(+)-Discodermolide binds to microtubules in stoichiometric ratio to tubulin dimers, blocks taxol binding and results in mitotic arrest

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Background: The marine natural product (+)-discodermolide has potent immunosuppressive activity. It inhibits proliferation of a wide range of human and murine cells, induces cell cycle arrest in the G2 or M phase and was recently shown to stabilize microtubules. Total synthesis of discodermolide has made it possible to generate variants of the compound to study its intracellular function in detail.

Results: We have determined that (+)-discodermolide arrests MG63 cells at M phase, and has a stabilizing effect on microtubules. *In vitro* studies show that discodermolide induces polymerization of purified tubulin in the absence of microtubule-associated proteins, and that it binds to tubulin dimers in microtubules at 1:1 stoichiometry. Discodermolide binds taxol-polymerized microtubules at near stoichiometric level, whereas taxol binds discodermolide-induced microtubules poorly. Competition data show that the binding of microtubules by discodermolide and taxol are mutually exclusive; discodermolide binds with higher affinity than taxol. The results of binding assays carried out *in vivo* or in cell lysates also suggest that the microtubule network is discodermolide's cellular target.

Conclusions: (+)-Discodermolide causes cell cycle arrest at the metaphase–anaphase transition in mitosis, presumably due to its stabilizing effect on microtubules. *In vitro*, discodermolide polymerizes purified tubulin potently in the absence of MAPs. It binds microtubules at one molecule per tubulin dimer with a higher affinity than taxol, and the binding of microtubules by discodermolide and taxol are mutually exclusive. In total cell lysates discodermolide displays binding activity that is consistent with its effects on microtubules.

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Introduction

Lipophilic natural products that penetrate the plasma membrane of cells and interfere with cellular functions can serve as excellent tools for probing intracellular signal transduction pathways. The marine natural product (+)-discodermolide, originally isolated from the sponge *Discodermia dissoluta*, is a promising candidate for this purpose. Discodermolide is immunosuppressive; it has an IC_{50} of 9 nM for inhibition of purified murine T cell proliferation, inhibits the mixed leukocyte reaction, and suppresses graft-versus-host disease in transplanted mice [1,2]. Recently, this compound has also been shown to cause cell cycle arrest in the gap 2 (G2) or mitosis (M) phase in a variety of human and murine cell lines, with IC_{50} values ranging from 3 nM to 80 nM [3,4]. The exact arrest point and the mechanism of arrest are not known, however. Total syntheses of both (+)- and (–)-discodermolide have been accomplished [3,5], and it is now possible to make discodermolide derivatives to probe the target of its action.

To understand the mechanism of discodermolide's action better, we have investigated its cellular effects in detail. We initiated the characterization of discodermolide's cellular target using tritiated discodermolide, and identified a cellular binding activity [3]. Here we report that discodermolide arrests cells at a stage after entering mitosis. Entry into, and exit from, the M phase of the cell cycle is controlled by the synthesis and degradation of M-phase-promoting factor (MPF). Cyclin B is an essential component of MPF, and its synthesis and degradation are an important part of the control of MPF activity. Here we show that prolonged cyclin B expression is observed in the presence of discodermolide, and that treated cells eventually exit mitosis with micronucleated nuclei. Abnormal microtubule formation is observed in discodermolide-treated cells. The microtubules form concentrated bundles instead of the normal network and show increased stability against cold-induced depolymerization. These results indicate that discodermolide may have a direct effect on microtubules, consistent with the recent report that discodermolide stabilizes microtubules in breast cancer cells [6]. In accordance with these observations, fractionation of cells treated with [^{3}H]discodermolide and lysed with non-ionic detergent shows that

over 90 % of radioactivity is associated with cell pellets, which include nuclei and cytoskeletons. Since the mitotic spindle, which is composed of microtubules, is essential for M phase progression of the cell cycle, our results strongly suggest that discodermolide arrests cells at M phase by causing aberrant spindle formation.

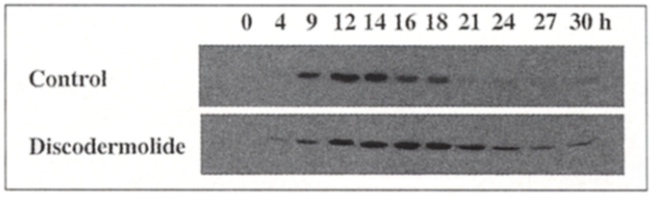
This effect of discodermolide on microtubules is reminiscent of the effect of taxol, which has been reported to polymerize, bind and stabilize microtubules [7–9]. We therefore examined the effect of discodermolide on purified tubulin *in vitro*. Indeed, discodermolide polymerizes tubulin into microtubules in the absence of microtubule-associated proteins (MAPs) and binds to microtubules at one molecule per tubulin dimer. Discodermolide binds either to the same site on microtubules as taxol or to an overlapping site, and has a significantly higher affinity for its site than does taxol.

Results

Discodermolide arrests cells in mitosis

Discodermolide has previously been shown by flow cytometry to arrest MG63 human osteosarcoma cells [3] and murine DO11.10 T hybridoma cells [4] at G2 or M phase. To define the arresting mechanism further, we examined the effect of discodermolide on cyclin B expression. In a variety of eukaryotic cells, expression of cyclin B and its subsequent association with and activation of cdc2 kinase result in active MPF, which drives the cell cycle into mitosis [10–12]. Degradation of cyclin B and the resulting inactivation of MPF occur at anaphase of mitosis, followed by sister chromatid segregation, chromosome decondensation, cytokinesis, and nuclear envelope reformation [13–15]. MG63 cells synchronized at the G1/S boundary by hydroxyurea, an inhibitor of ribonucleotide reductase, were released from the block by removing hydroxyurea and incubated in the presence or absence of 100 nM discodermolide. Cyclin B1 expression was examined by lysing the cells at different time points and subjecting the lysates to Western analysis. As shown in Figure 1, discodermolide treatment did not affect the onset of cyclin B1 expression. The subsequent degradation of cyclin B1 was delayed by ~6–12 h in the presence of discodermolide, however. A similar delay was observed when serum-starved cells were synchronized at G0 (a quiescent state outside the normal cell cycle) and stimulated to re-enter the cell cycle (data not shown). The discodermolide-treated cells had a morphology characteristic of mitotic cells (detached and rounded) during the prolonged period of cyclin B1 expression, and re-adhered when cyclin B1 eventually dropped to basal level. The re-adhered cells, however, were mostly micronucleated and often larger in size than the control cells (see Fig. 2c,d). During the preparation of this manuscript, ter Haar *et al.* [6] reported that discodermolide arrests Burkitt lymphoma CA46 cells at mitosis, determined by mitotic index. Our observations using hydroxyurea-released MG63 cells confirm and extend their findings.

Figure 1



Discodermolide delays the degradation of cyclin B. MG63 cells synchronized at S phase by 2 mM hydroxyurea were released into the cell cycle in the absence or presence of 100 nM discodermolide. Cyclin B expression at 0–30 h after the release was analyzed by Western blotting with anti-human cyclin B1 antibody.

Discodermolide stabilizes microtubules *in vivo*

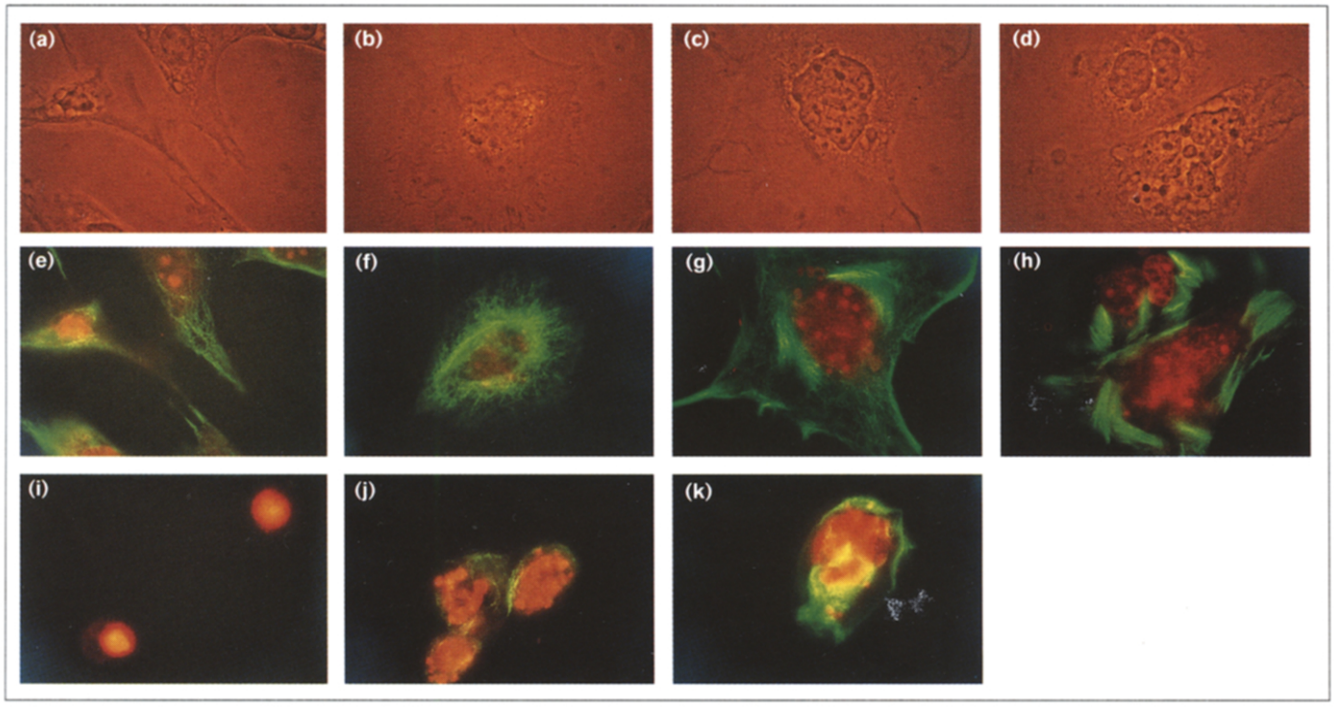
Almost all anti-mitotic reagents interact with microtubules. Classic examples include colchicine and colcemid, both microtubule-destabilizing reagents [16], and taxol, a microtubule-stabilizing drug [7–9]. These molecules arrest or prolong the cell cycle at the metaphase–anaphase transition of mitosis, and cells that eventually escape the block are mostly micronucleated. These observations therefore prompted us to examine the effect of discodermolide on microtubules. Asynchronous Swiss 3T3 cells treated with 100 nM or 1 μM discodermolide for 24–36 h were stained using anti-tubulin antibodies. Instead of the continuous network of microtubules seen in control cells, discodermolide-treated cells contained dense and discontinuous bundles of microtubules, mostly concentrated around the nucleus or edges of the cell (Fig. 2), whereas taxol-treated cells displayed an abnormally dense network of microtubules. The staining pattern of discodermolide-treated cells was independent of the phase of the cell cycle (data not shown).

We also examined the effect of discodermolide on the stability of microtubules upon cold treatment *in vivo*. Whereas the microtubule network in control Swiss 3T3 cells disappeared completely upon incubation at 4 °C for 16 h (Fig. 2k), discodermolide preserved microtubules even better than taxol (Fig. 2i,j), although in all cases cells were considerably diminished in size. We therefore conclude that discodermolide, like taxol, acts to stabilize microtubules *in vivo*.

Discodermolide binds to microtubules *in vitro*

Since taxol induces tubulin polymerization *in vitro* [8], we next investigated the effect of discodermolide on purified tubulins. In the absence of microtubule-associated proteins (MAPs), which are normally required for tubulin polymerization *in vitro*, 10 μM discodermolide alone rapidly induced polymerization of tubulin at 37 °C (data not shown). This is consistent with the results of ter Haar

Figure 2



Effects of discodermolide on microtubules in cells. In panels **(a)–(h)**, asynchronous Swiss 3T3 cells were treated with discodermolide or taxol for 36 h before fixing and staining as described in Materials and methods. In panels **(i)–(k)**, cells were treated with drugs for 24 h at 37 °C, then incubated at 0 °C for 16 h. Panels (a)–(d) show views from microscope light field, while panels (e)–(k) are from fluorescent field. Green, microtubules; red, DNA. (a), (e), (i) control, (b), (f), (j) 100 nM taxol, (c), (g), (k) 100 nM discodermolide, (d), (h) 1 μM discodermolide. All micrographs are shown at the same magnification.

et al. [6], who further showed that discodermolide polymerizes tubulin more potently than taxol at 0 °C or in the absence of MAPs and GTP.

We then studied the binding of tritiated discodermolide to microtubules. Pure tubulins (10 μM dimer, containing no MAPs) were polymerized by 10 μM taxol in the presence of 1 mM GTP at 37 °C for 30 min, then incubated with various concentrations of [^{3}H]discodermolide. Binding was assessed by sedimenting microtubules and determining radioactivity in the pellet by scintillation counting. Data presented in Table 1 show that the binding to 10 μM tubulin is saturated by 10 μM discodermolide, indicating a binding stoichiometry of one discodermolide per tubulin dimer. Under the above conditions, essentially all the tubulin dimers are occupied with taxol prior to the addition of discodermolide. Therefore, discodermolide must be binding to the same tubulin dimers that originally bound to taxol. These results did not determine whether discodermolide competes with taxol for binding to microtubules, however; discodermolide could bind at a site distinct from that for taxol, or to the same site but with a higher affinity. There is no obvious structural similarity between taxol and discodermolide (Fig. 3a), and therefore no *a priori* reason to suspect that they would bind to the same site.

To determine whether taxol and discodermolide bind to the same or overlapping sites on microtubules, we performed several competition binding experiments. Tubulin (10 μM) was polymerized in the presence of 10 μM radiolabeled taxol (Fig. 3b) or discodermolide (Fig. 3c), and then various concentrations of unlabeled competitor were added. At the end of the incubation, the radioactivity

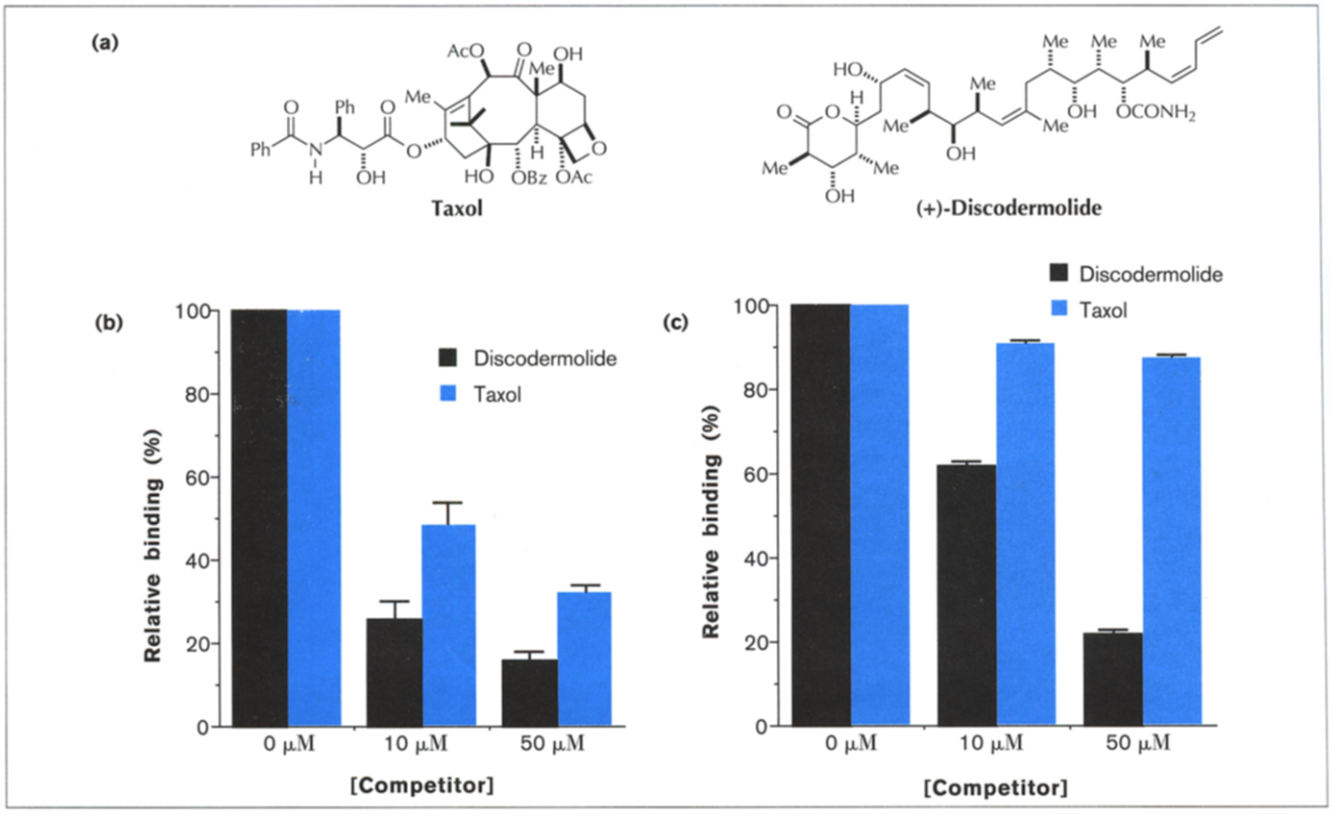
Table 1

Stoichiometry of discodermolide binding to taxol-polymerized tubulin.

[discodermolide]:[tubulin]	Fractional saturation of binding
0.1	0.113 ± 0.004
0.5	0.563 ± 0.016
1	0.994 ± 0.070
2	0.997 ± 0.062
5	1.000 ± 0.070

Pure tubulin (10 μM dimer) was polymerized in the presence of 10 μM taxol and 1 mM GTP at 37 °C for 30 min. [^{3}H]discodermolide was then added at 1 μM, 5 μM, 10 μM, 20 μM and 50 μM, respectively, and incubated at 37 °C for 30 min, followed by sedimentation and scintillation counting of the pellets. Fractional saturation of binding was calculated as the ratio of counts at each discodermolide concentration versus counts at saturating concentrations.

Figure 3



Competition of discodermolide and taxol binding of microtubules. **(a)** Discodermolide and taxol show no obvious structural similarity. **(b)** Purified tubulin (10 μM dimer) was polymerized in PEM buffer containing 1 mM GTP and 10 μM [^{3}H]taxol or **(c)** 10 μM [^{3}H]discodermolide. After incubation with various concentrations of drugs (competitors), microtubule-bound radioactivities were measured. Black bars, discodermolide as competitor; blue bars, taxol as competitor. Relative binding is defined as the percentage of binding in the presence of competitors relative to binding in the absence of competitors. Average of the data from at least three independent experiments are presented. Error bars indicate standard deviations.

remaining bound to the microtubules was counted. As shown in Figure 3, discodermolide is a more effective competitor for [^{3}H]taxol binding than taxol itself, and taxol was not able to compete effectively with [^{3}H]discodermolide, even at higher concentrations (50 μM). Both discodermolide and taxol competed with themselves as expected, showing ~50% competition at a concentration of 10μM. Therefore, discodermolide and taxol bind to mutually exclusive sites on microtubules, and discodermolide has higher affinity than taxol. When competition experiments were carried out for taxol versus taxotere by Diaz and Andreu [17], it was observed that taxotere competed with taxol for the same binding site with 1.9 times higher affinity, consistent with the fact that these two compounds are structurally related. In this case, binding is competitive in the absence of obvious structural similarity.

In vivo target of discodermolide

Previously we have shown that a specific binding activity for [^{3}H]discodermolide is present in MG63 cell total lysates [3]. To test whether this *in vivo* binding target corresponds to its *in vitro* effect, we crudely fractionated the cells that were treated *in vivo* with 20 nM [^{3}H]discodermolide by lysing the cells with non-ionic detergents (e.g., 1 % Triton X-100); the binding activity in the soluble fraction (containing cytoplasmic and membrane proteins) was precipitated by polyethylene glycol (PEG), and compared to the bound activity in the insoluble fraction (containing nuclei and cytoskeletons). Over 90 % of bound radioactivity was found in the insoluble fraction, which included the majority of microtubules. Co-treatment of the cells with colchicine, which disrupts microtubules, abolished the binding activity (Fig. 4a), indicating that the intact microtubule network was the binding target. This is consistent with the observed *in vitro* binding of discodermolide to microtubules.

The cellular discodermolide-binding activity seems highly unstable under the lysis conditions, if the cells are lysed before discodermolide treatment. When the cells were

lysed prior to addition of [^{3}H]discodermolide, very low binding activity was observed (Fig. 4b). Strikingly, when taxol (200 nM) was added at the time of lysis, binding activity comparable to the *in vivo* activity was observed and, again, at least 90 % of the binding activity was in the insoluble fraction (Fig. 4b). One possible explanation for this observation is that discodermolide does not effectively polymerize tubulins at the low concentration used for the assay, and that a pre-existing intact microtubule network is required for it to bind. Taxol serves to stabilize the microtubules, preserving the discodermolide binding site.

Discussion

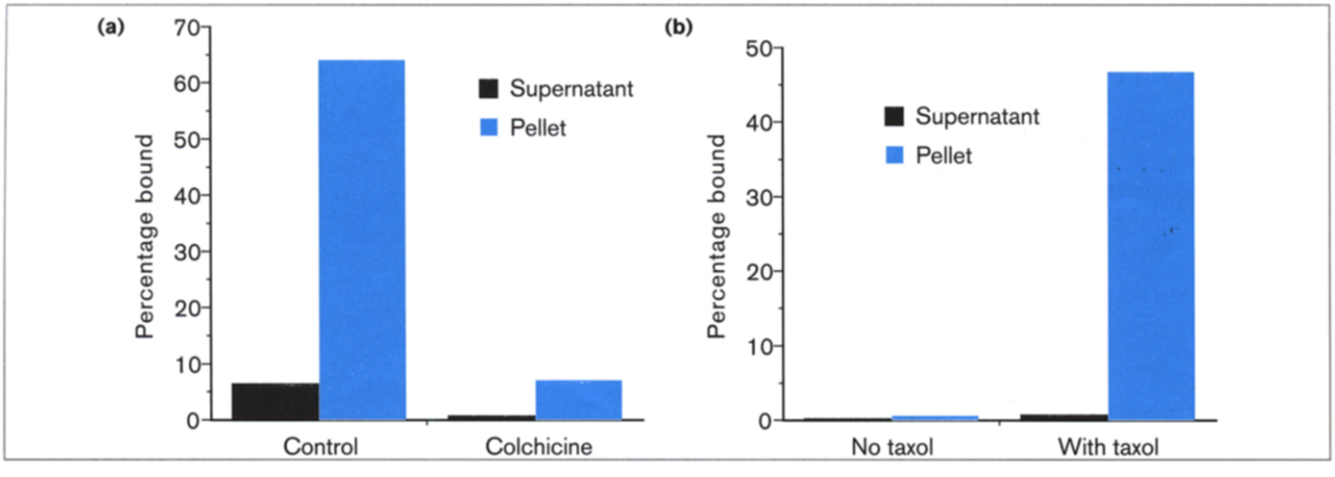
We have established that discodermolide has an effect on microtubules both *in vivo* and *in vitro*. Cells treated with discodermolide display abnormally intense staining of microtubule bundles that are cold-resistant (Fig. 2). *In vitro*, discodermolide potently induces the polymerization of purified tubulin in the absence of MAPs. During the preparation of this manuscript, ter Haar *et al.* [6] reported similar observations; consistent with our data, they have shown that discodermolide potently induces tubulin polymerization at 0 °C, or in the absence of both MAPs and GTP, conditions where taxol at an identical concentration has very little effect. Using radiolabeled discodermolide, we have further shown that this molecule binds to purified microtubules with an affinity that is higher than that of taxol. Despite the lack of structural similarities, the binding of discodermolide and taxol to microtubules is mutually exclusive (Fig. 3). It is possible that they bind to the same or overlapping sites. However, we cannot exclude the possibility that discodermolide and taxol each induce microtubule polymers to adopt different assembly configurations that preclude the simultaneous binding of the two ligands, and the competition by discodermolide may only reflect its higher affinity and polymerizing potency than taxol.

Discodermolide binding activity *in vivo* also appears to reside in the microtubule network. The integrity of this network appears to be crucial for *in vivo* binding, as treatment with colchicine or lysis prior to addition of [^{3}H]discodermolide results in total loss of binding activity, whereas addition of taxol upon lysis preserves the binding activity dramatically (Fig. 4).

Discodermolide treatment prolongs mitosis in MG63 cells, presumably due to its stabilizing effect on microtubules. It is believed that a checkpoint monitors the integrity of mitotic spindles and controls cell entry into anaphase during mitosis [12,18]; any disruption of the spindle will halt the cell cycle before the metaphase–anaphase transition, which coincides with the degradation of cyclin B. Our observation of prolonged cyclin B expression in the presence of discodermolide (Fig. 1) is consistent with the molecule's effect on microtubules (and therefore mitotic spindles). It has been shown that the stringency of the spindle formation checkpoint varies among different cell lines [19]. In other words, in different cell types the ability to survive (and exit) mitosis with disrupted spindles varies and is positively correlated with the cell's ability to degrade cyclin B during the prolonged mitotic period [19]. In this case, MG63 cells are only held at M phase for 6–12 h, rather than arrested permanently.

As reported by ter Haar *et al.* [6], the concentration of discodermolide required to induce 50 % assembly of 10 μM tubulin is 3.2 μM, almost three orders of magnitude

Figure 4



Discodermolide binding activity in MG63 cells is found in the fraction containing microtubules. **(a)** Cells treated with 20 nM [^{3}H]discodermolide were lysed and binding activity in the cell pellet as well as in the soluble fraction was measured as described in Materials and methods. Co-treatment with colchicine was carried out at 1 μM. Results are shown as percentage of total counts added. **(b)** Cells were lysed prior to addition of 20 nM [^{3}H]discodermolide, with or without 200 nM taxol.

higher than its IC_{50} (6 nM) for inhibition of proliferation [3]. A similar discrepancy has also been observed for taxol and many other microtubule-affecting reagents. Studies on the effects of taxol on the dynamics of microtubules have led to the suggestion that inhibition of cell proliferation by these drugs at their lowest effective concentrations involves stabilization of mitotic spindle microtubule dynamics, inhibiting the rate and extent of shortening, instead of changing the amount of polymerized tubulin [20,21]. It is likely that discodermolide exerts its antimitotic effect by the same mechanism; by stabilizing microtubules, discodermolide interrupts the dynamics of the mitotic spindle, preventing the spindle from aligning the chromosomes at the metaphase plate.

Significance

Discodermolide is a potent immunosuppressive agent, which inhibits T-cell proliferation. We have shown that discodermolide halts the cell cycle at the metaphase–anaphase transition in mitosis. Our findings suggest that discodermolide exerts its effect by binding and stabilizing mitotic spindle microtubules. Discodermolide induces the polymerization of purified tubulin *in vitro*, as does taxol, an antimitotic and antitumor reagent known to stabilize microtubules. Although they are structurally unrelated, discodermolide and taxol bind to mutually exclusive sites on the tubulin unit. Discodermolide's binding affinity for microtubules is much higher than that of taxol.

The *in vivo* binding activity of [^{3}H]discodermolide is also associated with the microtubule network. Although micromolar concentrations of discodermolide are required to induce microtubule assembly *in vitro*, discodermolide inhibits cell proliferation at low nanomolar concentrations. Discodermolide's cellular effect is probably attributable to an ability to alter mitotic spindle microtubule dynamics, and probably does not require a net increase of microtubule polymers. The fact that discodermolide and taxol have similar cellular actions, and the fact that discodermolide has higher potency than taxol, suggest that discodermolide could be an effective therapeutic agent for the treatment of cancer. Using discodermolide in this way will require a solution to the problem of insufficient supply, however, a situation reminiscent of the early phase of taxol studies.

Materials and methods

Cell cultures

Unless otherwise stated, MG63 cells and Swiss 3T3 cells were maintained at 37 °C with 5 % CO_2 in RPMI-1640 and Dulbecco's minimal Eagle's medium, respectively, both containing 10 % fetal bovine serum (FBS) and streptomycin/penicillin. These reagents were obtained from Gibco-BRL.

Analysis of cyclin B expression

To synchronize MG63 cells, the cells were first grown in 10 % fetal bovine serum to 50–70 % confluency. For G0 arrest, the cells were washed twice with serum-free medium, then incubated in media containing 0.2 % FBS for 48 h. For S-phase arrest, the cells were incubated in regular media containing 2 mM hydroxyurea for 24 h. When the synchronized cells were released from either arrest by changing back to regular medium, discodermolide or taxol was added to 100 nM. At various times cells were washed with phosphate-buffered saline (PBS) and lysed in sodium dodecyl sulphate (SDS) sample buffer. Aliquots of cell lysates equivalent in cell numbers were loaded on 10 % SDS gels, separated by electrophoresis, transferred to a membrane and analyzed using anti-human cyclin B1 monoclonal antibody (Santa Cruz Biotechnology).

Indirect immunofluorescence

Swiss 3T3 cells were plated on coverslips and grown to 50–80 % confluency under normal conditions, followed by drug treatment for 24–36 h. The cells were washed with PBS, fixed in 4 % formaldehyde for 15 min, then in 100 % methanol for 2 min. After extensive washing with PBS, the cells were incubated with anti-β-tubulin monoclonal antibody (Boehringer-Mannheim) in 3 % BSA (bovine serum albumin) for 1 h, washed three times in PBS, and incubated with fluorescein-conjugated anti-mouse IgG antibody (Gibco-BRL) in 3 % BSA for 30 min. Subsequently the coverslips were washed three times in PBS, and mounted on slides in 20 % glycerol saturated with 1,4-diazabicyclo[2,2,2]octane and containing 0.1 μg ml^{-1} propidium iodide.

In vitro *tubulin assembly and discodermolide binding*

Purified tubulin was a generous gift from Heather Deacon and Tim Mitchison's laboratory. A trace amount of GTP may be present in the tubulin samples. Polymerization of 10 μM purified tubulin in the presence of 10 μM discodermolide or taxol was carried out in PEM buffer (80 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$) at 37 °C and measured by turbidity change at 350 nm. To assess discodermolide binding to microtubules, 10 μM tubulin was polymerized with 10 μM taxol in the presence of 1 mM GTP at 37 °C. Upon completion of polymerization, [^{3}H]discodermolide [3] was added at various concentrations. Following incubation at 37 °C for 30 min, microtubules were sedimented in a microfuge at 14 000 rpm for 20 min. The pellets were resuspended in scintillation fluid and counted. The binding efficiency was ~80 %. For competition experiments, 10 μM tubulin was polymerized in the presence of 10 μM [^{3}H]discodermolide (specific activity 8 Ci mmol^{-1}) or [^{3}H]taxol (Moravek Biochemicals, Inc., specific activity 15.7 Ci mmol^{-1}) at 37 °C, and various concentrations of competitors were added subsequently. After incubation at 37 °C for an additional 30 min, the microtubules were collected as above and counted.

Discodermolide binding activity in cells

For *in vivo* binding assays, MG63 cells were treated with 20 nM [^{3}H]discodermolide for 30–36 h. As a control, an excess of cold discodermolide (2 μM) was added together with the tritiated compound. Treated cells were washed in PBS, trypsinized, and lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EGTA, 1 % Triton X-100). After 10 min centrifugation in a microfuge, the pellet and supernatant were separated. The total protein content from the supernatant was precipitated by incubation with 100 μg ml^{-1} γ-globulin and 12.5 % PEG-8000 on ice for 10 min, followed by centrifugation for 10 min. Both the protein pellet and the cell pellet were resuspended in scintillation fluid and counted. The binding assays were also performed in cell lysates as described above, except that untreated cells were lysed in lysis buffer with or without 1 μM taxol prior to the addition of [^{3}H]discodermolide.

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