaxol (Grover et al., 1995), a greater proportion of the discodermolide-induced polymer had a microtubule morphology in the absence of both MAPs and GTP than in the absence of MAPs only. Addition of $CaCl_2$ had no effect on polymer morphology under any reaction condition, nor was morphology altered when the reaction temperature was subsequently reduced to 0 °C. Thus, the discodermolide-induced polymer formed under all reaction conditions was stable to both Ca^{2+} and cold-induced disassembly when examined by turbidimetry (Figure 5) and by electron microscopy (data not presented).

DISCUSSION

The natural product (+)-discodermolide, isolated from the marine sponge *D. dissoluta*, acts both in cells and biochemically in a manner resembling that of taxol despite no apparent structural similarity between the compounds. With the recent description of epothilones A and B, isolated from the myxobacterium *Sorangium cellulosum*, as nontaxoid microtubule stabilizing agents (Bollag et al., 1995), discodermolide represents a third structurally distinct class of compounds that stabilize tubulin polymer and enhance assembly reactions.

Discodermolide appears to be substantially more active than taxol and the epothilones in terms of the morphological changes that occur in microtubules in cells and in terms of effects on *in vitro* tubulin assembly. On the other hand, the cytotoxicities of discodermolide versus taxol (reported here) and of the epothilones versus taxol (Bollag et al., 1995) in cells where direct comparisons have been made are quantitatively very similar. If confirmed in additional cell lines in further direct comparisons, this would suggest that it is more likely subtle effects on microtubule dynamics, assembly, and/or disassembly (Jordan et al., 1993; Derry et al., 1995) as opposed to major morphological alterations in microtubule arrangement that lead to cell death following treatment with this class of drug. Moreover, our data suggest that, in cells, discodermolide preferentially stimulates elongation of existing microtubules rather than the massive *de novo* nucleation that occurred in the *in vitro* assembly assays, since the submicrometer structures formed *in vitro* would be difficult to detect by immunofluorescence techniques.

Like 2-debenzoyl-2-*meta*-azidobenzoyltaxol and docetaxel (Taxotere) (Grover et al., 1995), discodermolide is more potent than taxol in stabilizing tubulin polymers to low temperatures. In fact, under no condition we examined did we find turbidimetric evidence for any polymer disassembly at 0 °C. With discodermolide, we also find that polymer is highly stable to the combined effects of Ca^{2+} and low temperature, which together destabilize taxol-induced polymers (Collins & Vallee, 1987). Substantial disassembly could be induced in all taxol polymers at 0 °C in the presence of 0.6 mM $CaCl_2$, while the discodermolide polymers persisted even with 5 mM $CaCl_2$, the highest concentration examined.

Perhaps the most striking *in vitro* effect of discodermolide is an extraordinary ability to hypernucleate assembly, much greater than the analogous effects of taxol, as evidenced by assembly at 0 °C in the presence of MAPs ± GTP and for assembly to occur in the absence of either MAPs or GTP or in the absence of both MAPs and GTP. This was particularly evident in the studies of the MAPs/GTP system, in which we determined polymer lengths. With discodermolide,

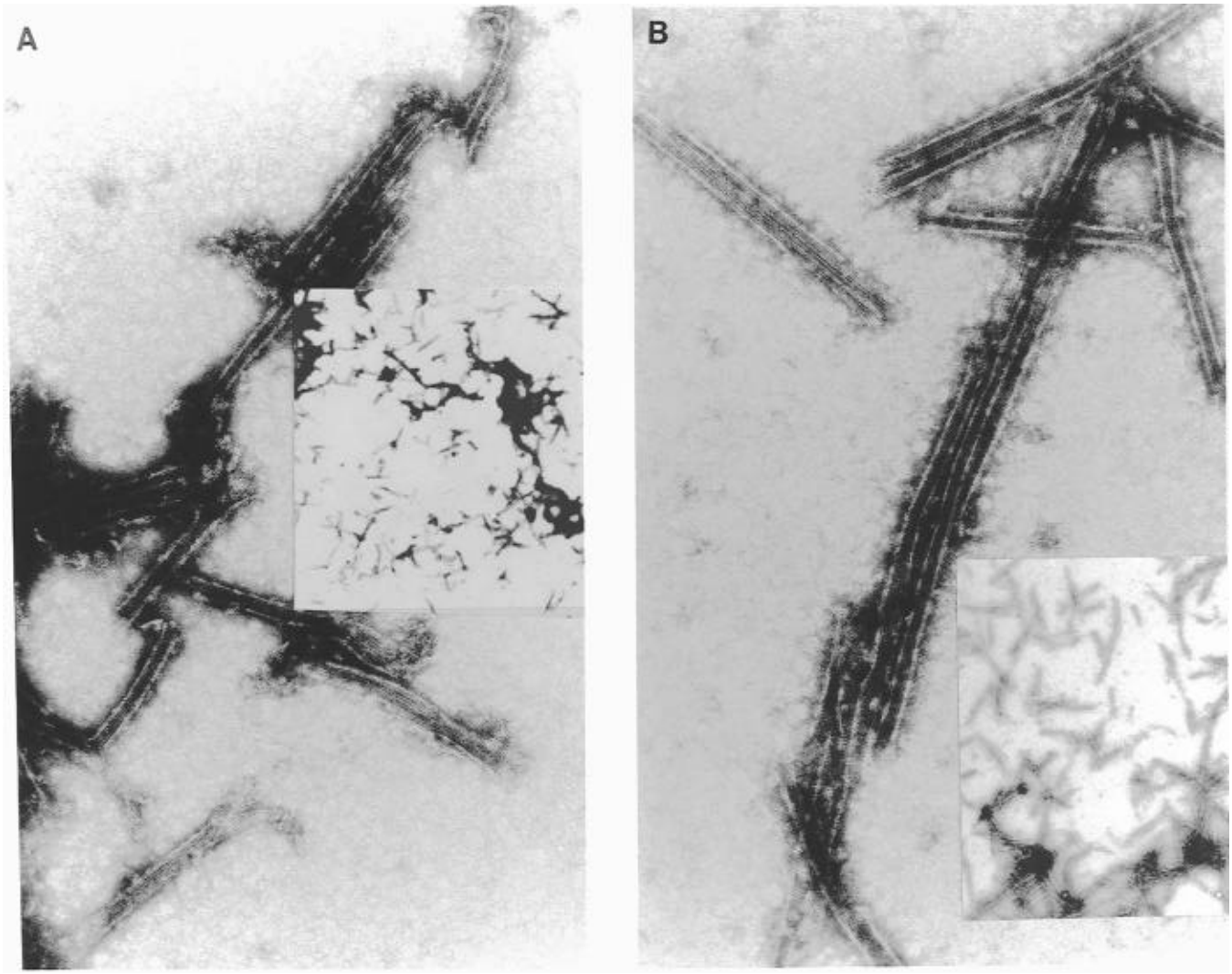


FIGURE 7: Morphology of discodermolide-induced tubulin polymer. The assembly reaction was followed spectrophotometrically and in temperature steps, as shown for Figure 4, with a control without drug and with samples containing 10 μM taxol or 10 μM discodermolide. Samples were incubated successively for 20 min at 0, 10, 20, and 37 °C. Aliquots were removed at the end of each temperature step from samples with significant assembly reactions and after 5 and 20 min at 0 °C from the discodermolide sample. (A) Polymer formed with discodermolide after 5 min at 0 °C. (B) Polymer formed with discodermolide after successive incubations at 0, 10, and 20 °C. Magnifications: ×106000 (main panels); ×11000 (insets).

average length was 0.6 μm, about one-third of the average length of the taxol polymers (1.7 μm) and one-sixth of the microtubules formed without drug (3.3 μm).

Discodermolide's behavior in the biochemical systems used here is similar to that observed with 2-debenzoyl-2-*meta*-azidobenzoyltaxol (Grover et al., 1995). Although we have not yet directly compared discodermolide to this taxol analog, the same protein preparations and reagents were used in both sets of experiments. Under all experimental conditions examined, discodermolide appears to be the more potent of the two compounds, with the EC_{50} values obtained in the 0.4 M glutamate system (3.2 μM for discodermolide, 4.7 μM for 2-debenzoyl-2-*meta*-azidobenzoyltaxol) perhaps representing a reasonable estimate of relative potencies. One major difference between discodermolide and 2-debenzoyl-2-*meta*-azidobenzoyltaxol is that microtubule lengths at steady state with the taxol analog appeared similar to those observed with taxol [see Grover et al. (1995)], although a quantitative comparison was not performed, in contrast to the much shorter polymers formed with discodermolide. This would suggest that the rate of nucleation is not closely related to the temperature required for the reaction to occur. Alternatively, it is possible that with taxol it is elongation, not nucleation, that is the rate-limiting step in the GTP/MAPs system.

In summary, (+)-discodermolide, obtained from a marine sponge, is a new and structurally unique antimitotic agent that stabilizes microtubules in cells and in biochemical systems more potently than any other agent yet described and is highly potent in causing the hypernucleation of tubulin assembly under normally unfavorable conditions. On the basis of its mechanism of action, discodermolide holds promise as a new therapeutic agent for neoplastic diseases and provides a new structural class for synthetic elaboration that could lead to improved compounds. One major advantage of discodermolide over taxol is its probable greater solubility in water. Supplies of discodermolide have been insufficient thus far to carefully evaluate its solubility, but taxol's aqueous solubility is only 5−9 μg/mL (6−11 μM).[3]

[3] Data of the Pharmaceutical Resources Branch, National Cancer Institute. We thank Dr. E. Tabibi for providing us with this information. Solubility in aqueous solution varied little with buffer component(s) or pH of the solution.

Using a fragment-based computational method to calculate aqueous solubility (Klopman et al., 1992), we found that discodermolide should be about 160-fold more soluble than taxol in water, and the value we calculated for taxol (4.4 μg/mL) was in good agreement with the empirical value of 7 μg/mL.

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