Synergistic Suppression of Microtubule Dynamics by Discodermolide and Paclitaxel in Non-Small Cell Lung Carcinoma Cells

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ABSTRACT

Discodermolide is a new microtubule-targeted antimitotic drug in Phase I clinical trials that, like paclitaxel, stabilizes microtubule dynamics and enhances microtubule polymer mass in vitro and in cells. Despite their apparently similar binding sites on microtubules, discodermolide acts synergistically with paclitaxel to inhibit proliferation of A549 human lung cancer cells (L. Martello et al., Clin. Cancer Res., 6: 1978–1987, 2000). To understand their synergy, we examined the effects of the two drugs singly and in combination in A549 cells and found that, surprisingly, their antiproliferative synergy is related to their ability to synergistically inhibit microtubule dynamic instability and mitosis. The combination of discodermolide and paclitaxel at their antiproliferative IC50s (7 nM for discodermolide and 2 nM for paclitaxel) altered all of the parameters of dynamic instability synergistically except the time-based rescue frequency. For example, together the drugs inhibited overall microtubule dynamic instability by 71%, but each drug individually inhibited dynamicity by only 24%, giving a combination index (CI) of 0.23. Discodermolide and paclitaxel also synergistically blocked cell cycle progression at G2-M (41, 9.6, and 16% for both drugs together, for discodermolide alone, and for paclitaxel alone, respectively; CI = 0.59), and they synergistically enhanced apoptosis (CI = 0.85). Microtubules are unique receptors for drugs. The results suggest that ligands that bind to large numbers of binding sites on an individual microtubule can interact in a poorly understood manner to synergistically suppress microtubule dynamic instability and inhibit both mitosis and cell proliferation, with important consequences for combination clinical therapy with microtubule-targeted drugs.

INTRODUCTION

Discodermolide (Fig. 1), currently in Phase I clinical trials, is a novel microtubule-targeted antimitotic drug isolated from the marine sponge Discodermia dissoluta (1). Like paclitaxel, discodermolide increases microtubule polymer mass and induces cold- and calcium-stable tubulin polymers (1, 2). In cells, it induces microtubule bundling, cell cycle arrest at the G2-M phase, aneuploidy, and apoptosis (1–4). Discodermolide competitively inhibits the binding of [3H]paclitaxel to microtubules, suggesting that discodermolide and paclitaxel have common or overlapping binding sites (5, 6). As with other microtubule-targeted drugs, low concentrations of discodermolide suppress microtubule dynamics, an action that appears responsible for its ability to inhibit the cell cycle transition from metaphase to anaphase (7). Surprisingly, Martello et al. (8) found that discodermolide and paclitaxel synergistically inhibit proliferation of four human tumor cell lines. However, combinations of paclitaxel with epothilones or eleutherobin (drugs that also bind to the paclitaxel binding site) did not inhibit cell proliferation synergistically (8). The mechanism of discodermolide also differs from that of paclitaxel in that discodermolide cannot substitute for paclitaxel in A549-T12 paclitaxel-resistant and paclitaxel-requiring cells, whereas epothilones and eleutherobin can (8).

Microtubules are highly dynamic cytoskeletal structures that alternate between phases of slow growth and rapid shortening, a process called dynamic instability (9–12). Dynamic instability is crucial for the ability of microtubules to participate in spindle formation and function during mitosis, including attachment of microtubules to the kinetochores of each chromosome, congression of chromosomes to the metaphase plate, signaling of the metaphase/anaphase transition, and anaphase chromosome separation (11).

Because discodermolide and paclitaxel both suppress microtubule dynamics at concentrations that disrupt mitosis and inhibit cell proliferation, we asked whether the synergistic antiproliferative actions of discodermolide and paclitaxel that have been observed in tumor cells might result from synergism in their effects on microtubule dynamic instability. We examined the effects of the two drugs singly and in combination on microtubule dynamic instability in living A549 human lung cancer cells during interphase at drug concentrations that inhibited proliferation. We found that the synergistic effects of discodermolide and paclitaxel on cell proliferation can be explained, at least in part, by a synergistic inhibition of microtubule dynamic instability. We found that the synergistic inhibition of microtubule dynamic instability correlated with synergistic increases in the percentage of G2-M-arrested cells and in apoptosis. The results indicate that drugs like discodermolide and paclitaxel that can bind to large numbers of binding sites on each individual microtubule target may act together in unique ways to alter microtubule architecture or tubulin conformation. In addition, as discussed, the binding of one drug to the microtubule may conceivably influence the binding of the other drug to the microtubule. The results have important consequences for clinical therapy with combinations of drugs that bind to the same or overlapping sites on microtubules.

MATERIALS AND METHODS

Agents. Discodermolide was provided as a gift from Frederick Kinder (Novartis Pharmaceuticals Corp., East Hanover, NJ); paclitaxel was from Sigma (St. Louis, MO), and [3H]paclitaxel (37 Ci/mmol) from Moravek (Brea, CA).

Cell Culture and Proliferation. A549 human non-small cell lung carcinoma cells were maintained in RPMI 1640 containing 10% fetal bovine serum. After incubation with drug for 72 h, cell proliferation was analyzed as described previously (7) using the sulforhodamine B assay (13).

Microinjection of Rhodamine-Labeled Tubulin. Time-Lapse Microscopy, and Image Acquisition. Rhodamine-labeled tubulin was prepared, stored, and microinjected into the cells as described previously (7). Injected cells were incubated for 2 h at 37°C to allow incorporation of the rhodamine-tubulin into the cellular microtubules. Cells were then incubated with drug for 4 h to allow attainment of an equilibrium intracellular drug concentration. The rhodamine-labeled microtubules were observed using a Nikon Eclipse E800
SYNERGISTIC SUPPRESSION OF MICROTUBULE DYNAMICS

Fig. 1. Structures of paclitaxel and discodermolide.

RESULTS

To determine the concentrations of discodermolide and paclitaxel to use to examine their potential synergistic actions on microtubule dynamics, we first determined the effects of the two drugs individually on cell proliferation and mitotic arrest in A549 cells over a range of drug concentrations. A549 cell proliferation was inhibited by 50% at 7 nM discodermolide (7) and at 2 nM paclitaxel (data not shown; 72-h drug incubation; see “Materials and Methods”). As determined by flow cytometry of DNA content after staining of cells with PI (see “Materials and Methods”), each drug singly induced G2-M arrest in a concentration-dependent manner, with half-maximal arrest occurring at 41.5 nM discodermolide and at 4 nM paclitaxel and maximal arrest occurring at 166 nM discodermolide and at 20 nM paclitaxel (Fig. 2).

To determine whether discodermolide and paclitaxel act synergistically to suppress microtubule dynamic instability, we determined the effects of each drug singly on microtubule dynamic instability over the concentration ranges, 7–166 nM for discodermolide and 2–20 nM for paclitaxel. We then determined the effects of the combination of the two drugs at their IC50s for inhibition of proliferation on the parameters of microtubule dynamic instability and calculated the CIs according to the method of Chou and Talalay (14).

Effects of Discodermolide and Paclitaxel on Dynamic Instability Parameters When Added Singly to A549 Cells. After rhodamine-tubulin was microinjected into cells and allowed to incorporate into microtubules, cells were incubated with a range of concentrations of either drug (7, 41.5, 83, and 166 nM discodermolide, or 2, 6, 12, and 20 nM paclitaxel, or a combination of 7 nM discodermolide and 2 nM paclitaxel, or no drug). The plus ends of microtubules in the lamellar regions of the cells were then imaged by time-lapse fluorescence microscopy (Fig. 3, A–C). Images were collected and analyzed as described in “Materials and Methods,” and life history traces of the

microscope maintained at 37 ± 1°C, with a Nikon plan apochromat 1.4 N.A. ×100 objective lens. Thirty to 41 images/cell were acquired at 3-s intervals using a Hamamatsu Orca II digital camera (Middlesex, NJ) driven by Metamorph software (Universal Imaging, Media, PA).

Analysis of Microtubule Dynamic Instability. The positions of the plus ends of individual microtubules in interphase cells over time were recorded and analyzed (7), and the lengths of individual microtubules were graphed as a function of time (life histories). Criteria for determination of rates, durations and lengths of growth and shortening events, transition frequencies, and dynamics were described in detail previously (7). As discussed previously (7), in control cells 69% of the microtubules were dynamic during the period of observation, and the fraction of dynamic microtubules decreased with increasing drug concentration. To determine the mechanistic effects of the drugs on the individual parameters of dynamic instability, only dynamic microtubules could be used for the measurements. Given the concentration-dependent nature of both suppression of dynamic instability and the degree of total stabilization, it is unlikely that adoption of this methodology significantly alters the conclusions concerning synergism at the level of microtubule dynamics.

Drug effects on dynamic instability of microtubules in interphase cells were used as an indication of drug effects in mitosis, the stage at which the cell cycle is arrested. Although we cannot be sure, all of our studies to date indicate that suppression of interphase microtubule dynamics that occurs at drug concentrations that arrest the cells in mitosis is accompanied by suppression of centromere dynamics in mitotic spindles (an indication of suppression of the dynamics of bundles of microtubules attached to the centromeres). In the one instance where mitotic arrest occurred in the absence of suppression of microtubule dynamics (by 2-methoxyestradiol), we also observed mitotic arrest in the absence of suppression of centromere dynamics. Thus, there appears to be a strong correlative relationship between drug effects on interphase microtubule dynamics and on mitotic microtubule dynamics.

Analysis of Drug Synergism. The combination index (CI) was calculated to determine whether the drugs interacted synergistically, additively, or antagonistically (14). The CI is defined by the following equation: CI = (D)1/(Dx)1 + (D)2/(Dx)2, in which (D)1 is the concentration of drug necessary to achieve a particular effect in the combination; (Dx)1 is the concentration of the same drug that will produce the identical level of effect by itself; (D)2 is the concentration of the second drug that will produce a particular effect in the combination; and (Dx)2 is the concentration of the second drug, which will produce the same level of effect by itself. CI > 1 indicates antagonism, CI < 1 indicates synergy, and CI = 1 indicates additivity.

G2-M Block and Apoptosis. After 48 h of drug treatment, adherent and floating cells were collected and incubated (15 min) with annexin-V-FITC (Euromedex, Souffelweyersheim, France) for determining surface exposure of phosphatidylserine in apoptotic cells (15) and propidium iodide (PI) for determining DNA content by flow cytometry (Ref. 16; FACScan, Becton Dickinson, San Diego, CA). DNA distribution was modeled as a sum of cells in G1, G2-M, S phase, and an aneuploid population close to the G1 peak, using ModFitLT software (3).

Intracellular Paclitaxel Concentration. The intracellular concentration of paclitaxel and its intracellular metabolites were determined chromatographically (16). Briefly, cells were incubated with 2 nM [3H]paclitaxel ± 7 nM discodermolide (4 h). Cells were harvested, washed several times in phosphate buffer, and lysed in 60% methanol. Radioactivity of cell lysates was determined by liquid scintillation counting (LS1707; Beckman, Fullerton, CA).


Fig. 2. Concentration-dependence for G2-M arrest (□) by discodermolide and paclitaxel, also showing G2-M arrest (●) induced by the combination of 7 nM discodermolide and 2 nM paclitaxel. Results are from three independent experiments; bars, ±SE. The results were obtained from flow cytometry as shown in Fig. 5.
changes in microtubule length versus time were constructed for 30 individual microtubules for each condition (Fig. 3, D–I). These traces were used to determine the individual dynamic instability parameters (Tables 1 and 2; Supplementary Data). In controls (in the absence of drug; Fig. 3, A–C), microtubules underwent episodes of slow growth and rapid shortening, and sometimes underwent periods of pause or attenuated dynamic instability during which the changes in length were below the resolution of the microscope (see “Materials and Methods”). As determined by analysis of the life history traces, control microtubules shortened at 24 ± 2.1 μm/min and grew at 13.8 ± 1.4 μm/min. The mean growing and shortening lengths in controls were 2.5 ± 0.2 μm and 5.0 ± 0.4 μm, respectively. Control microtubules spent 52% of their time in a paused state, neither growing nor shortening to a detectable extent.

Discodermolide and paclitaxel, when added singly to A549 cells, each suppressed microtubule dynamic instability in a concentration-dependent manner (Fig. 3, E and F). They affected nearly all of the parameters of dynamic instability to similar degrees with respect to their abilities to inhibit cell proliferation (Tables 1 and 2; Supplementary Data). For example, at the IC50 for inhibition of cell proliferation by discodermolide (7 nM), the mean shortening rate was reduced by 20%, the mean shortening length was reduced by 42%, and the dynamicity was reduced by 23%. In comparison, at the IC50 for inhibition of cell proliferation by paclitaxel (2 nM), the mean shortening rate was reduced by 14%, the mean shortening length was reduced by 30%, and the dynamicity was reduced by 25%.

Nearly all of the catastrophe and rescue frequencies were affected similarly by the two drugs at their individual IC50s for inhibition of proliferation. The frequencies of catastrophe and rescue are the frequency of switching from periods of microtubule growth or pause to shortening and from periods of shortening to growth or pause, respectively. They can be calculated either on the basis of time or of distance grown or shortened. At 7 nM discodermolide, the only significant effect was to greatly increase the length-based rescue frequency by 93%. Similarly, at 2 nM paclitaxel, the only significant effect was to greatly increase the length-based rescue frequency by 86%. At higher drug concentrations (>83 nM discodermolide or >6 nM paclitaxel; Supplementary Data), both discodermolide and paclitaxel, when added singly, reduced the time-based catastrophe frequency and increased the time-based and length-based rescue frequencies. Interest-
Table 1  Effects of discodermolide and paclitaxel, singly and in combination, on the parameters of microtubule dynamic instability in living A549 cells

Parameters of microtubule dynamic instability in A549 cells incubated with 7 nM discodermolide or 2 nM paclitaxel, singly, or with the combination of 7 nM discodermolide and 2 nM paclitaxel. The concentrations used singly are the IC_{50}s for inhibition of A549 cell proliferation by each drug applied singly. The percentage change from control values is shown for the parameters that were significantly different from controls. Data for 7 nM discodermolide are from (7).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>7 nM discodermolide</th>
<th>Change</th>
<th>2 nM paclitaxel</th>
<th>Change</th>
<th>7 nM discodermolide + 2 nM paclitaxel</th>
<th>Change</th>
<th>Combination Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate (µm/min)</td>
<td>13.8 ± 1.4</td>
<td>12.8 ± 1.0</td>
<td>2.3 ± 0.6</td>
<td>9.8 ± 0.8a</td>
<td>2.8 ± 3.2</td>
<td>7.4 ± 0.6</td>
<td>2.7 ± 0.3</td>
<td>0.30</td>
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<tr>
<td>Shortening rate (µm/min)</td>
<td>24 ± 2.1</td>
<td>19.1 ± 1.8b</td>
<td>20.7 ± 1.6b</td>
<td>14.2 ± 2.3b</td>
<td>10 ± 2.4</td>
<td>7.1 ± 2.4</td>
<td>2.6 ± 0.8</td>
<td>0.21</td>
</tr>
<tr>
<td>Growth duration (min)</td>
<td>0.18 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Shortening duration (min)</td>
<td>0.21 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Distance-based catastrophe</td>
<td>0.34 ± 0.06</td>
<td>0.29 ± 0.03</td>
<td>0.28 ± 0.03</td>
<td>0.45 ± 0.06b</td>
<td>3.2 ± 0.3</td>
<td>1.0 ± 0.1b</td>
<td>0.31 ± 0.1</td>
<td>0.41</td>
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<tr>
<td>Distance-based rescue</td>
<td>2.5 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>1.7 ± 0.1b</td>
<td>1.8 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>0.21</td>
</tr>
<tr>
<td>% time spent growing</td>
<td>5 ± 0.4</td>
<td>2.9 ± 0.2</td>
<td>3.5 ± 0.3b</td>
<td>1.8 ± 0.1b</td>
<td>7 ± 0.1</td>
<td>8 ± 0.1</td>
<td>6 ± 0.1</td>
<td>0.23</td>
</tr>
<tr>
<td>% time spent shortening</td>
<td>20 ± 26</td>
<td>24</td>
<td>24</td>
<td>12.7</td>
<td>19 ± 0.1</td>
<td>9 ± 0.2</td>
<td>9 ± 0.2</td>
<td>0.23</td>
</tr>
<tr>
<td>% time spent in pause</td>
<td>28 ± 20</td>
<td>20</td>
<td>20</td>
<td>14.3</td>
<td>14 ± 0.1</td>
<td>14 ± 0.1</td>
<td>14 ± 0.1</td>
<td>0.30</td>
</tr>
<tr>
<td>Distance-based catastrophe</td>
<td>9.4 ± 0.6</td>
<td>6.4 ± 0.6</td>
<td>23%</td>
<td>38%</td>
<td>1.1 ± 0.1</td>
<td>7.1 ± 2.5</td>
<td>2.7 ± 0.3</td>
<td>0.23</td>
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<tr>
<td>Time-based rescue frequency</td>
<td>0.08 ± 0.06</td>
<td>0.09 ± 0.06</td>
<td>0.15 ± 0.06</td>
<td>0.21 ± 0.06</td>
<td>0.28 ± 0.06</td>
<td>0.30 ± 0.06</td>
<td>0.36 ± 0.1</td>
<td>0.28</td>
</tr>
<tr>
<td>Distance-based rescue frequency</td>
<td>0.14 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>93%</td>
<td>93%</td>
<td>0.26 ± 0.01</td>
<td>86%</td>
<td>96%</td>
<td>0.28</td>
</tr>
</tbody>
</table>

* P < 0.05, estimate of significance by Student’s t test.
* P < 0.01, estimate of significance by Student’s t test.
* P < 0.001, estimate of significance by Student’s t test.

Table 2  Effects of discodermolide and paclitaxel, singly and in combination, on the frequency of catastrophe and rescue of microtubule dynamic instability in living A549 cells

Transition frequencies in A549 cells treated with 7 nM discodermolide or 2 nM paclitaxel, singly, or with the combination of 7 nM discodermolide and 2 nM paclitaxel. The concentrations used singly are the IC_{50}s for inhibition of A549 cell proliferation by each drug applied singly. The percentage change from control values is shown for the parameters that were significantly different from controls. Data for 7 nM discodermolide are from (7).

<table>
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<th>Change</th>
<th>Combination Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-based catastrophe frequency</td>
<td>1.68 ± 0.12</td>
<td>1.67 ± 0.09</td>
<td>0.01 ± 0.01</td>
<td>0.95 ± 0.06b</td>
<td>343%</td>
<td>7.1 ± 25%</td>
<td>2.7 ± 25%</td>
<td>0.23</td>
</tr>
<tr>
<td>Time-based rescue frequency</td>
<td>3.38 ± 0.30</td>
<td>4.62 ± 0.32</td>
<td>1.24 ± 0.30</td>
<td>4.7 ± 0.15</td>
<td>7 ± 0.1</td>
<td>7.1 ± 25%</td>
<td>2.7 ± 25%</td>
<td>0.23</td>
</tr>
<tr>
<td>Distance-based catastrophe frequency</td>
<td>0.48 ± 0.03</td>
<td>0.44 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>0.75 ± 0.05b</td>
<td>56%</td>
<td>7.1 ± 25%</td>
<td>2.7 ± 25%</td>
<td>0.23</td>
</tr>
<tr>
<td>Distance-based rescue frequency</td>
<td>0.14 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>%93</td>
<td>93%</td>
<td>0.26 ± 0.01</td>
<td>86%</td>
<td>96%</td>
<td>0.28</td>
</tr>
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* P < 0.01, estimate of significance by Student’s t test.
* P < 0.001, estimate of significance by Student’s t test.

Synergistic Inhibition of Microtubule Dynamic Instability by a Combination of Discodermolide and Paclitaxel at Their Antiproliferative IC_{50}s. When added together to A549 cells, the combination of 7 nM discodermolide and 2 nM paclitaxel (the concentrations of discodermolide and paclitaxel that inhibited proliferation by 50% at 72 h incubation) significantly suppressed microtubule dynamic instability (Fig. 3G). The traces in the life history graphs were analyzed, and the values of each parameter in Tables 1 and 2 and the Supplementary Data were graphed (data not shown). From these graphs, the concentration of each drug singly that produced an effect equal to the combination of 7 nM discodermolide and 2 nM paclitaxel was determined from the graphs. The CIs were calculated for each parameter as described in “Materials and Methods” according to the method of Chou and Talalay (14). For example, when cells are treated with both drugs at the same time, the dynamicity was reduced from 9.4 µm/min in controls to 2.7 µm/min (Table 1). In contrast, when the drugs were added singly, it required 132 nM discodermolide to suppress the dynamicity to 2.7 µm/min and 10.8 nM paclitaxel to suppress it to this same value (Supplementary Data). Thus, the CI is 2/10.8 + 7/132 = 0.23, indicating that the combination of discodermolide and paclitaxel acted synergistically to suppress the dynamicity (a CI < 1 indicates synergy, whereas a CI > 1 indicates antagonism). Discodermolide and paclitaxel acted synergistically on nearly all of the parameters of dynamic instability, as shown in the right-hand columns of Tables 1 and 2. The two drugs together induced a dramatic and synergistic decrease in microtubule dynamicity (−71%) as compared with a reduction of only 23–25% for either drug alone (CI = 0.23). In addition, they synergistically inhibited both the growing and the shortening rates (CI = 0.30 and 0.21, respectively), whereas each drug singly inhibited only the shortening rate (Table 1). The combination of the two drugs also reduced both the growing and the shortening lengths synergistically (CI = 0.41 and 0.21, respectively), whereas each drug singly reduced only the shortening length.

Fig. 4. Bar graph showing the percentage changes in several parameters of microtubule dynamic instability that were altered synergistically by 7 nM discodermolide and 2 nM paclitaxel. Values were obtained from data on Tables 1 and 2. GR, growth rate; SR, shortening rate; LBRF, length-based rescue frequency; TBCF, time-based catastrophe frequency; LBCF, length-based catastrophe frequency; OD, overall dynamicity.

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(Table 1). The combination synergistically increased the duration of pause (+32%), the percentage of time spent paused (+21%), the length-based catastrophe frequency (+56%), and synergistically decreased the time-based catastrophe frequency (−43%), whereas each drug singly exerted no significant effect on these parameters (Tables 1 and 2). The length-based rescue frequency was strongly and synergistically increased by 265% (CI = 0.28) by the combination, whereas each drug singly increased this parameter by only ~90% (Table 2). Fig. 4 illustrates the percentage changes in several parameters of microtubule dynamic instability that were altered synergistically by 7 nM discodermolide and 2 nM paclitaxel. The only parameter that was not synergistically altered was the time-based rescue frequency. (The CI was not calculated for this parameter because its value after incubation with the combination was not statistically different from its value with either drug alone.)

Do Discodermolide and Paclitaxel Act Synergistically to Induce G2-M Arrest and Apoptosis? To determine whether the synergistic inhibition of microtubule dynamic instability by the combination of paclitaxel and discodermolide was associated with a similar synergistic effect on G2-M block, we used flow cytometry to analyze the concentration dependence for the effects of each drug alone and for the combination of drugs on the DNA content of A549 cells. Cells were incubated for 20 h with 7–166 nM discodermolide or 2–20 nM paclitaxel or with the combination of 7 nM discodermolide + 2 nM paclitaxel. Fig. 5 shows representative flow cytograms for the distribution of DNA content in control cells and after incubation with 7 nM discodermolide, 2 nM paclitaxel, or the combination of both drugs. Fig. 2 shows the concentration dependence for G2-M arrest (□) as determined from the data of Fig. 5 and similar flow cytograms for each drug concentration. Both drugs increased the percentage of cells arrested in G2-M in a concentration-dependent manner. The percentage of cells in G2-M after incubation with the combination (41 ± 14%) was greater than after incubation with 7 nM discodermolide alone (16 ± 10%) or 2 nM paclitaxel alone (9.6 ± 4.5%). The CI (CI = 0.59 ± 0.04; 95% confidence interval) indicates that the combination of paclitaxel and discodermolide acts synergistically to induce a G2-M block.

We examined the potential ability of discodermolide and paclitaxel to induce apoptosis after 72-h drug incubation using flow cytometry of live A549 cells doubly stained with both annexin-V-FITC and PI (15, 16). Cells were distributed into three populations: (a) viable cells (low FITC and low PI signal) in gate R1; (b) early apoptotic cells (high FITC and low PI signal) in gate R3; and (c) cells that have lost membrane integrity as a result of very late apoptosis (high annexin-V labeling and high PI labeling, gate R4). Percentages of apoptotic cells (gate R3 and gate R4) are indicated on each cytogram. B, discodermolide and paclitaxel concentration dependence for induction of apoptotic cells as determined from cytograms like those shown in A. The percentages of apoptotic cells (R3 + R4) when the drugs were added individually are shown with □ and when added as the combination of 7 nM discodermolide and 2 nM paclitaxel, the results are shown as ■. Representative of four independent experiments; bars, ±SE.
a concentration-dependent manner (Fig. 6B). The percentage of apoptotic cells produced by the combination of discodermolide and paclitaxel (53.8 ± 5.8%) was the same as that produced by either 21 nM discodermolide or 3.8 nM paclitaxel when used singly. The CI (0.85 ± 0.05; 95% confidence interval) indicates that the combination acted with low synergy to induce apoptosis (discussed additionally below).

**Intracellular Paclitaxel Concentrations.** The intracellular paclitaxel concentration was measured with and without coincubation with discodermolide to determine whether the observed synergies might have resulted artifactually from an increase in the intracellular paclitaxel concentration after coincubation. After incubation with 2 nM [3H]paclitaxel (4 h), the intracellular concentrations of paclitaxel did not differ statistically in the presence or absence of 7 nM discodermolide (2.1 ± 0.4 × 10⁻⁸ and 3.4 ± 1.9 × 10⁻⁸ mol/g protein, respectively; P = 0.667). In addition, the intracellular inactive metabolites of paclitaxel were <1% with or without discodermolide (data not shown).

**DISCUSSION**

We have examined the effects of discodermolide and paclitaxel on microtubule dynamic instability in living A549 cells, both singly and in combination, to determine whether the synergistic antiproliferative effects of the drugs observed previously (8) might be because of a synergistic suppression of microtubule dynamics. Surprisingly, in view of the fact that both drugs appear to bind to the same or overlapping sites on microtubules, when added together, the two drugs synergistically suppressed nearly all of the dynamic instability parameters and synergistically induced mitotic block and apoptosis. The drug concentrations used, 7 nM for discodermolide and 2 nM for paclitaxel, were chosen because they half-maximally inhibited cell proliferation when added singly to the cells, and because Martello et al. (8) found that they synergistically inhibited A549 cell proliferation when combined at equipotent antiproliferative concentrations.

**Discodermolide Stabilizes Microtubule Dynamic Instability Similarly to Paclitaxel with One Exception.** It was first necessary to determine the effects of the drugs as single agents. Low concentrations of discodermolide alone (7 nM) or paclitaxel alone (2 nM; the IC₅₀ for proliferation) had nearly identical effects on dynamic instability, qualitatively and quantitatively. Consistent with previous work with paclitaxel (18, 19), the most potent actions of each drug were to suppress the rate and extent of shortening and induce a 23–25% reduction in dynamicity (Table 1). No other parameters were significantly affected except for the length-based rescue frequency (Table 2), which is directly dependent on the length a microtubule shortens during a shortening event. At higher drug concentrations (>7 nM for discodermolide and >2 nM for paclitaxel), each drug suppressed the growing and shortening rates, they increased the percentage of time spent in a paused state, they increased the durations of paused states, they increased the time- and length-based rescue frequency, they decreased the time-based catastrophe frequency, and they strongly suppressed overall dynamicity. When analyzed individually, the two drugs altered the dynamic instability parameters in similar ways, both qualitatively and quantitatively, relative to their ability to inhibit proliferation and block mitosis, except for the length-based catastrophe frequency (Supplementary Tables 1 and 2; Ref. 7).

The length-based catastrophe frequency was increased by 80% at the paclitaxel concentration that induced 80% (IC₅₀) of maximal G₂-M arrest (6 nM paclitaxel), whereas discodermolide had no significant effect on this parameter at the IC₅₀ for G₂-M arrest (83 nM; Supplementary Table 2). These results indicate that, although the mechanisms of action of discodermolide and paclitaxel are very similar, there is an important distinction between the two drugs. The paclitaxel-induced increase in the length-based catastrophe frequency is initially counterintuitive, but it may result from selective stabilization of the microtubule core without accompanying stabilization of the microtubule ends. Discodermolide may bind equally well to the microtubule core and the ends, whereas paclitaxel may bind less well to the extreme ends, thus, allowing “chatter” or frequent short catastrophes and rescues. Chatter was clearly visible in life history traces with paclitaxel (Fig. 3f) but not discodermolide (Fig. 3h). Such chatter was observed previously with epothilone B (20). Microtubules end differ from their cores in that tubulin-GTP or tubulin-GDP-P, (21) is located at the extreme ends, whereas tubulin-GDP makes up the core. Thus, differential actions of drugs on microtubule catastrophes might occur if, for example, paclitaxel and epothilone B, but not discodermolide, bind less well to tubulin-GTP than to tubulin-GDP. Reduced binding of paclitaxel to microtubule ends relative to the cores is suggested by previous experiments performed in vitro (18, 22).

**Synergistic Inhibition of Microtubule Dynamic Instability by the Combination of Discodermolide and Paclitaxel.** Competitive inhibition of [3H]paclitaxel binding to microtubules by discodermolide suggests that discodermolide and paclitaxel may share a common, high-affinity binding site (5, 6). Recent evidence also indicates that there is a second, low-affinity, paclitaxel-binding site on microtubules and that discodermolide binds noncompetitively to this low affinity site (6). Discodermolide may also bind to other distinct sites on microtubules that would not be discernible by competition with [3H]paclitaxel. The striking similarity in the effects of the two drugs on dynamic instability seems to favor a common binding site. Thus, it is surprising that these two drugs can synergize when used simultaneously. For example, other microtubule stabilizing agents that inhibit competitively [3H]paclitaxel binding to microtubules such as eleutherobin and epothilones A and B (23, 24) do not act synergistically with paclitaxel in cells (8).

Several possible mechanisms could explain the synergism between discodermolide and paclitaxel. Microtubules are unusual receptors for drugs in that an individual microtubule contains ~1690 tubulin dimers/μm of length and, thus, very large numbers of binding sites. In addition, occupancy of just a few sites on an individual microtubule exerts powerful suppression of dynamics (17, 25). The two drugs did exert different effects on the length-based catastrophe frequency. One possibility is that the two drugs, although they may bind to the same site, induce subtle differences in the conformation of the tubulins to which they are bound, which, in turn, differentially influences the stability of the microtubule lattice.

It is also conceivable that the binding of one of the drugs to a site on the microtubule enhances the binding of the second drug in a cooperative manner. For example, although paclitaxel and the endogenous microtubule-binding protein tau appear to compete for the same binding site on the interior of the microtubule (26), it was found recently that tau induces paclitaxel to bind to microtubules cooperatively in a tau concentration-dependent manner, indicating that tau induces a conformational change that enhances paclitaxel binding. Similarly, the steep accelerating concentration dependence for paclitaxel-induced G₂-M arrest and induction of apoptosis compared with the more linear curves for discodermolide (Figs. 2 and 6) suggest a possible cooperative binding of paclitaxel. Thus, it is conceivable that paclitaxel might enhance positively the binding of discodermolide to microtubules or enhance the effects of discodermolide binding to microtubules. In addition, calculation of the CI for dynamicity indicates that the major contribution (meaning smallest numerical contri-
bution) to the “less-than-one” CI comes not from the paclitaxel component (2/10.8 = 0.185) but rather from the discodermolide component (7/132 = 0.053; see sample calculation of CI in “Results”). In other words, paclitaxel has a much greater influence on the effects of discodermolide than vice versa.

The synergy may also result from differential binding of the two drugs to the various tubulin isotypes. Paclitaxel appears to have different binding affinities for the different tubulin isotypes, and it differentially affects the dynamic instability of isotopically different microtubules (27). If each drug binds preferentially to different tubulin isotypes, then in a microtubule composed of those isotypes, neighboring tubulin molecules with different drugs bound might affect each other’s conformations uniquely. The changes could lead to an altered microtubule lattice structure and synergy. If the tubulin isotype composition of tumor cells proves to be an important determinant of synergy between discodermolide and paclitaxel, then determination of tumor tubulin isotypes might play an important role in clinical therapeutically strategies. Synergy might also result from differential effects of the two drugs on overall microtubule architecture. The microtubule architecture (e.g., the number of protofilaments) varies under the influence of different drugs. The predominant number of protofilaments in a microtubule is reduced from 14 in controls in vitro to 12 by paclitaxel and eleutherobin, and to 13 by epothilone B, although it remains at 14 in the presence of docetaxel (28, 29).

Finally, microtubule dynamics are highly regulated in cells. Cellular regulators of dynamics (30, 31) may play an important role in the effects of microtubule-targeted drug in cells, as shown recently for stathmin (32, 33). Different conformational changes induced in tubulin by paclitaxel or discodermolide might differentially influence the accessibility of the regulators to the microtubule or their subsequent effects.

We note that, in addition to their synergistic effects on suppression of microtubule dynamic instability, there is recent evidence for additional antiproliferative mechanisms of the antiproliferative synergy of discodermolide and paclitaxel. Discodermolide was found recently to be a potent inducer of accelerated senescence in A549 lung carcinoma cells, whereas paclitaxel was not (34), thus indicating additional differences in their antiproliferative mechanisms.

Combination Therapy with Microtubule-Targeted Drugs. Combination therapies often use drugs with dissimilar mechanisms of action, with the rationale that targeting two independent pathways may result in synergistic efficacy and reduced side effects. Recent studies indicate that combinations of two microtubule-targeted drugs that suppress microtubule dynamics can act synergistically; these combinations include vinorelbine and paclitaxel (35–37), docetaxel and the colchicine analog CB980 (38), and paclitaxel and vinblastine (39). In addition, estramustine acts synergistically or additively in combination with vinblastine or paclitaxel (40, 41), and estramustine and vinblastine act synergistically on microtubule dynamic instability in vitro (42). These synergies are not surprising, in part because the drugs involved bind to different sites on microtubules and in part because they can affect different parameters of dynamic instability or have opposite effects on microtubule polymer mass at high drug concentrations. Unexpectedly, our results demonstrate that paclitaxel and discodermolide, two agents that appear to bind similarly to microtubules and increase microtubule polymer mass at high concentrations, act synergistically by suppressing microtubule dynamic instability in living cells. The results indicate a novel and important strategy for combining low doses of drugs in cancer therapy. They show that the synergistic action of low concentrations of discodermolide plus paclitaxel on microtubule dynamics directly contributes to synergistic G2-M arrest, inhibition of proliferation, and induction of apoptosis. Thus, because of the unique qualities of microtubules as drug receptors, drugs that bind to the same or overlapping sites on microtubules may offer an important new chemotherapeutic strategy.

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Synergistic Suppression of Microtubule Dynamics by Discodermolide and Paclitaxel in Non-Small Cell Lung Carcinoma Cells

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