Taxol and Discodermolide Represent a Synergistic Drug Combination in Human Carcinoma Cell Lines

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ABSTRACT

Recently, three natural products have been identified, the epothilones, eleutherobin, and discodermolide, whose mechanism of action is similar to that of Taxol in that they stabilize microtubules and block cells in the mitotic phase of the cell cycle. In this report, we have compared and contrasted the effects of these new agents in Taxol-sensitive and -resistant cell lines. We also have taken advantage of a human lung carcinoma cell line, A549-T12, that was isolated as a Taxol-resistant cell line and found to require low concentrations of Taxol (2–6 nM) for normal cell division. This study then examined the ability of these new compounds to substitute for Taxol in sustaining the growth of A549-T12 cells. Immunofluorescence and flow cytometry have both indicated that the epothilones and eleutherobin, but not discodermolide, can substitute for Taxol in this Taxol-resistant cell line. In A549-T12 cells, the presence of Taxol significantly amplified the cytotoxicity of discodermolide, and this phenomenon was not observed in combinations of Taxol with either the epothilones or eleutherobin. Median effect analysis using the combination index method revealed a schedule-independent synergistic interaction between Taxol and discodermolide in four human carcinoma cell lines, an effect that was not observed between Taxol and epothilone B. Flow cytometry revealed that concurrent exposure of A549 cells to Taxol and discodermolide at doses that do not induce mitotic arrest caused an increase in the hypodiploid population, thereby indicating that a possible mechanism for the observed synergy is the potentiation of apoptosis. Our results suggest that Taxol and discodermolide may constitute a promising chemotherapeutic combination.

INTRODUCTION

Taxol is an antimitotic agent that enhances the assembly of microtubules and stabilizes them against depolymerization (1) and has been used with success for the treatment of breast, ovarian, and lung carcinomas (2). The cellular target of Taxol is the microtubule, specifically the β-tubulin subunit in the polymer (3, 4). Incubation of cells with Taxol causes the formation of stable bundles or parallel arrays of microtubules, resulting in the arrest of cells in mitosis (5). Low concentrations of Taxol (10 nM) induce a mitotic block without microtubule bundle formation and initiate apoptosis in HeLa cells (6, 7). These observations have led to the concept that Taxol can cause cell death by different mechanisms, depending on the drug concentration (8).

The low aqueous solubility of Taxol and the development of clinical drug resistance have led to a search for new compounds that may have a greater or comparable efficacy relative to Taxol. Ideally, the new agents would be more soluble in aqueous solvents and would be poor substrates for P-glycoprotein, a known mediator of Taxol resistance (9). Several promising antimicrotubule drugs with unique structures unrelated to that of Taxol have been reported to have similar mechanisms of action as Taxol (Fig. 1). Discodermolide was isolated from a marine sponge and reported to induce the assembly of microtubules in vitro more rapidly than Taxol and cause mitotic arrest and microtubule bundling (10–12). Discodermolide has been predicted to be 100-fold more soluble than Taxol and to have a reduced affinity for P-glycoprotein (13). Epothilones A and B, which were isolated from a Myxobacterium fermentation broth, were also found to induce tubulin polymerization, arrest cells in mitosis, and cause the formation of microtubule bundles (14, 15). Epothilone B was reported to be more potent than Taxol and epothilone A in promoting microtubule assembly in vitro (15). The epothilones are 30 times more water soluble than Taxol (16). Structure-activity studies with the epothilones have demonstrated that the acyl region (C1-C8) is essential for Taxol-like activity (17). In addition, the epothilones retained sensitivity in P-glycoprotein-expressing cells that were resistant to Taxol (15). Recently, a fourth microtubule stabilizing agent, eleutherobin, was isolated from a marine soft
coral and shown to have activity comparable to that of Taxol (18). Structure-activity analysis of eleutherobin analogues concluded that the C8 urocanic acid moiety is required for Taxol-like activity (19). Eleutherobin displayed cross-resistance in multidrug resistant cell lines, an effect that was reversible by verapamil, suggesting that eleutherobin is a substrate of P-glycoprotein (19, 20). Although there are no clinical data available to date on the therapeutic activity of these new antimitotic agents, it is their distinct chemical structures and improved aqueous solubility that will influence their therapeutic activity, based on the pharmacokinetics, bioavailability, and metabolism of each drug.

Although Taxol has had clinical success as a single agent and in combination with cisplatin (21), its use in combination with other antitumor agents is now under intense evaluation, particularly for the treatment of advanced or recurrent cancers that are refractory to standard chemotherapy (22). The development of clinical drug resistance has highlighted the need for new chemotherapeutic drugs and new combinations and schedules for these agents. Classically, synergy is defined as greater than additive therapeutic effects when compared with the therapeutic efficacy of each drug alone. Many combination therapies now being tested use drugs with dissimilar mechanisms of action, with the rationale being that targeting two independent pathways will result in enhanced cytotoxicity, whether additive or synergistic (23–26). Nevertheless, one must not discount the use of agents with similar mechanisms of action or molecular targets (27–29).

The present study examined the effects of the three new classes of antimicrotubule agents on a Taxol-resistant human non-small cell lung carcinoma cell line, A549-T12. Although isolated as a Taxol-resistant cell line, it was later found that these cells required low concentrations of Taxol (2–6 nM) for normal growth. The mechanism of resistance/dependence has not yet been determined; however, it is known that these cells do not express P-glycoprotein and do have alterations in their β-tubulin isotype expression (30). The primary objectives of this study were to compare and contrast the cytotoxic profiles of these three new classes of antimitotic agents in Taxol-sensitive and -resistant cell lines and to investigate their ability to substitute for Taxol in the A549-T12 cell line. The results obtained led to an extensive evaluation of the interaction between Taxol and discodermolide in four human cancer cell lines using the CI method.

MATERIALS AND METHODS

Materials. Taxol was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD). Epothilones A and B and eleutherobin were synthesized as described previously (31–34), as was discodermolide (35). All drugs were dissolved in sterile DMSO and stored at −20°C.

Cell Culture. The drug-sensitive mouse macrophage-like cell line J774.2 and its Taxol-resistant cell line, J7-T3-1.6, were maintained as described previously (36). J7-T3-1.6 cells were grown in the presence of 1.6 μM Taxol. Drug-sensitive and vinblastine-resistant human ovarian carcinoma cell lines SKOV3 and SKVLB were from Dr. V. Ling (British Columbia Cancer Research Center, Vancouver, Canada) and were maintained as described previously (19). SKVLB cells were grown in the presence of 1 μM vinblastine. Both J7-T3-1.6 and SKVLB cells overexpress P-glycoprotein. The Taxol-resistant human

3 The abbreviation used is: CI, combination index.
non-small cell lung carcinoma cell line A549-T12 was derived from the drug-sensitive A549 cell line, and both cell lines were maintained as described previously (30). The A549-T12 cell line was grown in the presence of 12 nM Taxol. The human breast carcinoma cell lines MCF-7 and MDA-MB-231 were both maintained in Improved Modified Eagle Medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.

**Cytotoxicity Assays.** Cells were seeded at a density of 1 \( \times 10^4 \) (A549) or 3 \( \times 10^4 \) cells/ml (A549-T12) in triplicate 6-well plates and allowed to attach for 24 h. After incubation with the indicated drug concentrations for 72 h, adherent cells were trypsinized and counted (Coulter counter model ZF0031; Coulter Corp., Miami, FL), and the IC\(_{50}\) was determined. The SKOV3 and SKVLB cell lines were assayed in a similar manner. Cells were seeded in triplicate at a density of 6 \( \times 10^3 \) (SKOV3) or 12 \( \times 10^3 \) cells/ml (SKVLB) in 6-well plates and incubated with drug for 6 days. An accurate IC\(_{50}\) could only be derived after a 6-day incubation period due to the high resistance of these cell lines. J774.2 and J7-T3-1.6 cells were plated at a density of 2 \( \times 10^4 \) cells/well in 96-well plates and allowed to attach overnight. Serial dilutions of each drug were added, and the cells were incubated for 72 h. The IC\(_{50}\) was determined using the CellTiter 96 AQe uous nonradioactive cell proliferation assay (Promega, Madison, WI), which correlates with the number of live cells. Different methods to determine IC\(_{50}\) values were used, depending on the most accurate method for that set of cell lines.

For the drug combination assay, A549-T12 cells were seeded at a density of 4 \( \times 10^4 \) cells/ml in 96-well plates in the absence or presence of various Taxol concentrations plus serial dilutions of the test drug in triplicate. After a 72-h incubation, the plates were assayed using the colorimetric proliferation assay. To sustain the growth of A549-T12 cells in the absence of Taxol, cells were seeded at the higher densities described above.

**Methylene Blue Assay.** Cells were seeded into 24-well plates at a density of 400 (A549) or 500 cells/well (A549-T12) and treated with different concentrations of Taxol. After 8–11 days of growth, the medium was removed, and cells were stained with 0.5% methylene blue (Sigma) in 50% ethanol for 20 min and then rinsed with distilled water.

**Indirect Immunofluorescence.** A549-T12 cells, which were grown to subconfluence on glass coverslips in the absence of drug or in the presence of different drugs at the indicated concentrations, were prepared for immunofluorescence microscopy as described previously (8), with the following modifications: (a) nonspecific binding was blocked using 10% normal goat serum in PBS for 30 min at 37°C; (b) cells were incubated with a monoclonal antibody to α-tubulin (Sigma; 1:100 dilution; 5% normal goat serum in PBS) and then incubated with a Cy3-conjugated antimouse IgG secondary antibody (Amer sham, Arlington Heights, IL; 1:1000 dilution); and (c) slides were analyzed using a Zeiss Axioskop microscope (rhodamine filter) at \( \times 63 \) magnification.

**Flow Cytometry.** For the drug substitution experiments, A549-T12 cells were seeded at a density of 3 \( \times 10^4 \) cells/ml in 75-cm\(^2\) flasks and treated with various drugs at the indicated concentrations. After 48 h, both adherent and nonadherent cells were harvested, fixed in 70% ethanol for at least 20 min, permeabilized with 0.1% Triton X-100 in PBS for 3 min, and stained for 30 min at 37°C with 10 \( \mu \)g/ml propidium iodide (Sigma) in a PBS solution containing 1 \( \mu \)g/ml RNase A (Boehringer Mannheim, Indianapolis, IN). Cell cycle analysis was performed using the Becton Dickinson FACScan and the CellQuest program. For the drug combination studies, A549 cells were seeded at a density of 3 \( \times 10^4 \) cells/ml in 75-cm\(^2\) flasks and grown in the presence of either Taxol, discodermolide, or both at their equipotent ratios of 1:5, respectively, for 24 h. The cells were then prepared as described above.

**Multiple Drug Effect Analysis.** Cells were seeded in triplicate into either 96-well or 24-well plates, and after adherence, the different drugs were added (alone or in combination) for 72 h (96 h for MDA-MB-231 cells). Tenfold serial dilutions were performed for both single drugs and the combinations to obtain good dosage ranges. The doses evaluated were all based on the IC\(_{50}\) values of each individual drug, and combined drug regimens were evaluated at their equipotent ratios, i.e., equivalent to the ratio of their IC\(_{50}\). Dose-response curves were determined from cell survival data obtained using the colorimetric cell proliferation assay (Promega) or cell counts. The CI method of Chou and Talalay (37) was used to analyze the nature of the interaction between Taxol and either discodermolide or epothilone B using Calcsyn software (Biosoft, Cambridge, United Kingdom). In summary, the interaction of the two drugs was quantified by determining a CI at various levels of cytotoxicity or cell kill. CI values of less than or greater than 1 indicate synergism or antagonism, respectively, whereas a value of 1 indicates additivity. The CIs were calculated using both the mutually nonexclusive assumption (dissimilar mechanism of action of both drugs) and the mutually exclusive assumption (similar mechanism of action of both drugs). Each data point represented is the mean ± SE of at least three independent experiments, each of which was performed in triplicate. One-sample t tests and Wilcoxon signed rank tests (two-tailed) were used to determine whether the means and medians, respectively, of the CI values were significantly different from 1.

**RESULTS**

*Cytotoxicity of Antimitotic Agents in Both P-Glycoprotein-expressing and Non-P-Glycoprotein-expressing Cell Lines.* The mouse macrophage-like cell line J774.2 was most sensitive to epothilone B and quite insensitive to eleutherobin (Table 1). The J7-T3-1.6 cell line, which was selected with Taxol and overexpresses P-glycoprotein, displayed significant cross-resistance to vinblastine and low resistance to epothilone A, epothilone B, and discodermolide. Because of the insensitivity of J7-T3-1.6 cells to eleutherobin, it was not possible to accurately determine its cross-resistance in this cell line. A human ovarian carcinoma cell line, SKOV3, displayed the highest sensitivity to epothilone B and vinblastine, with decreased but similar responses to Taxol, epothilone A, discodermolide, and eleutherobin. The resistance pattern for the vinblastine-resistant SKVLB cells, which also overexpress P-glycoprotein, indicated low-level cross-resistance to epothilone A, epothilone B, and discodermolide. However, definite cross-resistance to eleutherobin was exhibited. Experiments examining the steady-state accumulation of \(^{[3]H}\)Taxol indicated that eleutherobin, in contrast to the epothilones and discodermolide, was a substrate for P-glycoprotein (data not shown).
In contrast to Taxol-resistant cells that overexpress P-glycoprotein, the A549-T12 cell line does not express P-glycoprotein and is 9-fold resistant to Taxol. This cell line has a requirement for low levels of Taxol (2–6 nM) for normal growth, whereas the growth of the parental A549 cell line was unaffected by subnanomolar concentrations of Taxol (Fig. 2). At 6 nM, Taxol was cytotoxic in A549 cells; however, A549-T12 cells grew normally in 6 nM Taxol. Epothilone A, eleutherobin, and discodermolide displayed comparable activities in A549 cells, whereas epothilone B was more potent (Table 2). The A549-T12 cell line exhibited cross-resistance to epothilone A, epothilone B, and eleutherobin but no cross-resistance to discodermolide, vinblastine, or colchicine. In the absence of 2 nM Taxol, A549-T12 cells were 20-fold less sensitive to discodermolide (Fig. 3). When Taxol was titrated with a range of discodermolide concentrations, the potency of discodermolide was maximal in the presence of 2 nM Taxol (Fig. 3). At concentrations of Taxol above 2 nM, the combination of discodermolide and Taxol became significantly cytotoxic. Of the drugs evaluated in this study, this effect was seen only with the combination of Taxol and discodermolide.

The A549-T12 Cell Line Exhibits a Normal Microtubule Cytoskeleton in the Presence of the Epothilones and Eleutherobin. When A549-T12 cells were grown in the absence of Taxol for 48 h, the microtubule cytoskeleton appeared diminished when examined by immunofluorescence (Fig. 4E). In contrast, A549-T12 cells exhibited a normal microtubule cytoskeleton in the presence of Taxol, epothilone A, epothilone B, and eleutherobin (Fig. 4, A–D), indicating that the epothilones and eleutherobin were able to replace Taxol in this cell line. The different concentrations of the various drugs used reflect their distinct cytotoxic potencies. Neither discodermolide nor vinblastine was able to substitute for Taxol in A549-T12 cells (Fig. 4, F and H). At higher concentrations of discodermolide (≥12 nM), the microtubules became arranged in bundle-like formations at the periphery of the cell (Fig. 4G), an effect that was not observed with higher doses of the other drugs tested (data not shown).

The Epothilones and Eleutherobin Can Substitute for Taxol in the Taxol-dependent Cell Line and Reverse the Mitotic Block Caused by Taxol Removal. Cell cycle analysis by flow cytometry revealed a normal cell cycle profile.

### Table 1 Cytotoxicity of antimitotic agents in drug-resistant cell lines that overexpress P-glycoprotein

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Taxol (IC₅₀ (nM))</th>
<th>VBL (IC₅₀ (nM))</th>
<th>EpoA (IC₅₀ (nM))</th>
<th>EpoB (IC₅₀ (nM))</th>
<th>Disco (IC₅₀ (nM))</th>
<th>Eleu (IC₅₀ (nM))</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774.2</td>
<td>67 ± 7.0³</td>
<td>6.0 ± 2.7</td>
<td>10.6 ± 2.0</td>
<td>0.4 ± 0.1</td>
<td>36 ± 5.5</td>
<td>394.2 ± 5.8</td>
</tr>
<tr>
<td>J7-T3-1.6</td>
<td>18,000 ± 2,645</td>
<td>767 ± 351</td>
<td>170 ± 14</td>
<td>12 ± 4.0</td>
<td>1,050 ± 70</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Fold resistant</td>
<td>268.7</td>
<td>127.8</td>
<td>16</td>
<td>30</td>
<td>29.2</td>
<td>&gt;12.7</td>
</tr>
<tr>
<td>SKOV3</td>
<td>2.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>10.3 ± 4.5</td>
<td>0.5 ± 0.09</td>
<td>9.7 ± 0.3</td>
<td>8.2 ± 3.8</td>
</tr>
<tr>
<td>SKVLB</td>
<td>13,333 ± 1,154</td>
<td>1,880.3 ± 347.5</td>
<td>190 ± 14</td>
<td>9.5 ± 2.1</td>
<td>575 ± 233</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Fold resistant²</td>
<td>6,666.5</td>
<td>1,709.4</td>
<td>18.5</td>
<td>19.0</td>
<td>59.3</td>
<td>&gt;609.8</td>
</tr>
</tbody>
</table>

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³ IC₅₀, drug concentration that inhibits cell division by 50% after 72 h (J774.2 and J7-T3-1.6) or 6 days (SKOV3 and SKVLB). VBL, vinblastine; EpoA, epothilone A; EpoB, epothilone B; Disco, discodermolide; Eleu, eleutherobin.

² Mean ± SE.

¹ Ratio of IC₅₀ for the resistant cell line (J7-T3-1.6) to that for sensitive cell line (J774.2) after 72 h.

² Ratio of IC₅₀ for the resistant cell line (SKVLB) to that for sensitive cell line (SKOV3) after 6 days.
for A549-T12 resistant cells in the presence of 2 nM Taxol (Fig. 5A). When Taxol was removed, the resistant cells became blocked at the G2-M-phase transition (Fig. 5E). In the presence of epothilone A, epothilone B, or eleutherobin, the A549-T12 cells also exhibited a normal cell cycle profile (Fig. 5, B–D). In contrast, the addition of discodermolide (2 or 6 nM) did not prevent the development of a G2-M-phase block in the resistant cell line (Fig. 5, F and G). These data corroborate the immunofluorescence results and show that discodermolide is unable to substitute for Taxol in A549-T12 cells. At lower concentrations of discodermolide (0.001–1 nM), the cells also displayed a G2-M-phase block. At higher concentrations of discodermolide (12–24 nM), there was a substantial increase in the hypodiploid population, resulting in the loss of the cell cycle profile (Fig. 5H). A549-T12 cells also demonstrated a mitotic block in the presence of vinblastine (data not shown).

Flow cytometry was used to investigate whether the sustained G2-M-phase block induced by Taxol removal could be reversed in the presence of the epothilones, eleutherobin, and

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**Table 2** Cytotoxicity of antimitotic agents in a Taxol-resistant cell line that does not express P-glycoprotein

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Taxol</th>
<th>EpoA</th>
<th>EpoB</th>
<th>Eleu</th>
<th>Disco</th>
<th>VBL</th>
<th>CLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>2 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.4 ± 2.0</td>
<td>0.7 ± 0.1</td>
<td>14 ± 2.8</td>
<td>8.1 ± 0.14</td>
<td>1.8 ± 0.21</td>
<td>33 ± 10.6</td>
</tr>
<tr>
<td>A549-T12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.7 ± 0.9</td>
<td>34 ± 3.1</td>
<td>3.3 ± 0.72</td>
<td>69 ± 11.3</td>
<td>6.5 ± 2.4</td>
<td>1.8 ± 0.18</td>
<td>33 ± 12.7</td>
</tr>
<tr>
<td>Fold resistant&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.4</td>
<td>5.3</td>
<td>4.7</td>
<td>4.9</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> IC<sub>50</sub>, drug concentration that inhibits cell division by 50% after 72 h. EpoA, epothilone A; EpoB, epothilone B; Eleu, eleutherobin; Disco, discodermolide; VBL, vinblastine; CLC, colchicine.

<sup>b</sup> Mean ± SE.

<sup>c</sup> Cells were maintained in 2 nM Taxol during cross-resistance experiments.

<sup>d</sup> Ratio of IC<sub>50</sub> for A549-T12 resistant cell line to that for the A549 sensitive cell line.
discodermolide (data not shown). A549-T12 cells were grown in the absence of Taxol for 48 h and then incubated with various concentrations of Taxol, epothilone A, epothilone B, and eleutherobin for 48 h, which resulted in the return of a normal cell cycle profile. There also was an increase in the hypodiploid population after reversal of the G2-M-phase block. The mitotic block in the Taxol-dependent cells was irreversible in the presence of vinblastine or a range of discodermolide concentrations (0.001–24 nM).

**Fig. 4** A549-T12 cells display a normal microtubule cytoskeleton in the presence of the epothilones and eleutherobin. A549-T12 cells were grown on coverslips and treated with the different compounds for 48 h. Cells were then permeabilized, fixed, and incubated with an α-tubulin antibody, as described in “Materials and Methods.” A, 2 nM Taxol; B, 2 nM epothilone A; C, 0.1 nM epothilone B; D, 18 nM eleutherobin; E, no drug; F, 2 nM discodermolide; G, 12 nM discodermolide; H, 2 nM vinblastine.

discodermolide (data not shown). A549-T12 cells were grown in the absence of Taxol for 48 h and then incubated with various concentrations of Taxol, epothilone A, epothilone B, and eleutherobin for 48 h, which resulted in the return of a normal cell cycle profile. There also was an increase in the hypodiploid population after reversal of the G2-M-phase block. The mitotic block in the Taxol-dependent cells was irreversible in the presence of vinblastine or a range of discodermolide concentrations (0.001–24 nM).

**Taxol and Discodermolide Are a Synergistic Drug Combination in Various Human Carcinoma Cell Lines.** To extend the observation made in A549-T12 cells that the cytotoxicity of discodermolide was potentiated in the presence of Taxol (Fig. 3) and to fully evaluate the nature of the interaction of Taxol with discodermolide, we analyzed the combination of both drugs using flow cytometry and multiple drug effect analysis. Flow cytometric analysis revealed an increase in the hypodiploid population of A549 drug-sensitive cells when the cells were exposed concurrently to nanomolar concentrations of Taxol and discodermolide (up to 5 nM Taxol/25 nM discodermolide; Fig. 6). At these concentrations, there was no subsequent rise in the number of cells in the G2-M phase of the cell cycle. Only at high concentrations of both Taxol and discodermolide (10 nM Taxol/50 nM discodermolide) was there a
concomitant increase in the number of cells in the mitotic phase (data not shown).

Multiple drug effect analysis used the method of Chou and Talalay (37), which resolves the degree of synergy, additivity, or antagonism at various levels of cell kill. For these experiments, the interaction of Taxol with epothilone B was used as a positive control to verify that other microtubule-stabilizing agents did not have the same interaction with Taxol as discodermolide.

Fig. 5 The epothilones and eleutherothin, but not discodermolide, can substitute for Taxol in the A549-T12 Taxol-requiring cell line. Cells were incubated with the different agents for 48 h, fixed, stained with propidium iodide, and analyzed by flow cytometry as described in “Materials and Methods.” A, 2 nM Taxol; B, 2 nM epothilone A; C, 0.5 nM epothilone B; D, 18 nM eleutherobin; E, no drug; F, 2 nM discodermolide; G, 6 nM discodermolide; H, 12 nM discodermolide.

Fig. 6 Concurrent Taxol and discodermolide exposure caused an increase in the hypodiploid fraction of A549 cells. Cells were incubated with either Taxol, discodermolide, or a combination of the two for 24 h; fixed; stained with propidium iodide; and analyzed by flow cytometry as described in “Materials and Methods.” A, 0.1 nM Taxol; B, 1 nM Taxol; C, 5 nM Taxol; D, 0.1 nM discodermolide; E, 5 nM discodermolide; F, 25 nM discodermolide; G, 0.1 nM Taxol + 0.5 nM discodermolide; H, 1 nM Taxol + 5 nM discodermolide; I, 5 nM Taxol + 25 nM discodermolide.

Fig. 7 summarizes the multiple drug effect analysis of four human cancer cell lines, which is represented as fractional cell growth inhibition (FA) as a function of the CI. Because it could not be determined whether the interactions between the various classes of microtubule-binding agents were mutually exclusive or nonexclusive, the CI values were routinely calculated using both methods, which gave almost identical results in all experiments. The data presented here summarize the CI values based
on the more conservative assumption of mutual nonexclusion. CI values for the combination of concurrent Taxol with discodermolide were significantly less than 1 in all four cell lines, indicating a synergistic drug interaction. In all cell lines tested, this interaction was effective over a 3–4 fold concentration of either drug. We also evaluated the effects of sequential drug exposure, in which either Taxol or discodermolide was administered alone for 24 h before administration of the second drug. Sequencing of the drugs also resulted in the same magnitude of synergism as concurrent exposure and was independent of drug schedule (data not shown). Conversely, additive interactions were observed in all four cell lines after concurrent exposure to both Taxol and epothilone B, indicating that the synergism observed between Taxol and discodermolide was not shared by other microtubule-polymerizing agents.

DISCUSSION

Taxol has proven to be one of the most interesting antitumor agents of the past decade. In addition to its clinical activity in a variety of human malignancies, its mechanism of action, which includes stabilization of microtubules, formation of parallel arrays of microtubules, and activation of a number of signal transduction pathways, has encouraged scientists to continue investigation of this drug. Despite its considerable clinical success, there are serious problems with Taxol. One problem is related to the extreme hydrophobic nature of the compound, which has made its formulation a continual problem. A second problem is the development of drug resistance in human tumors, some of which is related to the overproduction of P-glycoprotein. Based on the success of Taxol in the clinic, there has been a continual search for new small molecules with Taxol-like activity.

During the past few years, new natural products from diverse sources and with distinct chemical structures have emerged. Despite their structural diversity, a model has been proposed that depicts a common pharmacophore linking these agents (38). The epothilones, eleutherobin, and discodermolide all have mechanisms of action with definite similarities to those of Taxol. In our in vitro microtubule polymerization assay, Taxol, the epothilones, eleutherobin, and discodermolide all enhance the assembly of stable microtubules in the absence of GTP, which is normally required for normal in vitro microtubule assembly.

Here we report a comprehensive analysis of the cross-resistant profiles of the epothilones, eleutherobin, and discodermolide in Taxol-sensitive and -resistant cell lines, which have different mechanisms of resistance. The data indicate little, if any, cross-resistance of the epothilones and discodermolide in Taxol-resistant cell lines that overproduce P-glycoprotein. These data are consistent with the results obtained from the Taxol accumulation study in a Taxol-resistant murine cell line in which the epothilones and discodermolide were unable to reverse the reduction in Taxol accumulation, reinforcing the finding that they are not substrates for P-glycoprotein.

Our study has taken advantage of an interesting cell line, A549-T12, that was isolated in our laboratory as a Taxol-resistant cell line and is maintained in 12 nM Taxol. A549-T12 cells are approximately 9-fold resistant to Taxol and do not
overproduce P-glycoprotein. On further examination, it was observed that the cells grew poorly, if at all, in the absence of Taxol. We report that after 48 h in the absence of Taxol, the microtubule cytoskeleton of A549-T12 cells is diminished, and the cells undergo a mitotic block; however, the addition of Taxol after 48 h reverses these abnormalities. It appears that the cells have modified their normal biochemistry to survive in the presence of the drug to such an extent that they have become dependent on the drug for normal growth. A possible mechanism for this phenomenon may be the selection of a variant form of tubulin whose microtubules are normally unstable but thrive in the presence of low concentrations of Taxol. Taxol-requiring Chinese hamster ovary cell lines have been described previously (39).

The availability of the A549-T12 cell line has allowed us to distinguish discodermolide from the other antimitotic compounds described in this study. The epothilones and eleutherobin demonstrated cross-resistance in A549-T12 cells and could substitute for Taxol, reversing the G2-M-phase block that is induced after Taxol withdrawal. This is in contrast to what we observed with discodermolide, which did not exhibit cross-resistance and is unable to substitute for Taxol. In fact, it is clear that in A549-T12 cells, low concentrations of Taxol are required for discodermolide to exert its maximum cytotoxic effects, thereby suggesting that the cellular substrate for discodermolide is a microtubule whose conformation has been modified by Taxol. A previous report has alluded to the requirement of an intact microtubule cytoskeleton for discodermolide binding to occur in cells (11). Vinblastine, like Taxol, is an antimitotic agent whose cellular target is also the tubulin/microtubule system. In contrast to Taxol, its major binding site is the tubulin dimer and in vitro, the drug promotes microtubule depolymerization (40). Although vinblastine is an excellent substrate for P-glycoprotein, and we observed high cross-resistance in the cell lines overproducing P-glycoprotein, there is no cross-resistance in the A549-T12 cell line. The cross-resistance data with the various antimitotic agents in A549-T12 cells are intriguing and require a thorough understanding of the mechanisms of resistance and dependence in the A549-T12 cell line.

Median effect analysis using the CI method of Chou and Talalay (37) confirmed a synergistic interaction between Taxol and discodermolide. In contrast, the combination of discodermolide and epothilone B was additive, indicating that there is a specific relationship between Taxol and discodermolide resulting in synergistic cytotoxicity. We have also shown that low concentrations of Taxol and discodermolide caused an increase in the hypodiploid population of cells without a corresponding increase in the G2-M-phase cell population. We therefore speculate that the synergism observed is probably due to the potentiation of apoptosis by this drug combination, although the precise mechanism remains to be determined.

Previous work has shown a schedule-dependent synergistic interaction between Taxol and vinblastine, both of which bind to separate distinct targets on the tubulin macromolecule (41). Here we describe synergy between Taxol and discodermolide, two drugs that apparently bind to the same or overlapping sites on β-tubulin (11). It is uncommon for two drugs that bind to identical sites on the same target to synergize when administered concurrently or in sequence. More often, this type of drug combination results in additivity (similar to what we observe with Taxol and epothilone B) or antagonism because both drugs cannot bind the same site simultaneously. In A549-T12 cells, discodermolide does not exhibit cross-resistance, unlike the epothilones and eleutherobin. Furthermore, it has been reported that epothilone A-resistant ovarian carcinoma cells that do not express P-glycoprotein exhibit cross-resistance to Taxol, baccatin, and taxotere but do not exhibit cross-resistance to discodermolide (42). Taken together, these findings imply that the Taxol and discodermolide binding sites may be overlapping rather than identical.

Alternatively, the mechanism of synergy may be completely unrelated to the tubulin-binding properties of discodermolide, which was originally described in the literature as an immunosuppressant (43). In addition, discodermolide has been shown to modulate the expression of interleukin 2 receptors, which in turn regulate Fas-induced and nuclear factor κB-induced apoptosis, suggesting a hypothetical mechanism by which the synergy we have observed with Taxol and discodermolide could potentiate apoptosis (44).

At the present time, there is no information available on the antitumor activity of these new drugs in human tumors, and it will be of great interest to compare them with Taxol. Our data suggest that Taxol and discodermolide may represent a synergistic drug combination that merits exploration.

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Taxol and Discodermolide Represent a Synergistic Drug Combination in Human Carcinoma Cell Lines
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