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Peloruside A Synergizes with Other Microtubule Stabilizing Agents in Cultured Cancer Cell Lines

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Abstract: The microtubule stabilizing agent peloruside A binds to a unique site on the tubulin α,β-heterodimer compared to taxoid site drugs such as paclitaxel (Taxol), docetaxel (Taxotere), epothilone A, and discodermolide. Because the binding sites differ, peloruside A may be able to synergize with these taxoid site drugs when added in combination to cultured cells. Ovarian carcinoma cells (1A9) and myeloid leukemic cells (HL-60) were treated with different concentrations of peloruside A and taxoid site drugs, both compounds given singly and in combination in the nanomolar range, and the antiproliferative activity, G_2/M blocking potency, and microtubule stabilizing activity of the treatments assessed. Cell proliferation was monitored using the MTT cell proliferation assay, cell cycle block was determined by flow cytometry, and stabilization of the tubulin polymer was assessed by Western blotting for β-tubulin distributions in supernatant and pellet fractions of cell lysates. A combination index (CI) was calculated from the equation $CI = D_1/Dx_1 + D_2/Dx_2$ in which D_1 and D_2 are the concentrations of drug 1 and drug 2 that in combination give the same response as drug 1 alone (Dx_1) or drug 2 alone (Dx_2). A CI of less than 1 indicates synergy, equal to 1, additivity, and greater than 1, antagonism. Confidence intervals for each CI value were obtained using a bootstrapping procedure. In cell proliferation assays, statistically significant synergy was found between peloruside A and paclitaxel and epothilone A. Combinations of these two taxoid site drugs, however, also showed synergy in their effects on cell proliferation. These results confirm that peloruside A, when added in combination with other microtubule stabilizing agents, acts synergistically to enhance the antimitotic action of the drugs, but also highlight the complexity of drug interactions in intact cells.

Keywords: Peloruside; paclitaxel; epothilone; synergy; anticancer drug; G_2/M arrest

Introduction

Peloruside A (PelA) is a secondary metabolite of the New Zealand marine sponge *Mycale hentscheli,* found in Pelorus Sound, New Zealand.[1] PelA has a 16-membered macrolide ring similar to the epothilones (Epo), and is cytotoxic at nanomolar concentrations.[2,3] Like paclitaxel (Ptx; Taxol) and other microtubule stabilizing agents, PelA binds to and

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stabilizes the polymerized form of tubulin, thereby blocking cells in G_2/M of the cell cycle.[4] PelA is a good candidate for development as an anticancer drug. It has a similar mechanism of action to Ptx and docetaxel (Taxotere), but has a number of distinct advantages compared to the taxanes. For example, it is less lipophilic and therefore easier to solubilize and deliver as a drug. Since cremophor vehicle (Cremophor EL, a polyoxyethylated castor oil) would not be needed to deliver PelA to the body, the cremophor-induced hyperallergic reactions seen with Taxol therapy[5] should not be present. PelA is also more likely to be effective against cells that express the multidrug resistance (MDR) phenotype.[6] Most microtubule stabilizing drugs like Ptx, docetaxel, Epo, and discodermolide bind to a well-defined site (taxoid site) on β-tubulin.[7] However, like laulimalide, another microtubule stabilizing agent from a marine sponge,[8] PelA has a unique binding site on tubulin that differs from the taxoid site;[6] therefore, β-tubulin mutant cell lines with amino acid substitutions in the taxoid binding site are more susceptible to PelA[6] and laulimalide[8] than to Ptx or Epo. Using docking and QSAR studies, Pineda et al.[9] provided evidence that both PelA and laulimalide bind to a distinct site on the α-tubulin monomer, not the β-tubulin monomer like the taxoid drugs. Further support for an α-tubulin binding site was recently presented for PelA in an independent study using tr-Noesy NMR.[10] The uniqueness of the laulimalide/PelA binding site offers an opportunity for synergistic interactions in the combined action of PelA with one or more of the taxoid drugs. The use of combinative therapy in cancer treatment is an approach with great potential for improving the efficacy of anticancer drugs. Combining two drugs that act synergistically would allow treatment with lower doses of each drug, yet deliver greater effects of the drugs at these reduced concentrations. A major problem of a water insoluble drug like Ptx is the difficulty of delivering the high plasma levels that are needed to have an effect on cancer cells. A combination of Ptx and a more soluble drug like PelA might overcome this problem. Administration of less Ptx would also reduce the side effects caused by the cremophor. Previous studies by Hamel et al.[11] have shown that PelA, as well as laulimalide,[12] reacts synergistically with a range of taxoid drugs in its ability to polymerize isolated, purified tubulin. In intact cell cytotoxicity studies, laulimalide was unable to synergize with taxoid site drugs in one study,[8] but was later shown to synergize with Ptx and 2-methoxyestradiol in a lung (A549) and breast (MDA-MB-435) cancer cell line.[13] Interestingly, two synthetic analogues of laulimalide gave better synergy than the parent compound in this study.

The chemical interactions between combinations of drugs are complex, and the mechanisms behind synergism are largely unknown. For example, Ptx has been shown to act synergistically with another taxoid site drug discodermolide,[14] and this synergy appeared to involve an inhibition of the dynamic instability of microtubules.[15] It was suggested that discodermolide bound to a second, low affinity, binding site on tubulin in addition to the classic taxoid binding site. Docetaxel and Ptx have also been reported to act synergistically in breast cancer cells, but only if the cell line overexpressed the Pgp efflux pump (MCF7ADR+ cells).[16]

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In this same study, a combination of the two drugs acted antagonistically in the parental cell line (MCF7WT), and another breast cancer cell line that lacked the MDR phenotype (BT 474) showed only an additive effect of Ptx and docetaxel. A more recent study by Huang et al.[17] showed synergy in vivo between Ptx and discodermolide in SKOV-3 human ovarian carcinoma xenograft-bearing mice with regard to tumor growth, but found that Ptx and EpoB combination therapy was no more effective than EpoB treatment on its own, despite these two drugs being additive when added to cultured cells in vitro. It is also possible that synergy between taxoid site agents could arise as a result of differential expression of tubulin isoforms. For example, it has recently been shown using computer modeling that three taxoid site drugs have different affinities for the β-tubulin isoforms βI- and βIII-tubulin, with Ptx having a higher affinity for the most common isoform βI; whereas a taxane analogue IDN5390 binds better to βIII, and EpoA binds equally well to both isotypes.[18] These differences in binding reflect changed conformation of the M loop as a result of the amino acid in position 277 being either serine (β-I) or alanine (β-III).

The aim of the present study was to investigate synergy between PelA, a microtubule stabilizing agent that does not bind to the taxoid site on β-tubulin, and selected taxoid site drugs in cultured cells by testing for the combined effects of the drugs at three levels of complexity: cell growth inhibition, G_2/M arrest, and in situ tubulin polymerization. Two different cell lines, a human ovarian carcinoma cell line (1A9) and a human promyelocytic leukemic cell line (HL-60), were used. Drug interactions were measured with Berenbaum's combination index;[19] variability in this index due to natural variation characteristic of drug−target interactions was estimated with a bootstrap confidence interval.[20,21]

Materials and Methods

Materials. Peloruside A (PelA) was isolated and purified from the marine sponge *M. hentscheli*[1] and stored as a 1 mM solution in absolute ethanol at −20 °C. Paclitaxel (Ptx) was purchased from Sigma Chemical Co. (St. Louis, MO), and epothilone A (EpoA) was purchased from Calbiochem (La Jolla, CA). The 1A9 human ovarian cancer cell line was a gift from Dr. Paraskevi Giannakakou (Cornell University, Ithaca, NY), and the HL-60 human promyelocytic leukemic blood leukocyte cell line was a gift from Dr. Michael Berridge, Malaghan Institute of Medical Research, Wellington, New Zealand.

Cell Culture and Cell Proliferation Assay. 1A9 cells and HL-60 cells were cultured at 37 °C in a 5% CO_2 in air atmosphere using standard cell culture techniques. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% FCS, 100 units/mL penicillin, and 100 units/mL streptomycin. 1A9 cells were additionally supplemented with 0.25 units/mL insulin (Sigma). A cell proliferation assay was used that involved the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide (MTT) by viable cells,[22] as previously described.[2] For growth inhibition studies, cells were treated with drugs for 48 h in 96-well plates and then incubated with MTT solution (5 mg/mL in PBS) for 2 h. The blue crystals were then solubilized in 10% SDS, 45% dimethylformamide (pH 4.5, adjusted with acetic acid), and MTT reduction was measured the next day by absorbance at 570 nm in a multiwell plate reader (Versamax, Molecular Devices, Sunnyvale, CA).

G_2/M Block. 1A9 cells were treated with drugs for 18 h to optimize the percentage of cells in G_2/M arrest. Cells were collected after detachment in trypsin−EDTA (0.05% trypsin, 0.53 mM EDTA) and were washed in PBS, and 250 μL of propidium iodide (PI) solution (0.05 mg/mL PI, 0.1% sodium citrate, 0.1% Triton X-100) was added. After staining for 15 min, DNA content was determined in a flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell cycle analysis was performed using BD Cell Quest Pro software (Becton Dickenson).

Tubulin Polymerization Assay. An in situ cellular assay was used to monitor the shift in tubulin from its depolymerized form to its polymerized form, as described originally by Giannakakou et al.[23] HL-60 cells were treated with drugs for 16 h, harvested, and lysed on ice for 1 h in 100 μL of cell lysis buffer consisting of 1 mM $MgCl_2$, 2 mM EGTA, 1% NP40, 50 mM Tris-HCL pH 6.8, and 10 μL/mL protease inhibitor cocktail (Sigma). Supernatant and pellet fractions were collected by centrifugation at 16 000 rpm for 10 min at 4 °C in a refrigerated benchtop centrifuge (Eppendorf model 5415R). The supernatants were transferred into new tubes and kept on ice. The pellets were resuspended in 100 μL of sample buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS) and sonicated (20 pulses at a 20% duty cycle over 2 s intervals; power setting 2) with an ultrasonic

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processor microtip probe (model W-380, Heat Systems Ultrasonics, Biologics-Inc, Manassas, VA). To each 100 μL sample, 20 μL of loading buffer was added consisting of 4 M SDS, 6.1 M glycerol, 0.22 M Tris-HCL, pH 6.8, and 0.75 mM bromophenol blue, supplemented with 10% β-mercaptoethanol just prior to use. The samples were boiled for 5 min, and then 30 μL was added to each lane of a 10% SDS polyacrylamide gel. After electrophoretic separation, the proteins were transferred to low background fluorescence Immobilon FL membrane (Millipore Corp, Billerica, MA), and β-tubulin bands were identified by immunoblotting with mouse anti-β-tubulin monoclonal antibody (1:1000) (Becton-Dickinson) and goat anti-mouse IgG secondary antibody labeled with either Cy3 or Cy5 (1:2500) (Amersham, Buckinghamshire, U.K.). The transfer membrane was scanned on a fluorescence scanner (Image Reader FLA-5000, Fuji Photo Film Co, Tokyo, Japan), and bands were quantified using Image J program (Wayne Rasbond, National Institutes of Health, U.S.A.).

Statistical Analysis. In the cell growth inhibition studies, a family of four-parameter logistic curves was fitted to dose−response data for each drug given singly. Each plate of cells was assumed to have a different average number of cells before administration of drug, so that the response to control varied from plate to plate. We also assumed that the effect of each dosage of drug reduced this number by a certain percentage, which was the same for all plates. The IC_{50} and the slope of the dose−response curve, β, were assumed to be the same for all plates. Specifically, the following equation was fitted to dose−response data from each drug:

$$y_{ij} = p \max_i + \frac{\max_i(1-p)}{1+\exp\{\beta(\log(x_j) - \log(IC_{50}))\}}$$

where y_{ij} is the response in plate i to dose x_j, $\max_i$ is the maximal response in plate i, and p is the ratio of the minimum to maximum responses, which is assumed to be a constant for each drug over all plates.

Data from 4, 10, and 12 plates (1A9 cells) and 4, 8, and 8 plates (HL-60 cells) were used to estimate the family of dose−response curves for EpoA, PelA, and Ptx, respectively. Data were log-transformed before analysis to stabilize the variance across dosages. The fit to this model was very good, with the percent of variation explained by the model (R^2 values) ranging from 88% (Ptx) to 93% (EpoA) for the 1A9 cells and from 90% (Ptx) to 97% (peloruside and EpoA) for the HL-60 cells. Examples of typical dose−response curves generated for a set of plates are presented in Figure 1.

In the G_2/M block studies, a single four-parameter logistic curve was fitted to all dose−response data from each drug (see for example R documentation http://rss.acs.unt.edu/Rdoc/library/drc/html/l4.html). The fit of this model to the data was moderate to very good with R^2 values ranging from 62% (EpoA) to 76% (Ptx) to 94% (PelA) for the 1A9 cells and ranging from 83% (Ptx) to 94% (PelA) to 99% (EpoA) for the HL-60 cells.

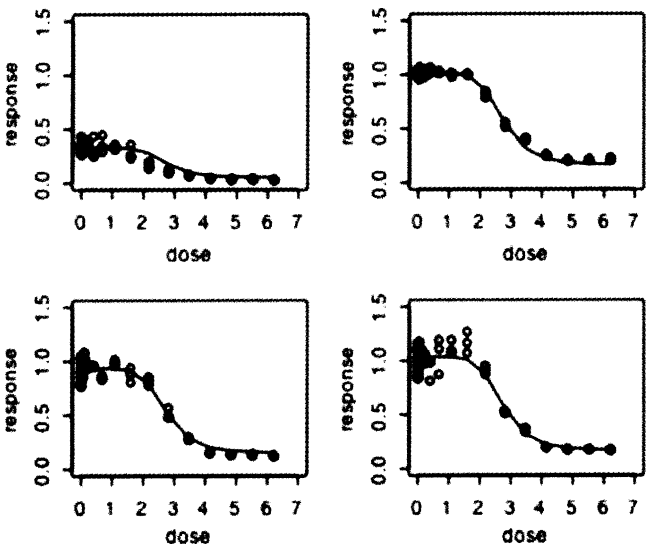

Figure 1. Bootstrapped dose−response curves for the MTT response of 1A9 cells to drugs. Examples of dose−response curves are shown to illustrate the bootstrapping process of analyzing each curve. Each plate has a unique maximum, and the minimum is a constant percentage of that for all plates. The *y*-axis is the natural log (ln) of the MTT response.

Drug interactions were assessed using Berenbaum's combination index:[19]

$$\mathrm{CI} = \frac{D_1}{\mathrm{Dx}_1} + \frac{D_2}{\mathrm{Dx}_2} \qquad (1)$$

in which a CI value equal to 1 indicates additivity, values less than 1 indicate synergy, and values greater than 1 indicate antagonism. The doses Dx_1 and Dx_2 are those used in the combination dose studied, and the doses D_1 and D_2 are the amounts of each drug given alone that would produce the same response as the combination dose, as determined by the estimated dose−response curve. For the growth inhibition studies on 1A9 cells, between 8 and 20 replicates from 3−6 different plates were used to estimate the response to each combination dose. For the growth inhibition HL-60 data, between 21 and 24 replicates from 7 or 8 different plates were used to estimate the response to each combination dose.

Statistical tests of synergy were carried out via bootstrapped confidence intervals for the CI values.[20,21] Five thousand resampled data sets (bootstrapped samples) were generated by sampling with replacement the residuals of each fitted dose−response curve (for each drug), as well as sampling with replacement the responses to the combination data. In this way, random noise is added to both marginal and combination drug data. New dose−response curves were fitted to each resampled data set, and these were used to calculate a CI value for the resampled combination data. In this way, 5000 bootstrapped CI values were generated; these were sorted and the α and $100 - \alpha$ percentiles gave the lower and upper bootstrapped confidence intervals for the CI values, respectively. For each drug combination (e.g., Ptx and PelA), α was chosen to give an overall 95% confidence,

given that *k* combination doses were evaluated, using a Bonferroni multiple-comparison adjustment. Both intra- and interplate variability is accounted for in this statistical analysis.

Results

Effects of Combination Doses of Drugs on Cell Proliferation (MTT Assay). To test for synergy between PelA and other microtubule stabilizing agents on cell proliferation, two cancer cell lines, 1A9 and HL-60, were incubated with drugs alone or in combination for 48 h. Drug concentrations that inhibit cell proliferation by 50% (IC_{50}) were determined for each drug. For 1A9 cells, the IC_{50} values for PelA, Ptx, and EpoA were 27 nM, 11 nM, and 10 nM, respectively. The IC_{50} values for PelA, Ptx, and EpoA in HL-60 cells were 32 nM, 34 nM, and 12 nM. Various concentrations were chosen to look for a possible synergistic effect of two of the drugs when given in combination. In 1A9 cells, doses below the IC_{50} values gave the best synergistic responses. Often, at doses above the IC_{50}, no synergy could be detected. There was a certain minimum concentration needed for each drug to have an effect as well as a maximum concentration, above which the response was likely to be maximal and therefore could not be increased significantly by adding a second drug.

In initial experiments, good synergy was seen between PelA and the two taxoid binding site drugs, Ptx and EpoA, at low nM concentrations (Figure 2). PelA had no effect on its own at 10 nM; however, when given in combination with Ptx at 5 nM and 10 nM, cell growth was reduced 28% and 85%, respectively (Figure 2A). PelA at 12 nM in combination with EpoA at 2 nM decreased proliferation to 44% relative to the control (Figure 2B). In these initial experiments, no obvious synergy was seen between the two taxoid site drugs, Ptx and EpoA, when given in combination at 5 nM each (84% of control) (Figure 2C); however, a more thorough analysis showed that synergistic interactions could also be obtained with these two drugs (see Figure 3C).

Bootstrapping the CI Values for Cell Proliferation. A rigorous bootstrap analysis of all the cell proliferation data on 1A9 cells (Figure 3) and HL-60 cells (Figure 4) showed that significant synergy existed between all the drugs to some extent, and the presence or absence of synergy was dependent on the particular combination used. Significant synergy was seen between Ptx and PelA as well as EpoA and PelA at most combinations. CI values for PelA and Ptx ranged from 0.48 to 0.96 in 1A9 cells (Figure 3A) and 0.16 to 0.87 in HL-60 cells (Figure 4A). CI values for PelA and EpoA ranged from 0.41 to 0.96 in 1A9 cells (Figure 3B) and 0.08 to 1.04 in HL-60 cells (Figure 4B). CI values for Ptx and EpoA ranged from 0.43 to 1.04 in 1A9 cells (Figure 3C) and 0.27 to 1.21 in HL-60 cells (Figure 4C).

Effects of Combination Doses of Drugs on G_2/M Block. To test whether PelA was synergistic in combination with either Ptx or EpoA in arresting cells in the G_2/M phase of the cell cycle, flow cytometry experiments were performed with 1A9 cells. Cells were treated for 18 h with the drugs, as this time gave the greatest accumulation of cells in G_2/M

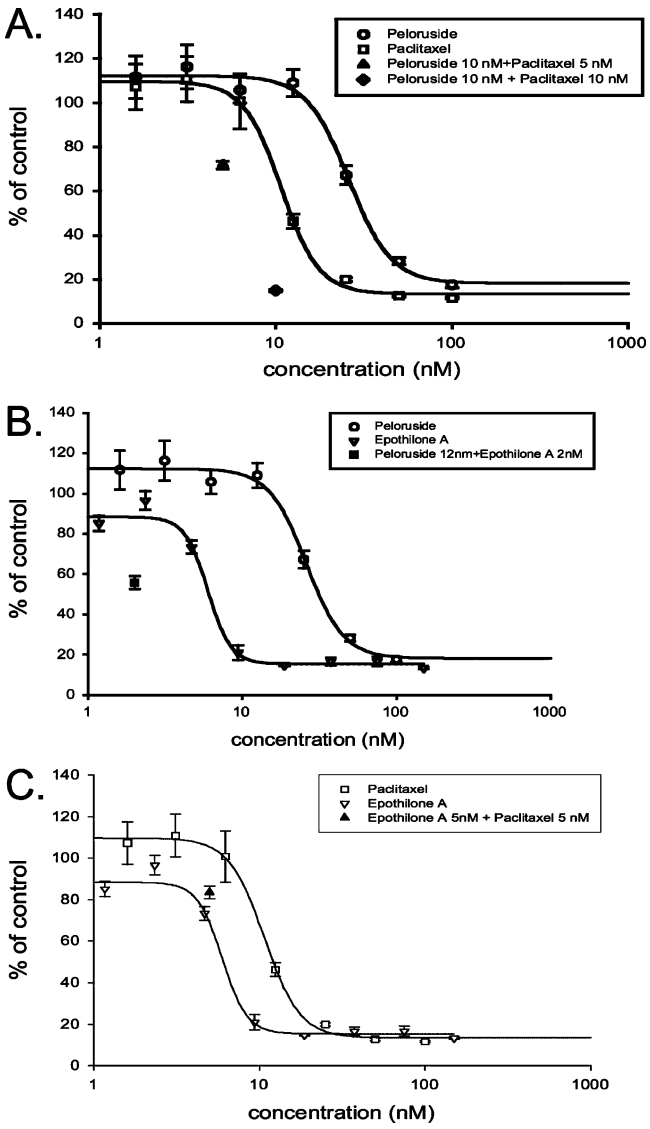

Figure 2. Synergistic effects of PelA, Ptx, and EpoA on growth of 1A9 cells. Dose−response curves for cell proliferation of 1A9 cells after 48 h treatment with individual drugs (clear symbols) and combinations of two drugs given at the same time (filled symbols) as determined by MTT assays. Panels A−C show one representative experiment. The IC_{50} value for PelA was 26.0 nM, for Ptx 10.7 nM, and for EpoA 6.0 nM.

of the cell cycle. At later time points, many of the cells had died, as evidenced by an increase in the proportion of cells in the aneuploid stage (DNA content less than 2n). A representative experiment is shown in Figure 5. At low concentrations (5 nM EpoA, 5 nM Ptx, and 15 nM PelA), cells treated singly with drugs (Figure 5A) showed no accumulation of cells in G_2/M of the cell cycle greater than that of the controls (24−30%). Combining PelA with Ptx or PelA with EpoA (Figure 5B) at those same concentrations led to significant increased accumulation of cells in G_2/M (to 53% and 56%, respectively). Combinations of Ptx and EpoA, however, did not increase the proportion of cells in

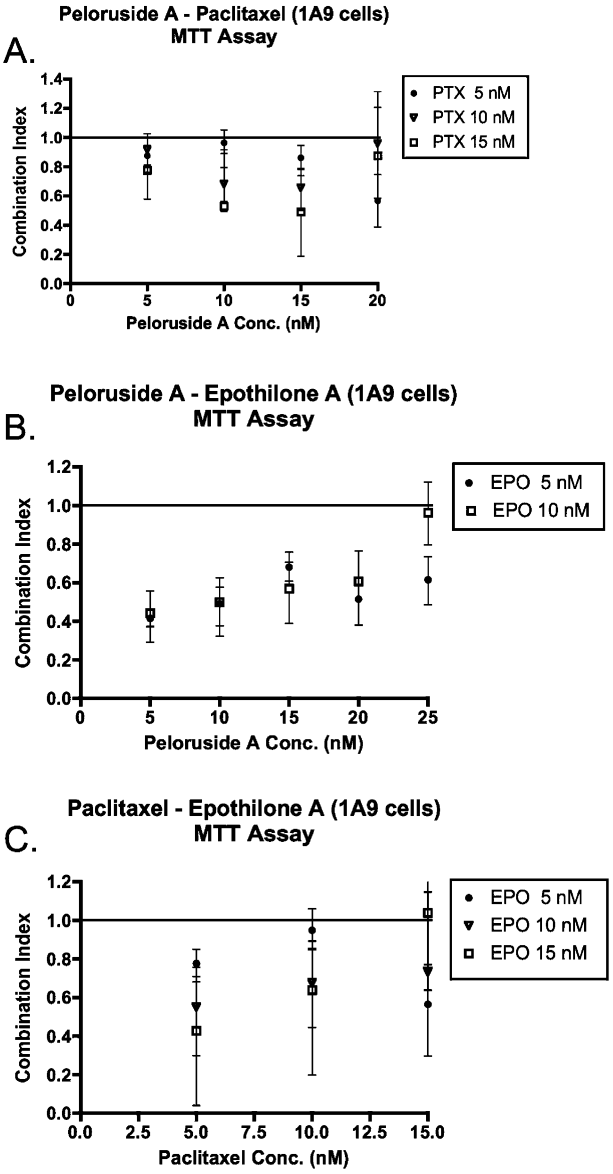

Figure 3. Combination index values for synergy on cell proliferation of 1A9 cells. The combination index (CI) values were calculated by bootstrapping for various combinations of drugs, and are presented along with the 95% confidence intervals for (A) PelA and Ptx, (B) PelA and EpoA, and (C) Ptx and EpoA.

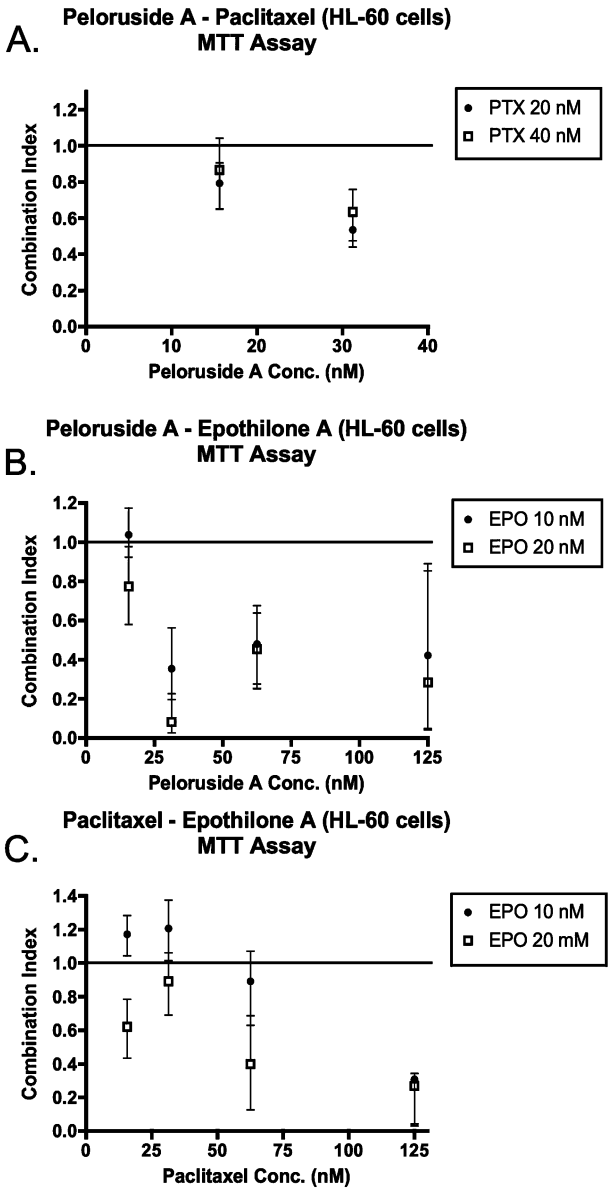

Figure 4. Combination index values for synergy on cell proliferation of HL-60 cells. The combination index (CI) values were calculated by bootstrapping for various combinations of drugs, and are presented along with the 95% confidence intervals for (A) PelA and Ptx, (B) PelA and EpoA, and (C) Ptx and EpoA.

G_2/M to the same extent (35%) at those same concentrations (Figure 5B).

Bootstrapping the CI Values for G_2/M Block. The flow cytometry experiments were analyzed by bootstrapping the CI values. PelA-treated 1A9 cells showed a similar percentage of cells in G_2/M at 15 nM (27 ± 3%) as in control cells (25 ± 3%). A maximum arrest of cells in G_2/M was seen at 80 nM PelA (79 ± 3%). 1A9 cells treated with EpoA did not show any G_2/M block at 3 nM (28 ± 3% in G_2/M), but at 11 nM a maximum arrest in G_2/M was seen (73 ± 3%). Ptx did not alter the percentage of cells in G_2/M at 5 nM (26 ± 5%) but caused the maximum accumulation of cells in G_2/M at 20 nM (65 ± 11%). Ptx-treated cells always showed a higher variation than the other two drugs, and this increased the difficulty in reproducing experiments. Combining PelA at either 15 or 20 nM with Ptx at 5 nM caused an increase of cells in G_2/M phase (to 45 ± 12% and to 58 ± 14%, respectively). CI values for those combinations were 0.85 and 0.77, indicating synergy (Figure 6A). PelA at 10 nM combined with EpoA at 3 nM and PelA at 15 nM combined with EpoA at 5 nM also increased the proportion of cells in G_2/M (to 36 ± 7% and 52 ± 7% respectively). CI values for those combinations ranged from 0.38 to 0.91 (Figure 6B). Thus, both Ptx and EpoA acted synergistically with PelA in causing G_2/M arrest. Although Ptx and EpoA acted synergistically on cell proliferation, CI values for G_2/M

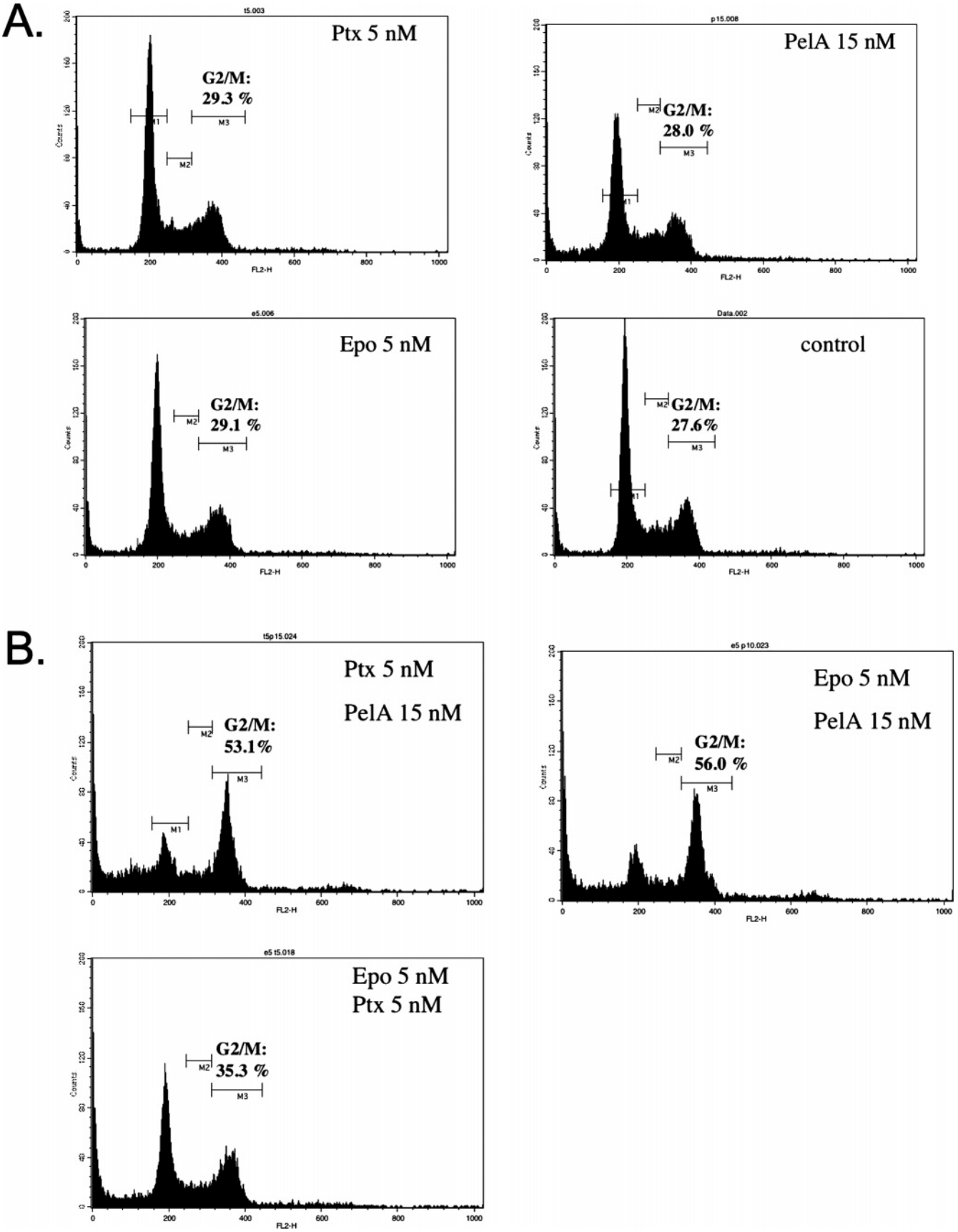

Figure 5. Synergistic effects of drugs on G_2/M block: representative experiment. After treatment with drugs for 18 h, the DNA content of 1A9 cells was determined by PI staining and flow cytometry. A representative experiment is shown. The percentages of cells in G_2/M phase are indicated on each graph. Drugs were given singly (A) or in combination at the same doses (B).

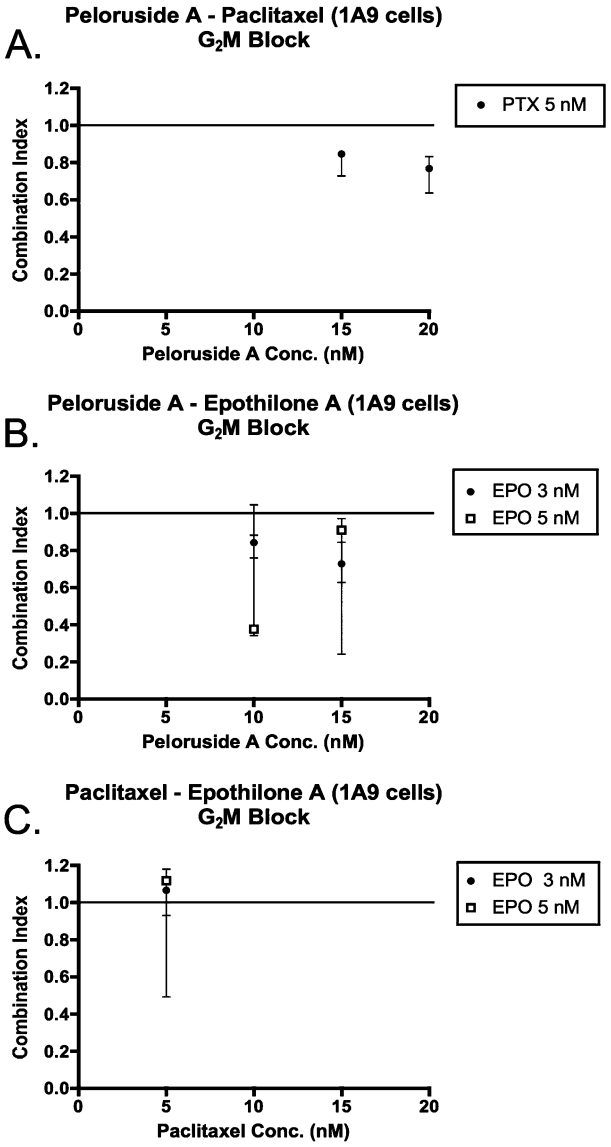

Figure 6. Combination index values for synergy on G_2/M block. All the 1A9 flow cytometry data were combined, and the combination index (CI) calculated for various combinations of drugs is presented along with the 95% confidence interval for (A) PelA and Ptx, (B) PelA and EpoA, and (C) Ptx and EpoA.

block (1.07 and 1.12) suggested only additivity for these two taxoid site drugs.

Effects of Combination Doses of Drugs on in Situ Tubulin Polymerization. Western blots of supernatant and pellet fractions from HL-60 cells treated with different combinations of tubulin drugs were analyzed for their percent microtubule or polymer fractions by densitometric analysis. A representative experiment with PelA and Ptx is shown in Figure 7A, and a summary of the results of three experiments is shown for combinations of PelA (10 nM), Ptx (10 nM), and EpoA (5 nM) (Figure 7B). At these doses, none of the drugs caused significant polymerization of tubulin on their own, but in combination, the percent of tubulin in the supernatant decreased for all three. Definitive conclusions based on bootstrapping analysis could not be drawn from these experiments because of the inherent problems in quantifying Western blotting results by densitometric analysis.

Discussion

Combinatorial therapy in the treatment of cancer has increased in recent years, particularly the use of two or more anticancer drugs that have essentially different mechanisms of action. An example is the microtubule stabilizing agent Ptx used in combination with the apoptotic agent amifostine,[24] or the farnesyl transferase inhibitor lonafarnib,[25] or the platinum containing agent nedaplatin.[26] These combinations have been shown to be synergistic rather than additive in tests on cultured cancer cells. In some cases, the order of addition of the drugs was critical in obtaining optimal effects. With the discovery that laulimalide bound to a different site on tubulin than the taxoid site drugs,[8] the possibility that laulimalide could synergize with other microtubule stabilizing agents that bind to the taxoid site was tested. Although laulimalide synergized with Ptx in its effects on isolated tubulin polymerization,[11,12] it did not synergize with Ptx in its cytotoxic effects on cultured cells[8] in one study, but a more recent study reported clear synergy between laulimalide and two of its analogues, C_{16}-C_{17}-des-epoxy-laulimalide and C_{20}-methoxy laulimalide in two cancer cell lines.[13] Hamel et al.[11] have recently demonstrated that PelA, which binds to the same or an overlapping site as laulimalide,[6] can also synergize with taxoid site drugs to polymerize isolated tubulin at subthreshold single-dose concentrations. PelA and laulimalide were unable to synergize with each other in vitro. The aim of the present study was to test whether PelA could synergize with the taxoid site drugs Ptx and EpoA in cultured cancer cells. We also wished to test for possible synergy between two taxoid site drugs given in combination, because of reports of substantial synergy between Ptx and discodermolide[14,15,27] and Ptx and docetaxel,[16] the latter combination, however, only in an MDR-overexpressing cell line.

Methods for Measuring Synergy. Evidence of synergistic effects between drugs requires quantitative statistical analysis

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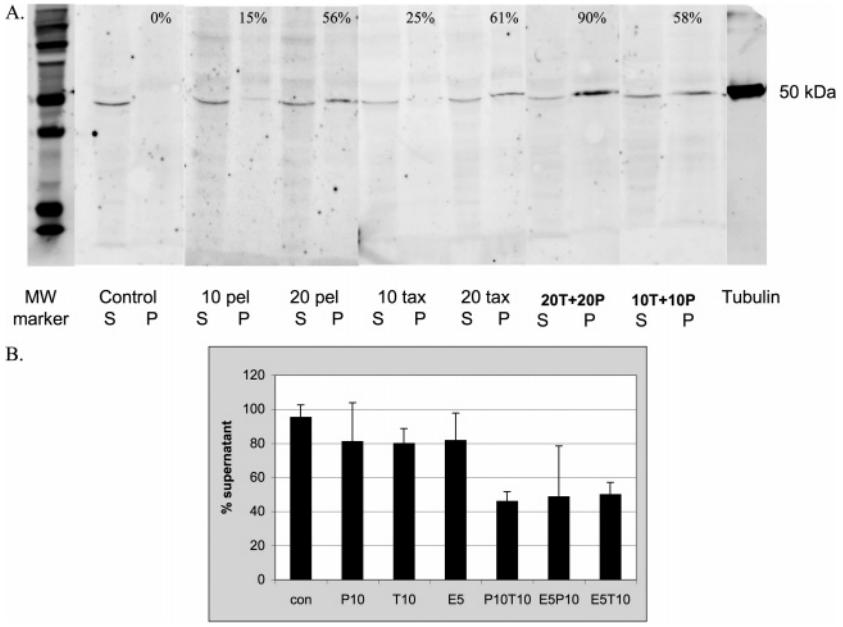

Figure 7. Synergistic effects on in situ tubulin polymerization. (A) Western blot analysis of a synergy experiment on cellular tubulin polymerization in the presence of PelA, and Ptx, alone and in combination. The percentage values at the top represent the percent of polymerized tubulin in the cell relative to total tubulin. Supernatant (S) and pellet (P) fractions are as marked. (B) Summary of results from 3 experiments. The percentage of tubulin in the supernatants following treatment of cells with different amounts of drug, PelA (P), Ptx (T), or EpoA (E), for 16 h are shown.

because of the normal variation seen in drug actions.[28] Cell culture studies often generate variable results because of slight differences in culture conditions between preparations, changes in cell biochemistry with passaging, and cell variation induced by alterations in cell density at the time of measurement. In addition, multiwell culture plates introduce positional variation as a result of gas and humidity heterogeneity in different parts of the plate. Therefore, accurate assessment of synergy requires accurate modeling of the dose−response curves for each drug, since these models are used to determine Dx_1 and Dx_2 in eq 1, i.e. the doses of the drugs given alone that produce the same response as the combination doses. The four-parameter logistic model used to calculate the dose−response relationship gave a good fit to the MTT data only when the model included a unique maximum response for each plate; then the fit was very good, with the percent of variation explained by the model (R^2 values) ranging from 88% to 97%.

In assessing synergistic interactions, often standard errors for CI values are obtained by calculating K CI values from K different experiments.[29,30] Intervals of the mean CI plus or minus the standard error of the mean obtained in this way should not be used to test synergy unless the number of experiments is large, since CI values by definition are apt to be skewed and non-normally distributed. Instead, the statistical methodology used to assess synergy should account for the (non-normal) distribution of the CI values, the error in estimating the individual dose−response curves, and the error in estimating the response to the combination dose. In the present study we used a bootstrapping simulation method that accounted for these unique features of the data.[19−21]

Synergy between PelA and Other Microtubule Stabilizing Agents. Synergy in Cells (Cytotoxic Effects). We found significant synergy in the cytotoxic effects of PelA with all the microtubule stabilizing agents tested in both cancer cell lines, 1A9 (Figure 2 and 3) and HL-60 (Figure 4). The extent of synergy was dependent on the particular combinations used, since too low a concentration would not achieve an effect, whereas too high a concentration would give the maximum response before addition of the second drug in combination. It has previously been recognized[28] that whether or not two drugs interact synergistically or antagonistically is dependent on the ratio of the drugs and the particular test measured. Unfortunately, in the present study, we were unable to test, as predicted from the in vitro studies, whether PelA was unable to interact synergistically in combination with laulimalide, a microtubule stabilizing agent that shares at least some aspects of the PelA binding site, because we could not obtain a sample of laulimalide for our study.

We found significant synergy between Ptx and EpoA, despite the fact that no synergy was reported between these

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two drugs in isolated tubulin polymerization assays[11,12] nor between Ptx and EpoB in their effects on cancer cell lines.[14] Using the Epo analogue BMS-247550, no synergy was seen with Ptx in cells, and additivity, rather than synergy, was seen between EpoB and Ptx in vivo.[17] The demonstration of synergy between two taxoid site drugs highlights the complexity of the cytotoxic response in cells and alludes to the possibility of other effects of the drugs that do not relate to their known biochemical mechanisms of action. The complexity of cytotoxic effects compared to purified binding studies is illustrated in a study on laulimalide analogues by Paterson et al.[31] who synthesized a number of analogues, and only one, 11-desmethyl laulimalide, showed a reasonable IC_{50} (50 nM) compared to the IC_{50} of the parent compound of 3.4 nM. All the other analogues lost most of their cytotoxicity, with IC_{50} values ranging from 430 nM to 40 μM, despite the fact that their ability to polymerize tubulin, as assessed by their critical concentration, was changed very little. A similar lack of correlation between the abilities of analogues to induce tubulin assembly and cytotoxicities was reported by Buey et al.,[32] although the binding affinity constants of the microtubule stabilizing agents correlated reasonably well with their IC_{50} values ($r^2 = 0.76$). In an earlier study by Lobert et al.,[33] vinca alkaloid-induced tubulin-spiral formation was significantly correlated with cytotoxicity against a leukemic cell line, but not with the ability to inhibit the formation of microtubles from purified tubulin. These authors discuss the stoichiometric nature of tubulin interactions that requires a tubulin binding drug to be at a concentration close to the tubulin concentration to be effective.

Synergy at the Level of Mitosis (G_2/M Block). Because of the complexity of drug actions in intact cells, we also looked for synergistic effects at a biochemical level closer to the known microtubule mechanism of action, namely, G_2/M arrest as a result of microtubule stabilization at the start of mitosis. In 1A9 cells, we were able to demonstrate synergy between PelA and the taxoid site drugs Ptx and EpoA (Figures 5 and 6), but not between Ptx and EpoA (Figure 6C). Thus, our results on G_2/M block were more comparable to the synergy experiments carried out at the level of isolated tubulin. Clark et al.[13] have also shown synergistic effects between laulimalide and Ptx on mitotic arrest. In the case of discodermolide, both Martello et al.[14] and Honore et al.[15] demonstrated synergy between Ptx and discodermolide in their effects on G_2/M block in the same non-small cell lung carcinoma (A549 cells). Huang et al.[17] report that although synergism was seen in SKOV-3 human ovarian carcinoma cells at higher doses than those needed to disrupt microtubule dynamics in other cell lines,[15] the synergism was lost at higher concentrations of drug required to induce G_2/M arrest.[17]

Synergy at the Level of in Situ Tubulin Polymerization (Western Blotting). In preliminary experiments, we looked for synergy between PelA and Ptx at the level of in situ tubulin polymerization, using the method originally described by Giannakakou et al.[23] These experiments proved to be difficult to reproduce consistently but showed some promise (Figure 7); however, no convincing evidence of synergy was obtained, mainly due to the variation characteristic of quantitative analysis of Western blot results.

Biochemistry of Synergy. The biochemical mechanism of the synergy observed between combinations of microtubule stabilizing agents is not known. Honore et al.[15] have proposed a number of possible mechanisms to explain the synergy between Ptx and discodermolide, some of which are reproduced below.

Interactions at the Level of Microtubules. An important question proposed by Honore et al.[15] was whether distinct binding sites were needed for synergistic interactions to occur between two drugs that bind to the same target. In the case of discodermolide and Ptx, there is some evidence that their tubulin binding sites, although almost certainly overlapping (because they compete with each other for binding to β-tubulin), are not identical. For example, β-tubulin mutations that markedly increase the IC_{50} of Ptx have no effect on the IC_{50} of discodermolide (cf. the mutation of amino acid β270 in Ptx10 cells and the mutation of amino acid β364 in Ptx22 cells).[34] In addition, mutations in other amino acids affect the IC_{50} of both Ptx and EpoB but again have no effect on discodermolide (cf. the mutation of amino acid β292 in A549.epoB40 cells and the mutation of amino acid β422 in HeLa.EpoB1.8 cells).[35] Although our study is the first to show synergy between Ptx and EpoA in intact cells, a similar explanation to that given above for discodermolide is possible for Epo since Giannakakou et al.[36] have developed β-tubulin mutant cell lines that affect the IC_{50} of Ptx without

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increasing the IC_{50} of EpoB (the mutation of amino acid β270 in Ptx10 cells), as well as tubulin mutations that affect the IC_{50} of EpoA and B (cf. the mutation of amino acid B274 in A8 cells and the mutation of amino acid β282 in B10 cells) that have very little effect on Ptx cytotoxicity. Thus, the concept of a single taxoid binding site needs to be reconsidered in view of the above evidence of unique binding interactions between purportedly similar taxoid site drugs. How this explanation fits in with the fact that these drugs compete with each other for binding to the microtubule is currently an open question.

Another explanation for synergy suggested by Honore et al.[15] is cooperativity between the two agents such that one binds and induces a small conformational change in tubulin that increases the binding affinity of the other agent. In the study by Hamel et al.[11] on isolated tubulin, the critical concentration needed to cause tubulin polymerization was lowered by combinations of the drugs, thus enhancing the amount of polymer formed, possibly through cooperativity between binding sites within the microtubular lattice. In this same study, Ptx and discodermolide were shown to not synergize in vitro, despite the evidence for synergy in intact cells by Martello et al.[14] and Honore et al.[15]

Other explanations that have been suggested to explain synergy[15,17,27] are that the agents show differential binding to different tubulin isotypes in the cell, and this interaction with unique tubulin isotypes would have distinct effects on microtubule dynamics,[37] perhaps interacting in a nonlinear manner, hence, a synergistic rather than additive response. In addition, each microtubule stabilizing agent may have different effects on the overall microtubule architecture, such as the number of protofilaments, or they may differentially affect the biochemical regulators of microtubule polymerization, such as the microtubule associated proteins MAP1a, MAP1b, MAP2, MAP4, XMAP215, and tau. Giannakakou et al.[27] suggested that low concentration effects, not high concentration effects, of the drugs might be contributing to the synergy, and Honore et al.[15] followed this up by demonstrating synergistic effects of microtubule stabilizing agents on tubulin dynamics at low nanomolar concentrations near the IC_{50} values, well below those necessary to cause large increases in tubulin polymer formation. Ptx and discodermolide both suppressed microtubule dynamic instability in living cells, and this "freezing" of the microtubules presumably contributed to their synergistic G_2/M arrest, thus explaining the synergistic interaction seen between drugs that bind to the same or overlapping sites on tubulin. In the study by Clark et al.,[13] laulimalide and two of its analogues were able to synergize with the tubulin destabilizer, 2-methoxyestradiol, and these authors concluded that the synergy between a tubulin stabilizer and destabilizer possibly occurs by effects on microtubule dynamics, similar to what was suggested by Honore et al.[15] Although most studies consider mitotic effects as the primary response to microtubule stabilizing agents, there is also a possibility that the target is not just the spindle microtubules but rather interphase microtubules. For example, laulimalide and docetaxel have been shown to interfere synergistically with endothelial cell migration through their respective effects on dynamic microtubular activation of signaling cascades.[38]

Transport or Metabolic Effects. Synergistic interactions could arise by competition for multiple drug resistance efflux pumps or by effects on metabolism or uptake of the drugs. For example, docetaxel and Ptx were shown by Budman and Calabro[16] to synergize in the MCF7/adr breast cancer cell line that overexpresses the P-glycoprotein efflux pump, but these two compounds did not synergize in the parental cell line. With regard to metabolism in cells, however, Honore et al.[15] found that discodermolide had no effect on the metabolism of Ptx by A549 lung carcinoma cells; thus, drug metabolism was ruled out as a cause of synergy in this particular case. The role of metabolism and drug efflux or uptake will need to be considered as possible sources of synergistic interactions in future studies.

Importance of Synergy in Cancer Chemotherapeutics. Two problems with clinical chemotherapeutics are the difficulty of achieving effective drug levels at the site of the target via systemic administration and the resultant toxicity secondary to systemic exposure. Since toxicity profiles vary between different drugs, using two drugs in combination may effectively present both drugs at subthreshold levels for development of toxicity. Huang et al.[17] reported the first synergy in vivo between microtubule stabilizing drugs using SKOV-3 human ovarian carcinoma xenograft-bearing mice. They used Ptx at 20 mg/kg iv and discodermolide at 5 mg/kg iv, and observed superior antitumor activity for the combination of drugs relative to either drug alone. In addition, no toxicity was seen with this dose combination. In preliminary studies in mice,[39] PelA has shown little in vivo toxicity at doses required to induce regression in established tumors; thus, in combination with other drugs, it would not be expected to greatly increase the toxicity of the treatment. The microtubule stabilizing agents Ptx and docetaxel are effective clinically used anticancer drugs,[5] and other microtubule stabilizing agents are currently in advanced clinical trials, including some Epo derivatives and discodermolide analogues.[40] Clinical trials by Novartis with discodermolide, however, were discontinued in 2004 due to

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unacceptable toxicity of the natural product. The success of Ptx and docetaxel in the treatment of solid tumors of the breast, ovary, and lung indicate that possible combinative therapy with other microtubule stabilizing agents could improve the overall effectiveness as well as decrease side effects of these drugs, particularly a problem with Ptx[5] and to a lesser extent docetaxel. The complicated downstream biochemical signaling pathways activated by microtubule stabilizing agents that lead to apoptosis of cancer cells have recently been reviewed,[41] and there is ample room in signaling pathways for explanations of synergistic interactions between drugs that work via the same upstream cellular targets.

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