molecular pharmaceutics

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Laulimalide and Synthetic Laulimalide Analogues Are Synergistic with Paclitaxel and 2-Methoxyestradiol

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Received February 15, 2006

Abstract: Some of the most significant therapeutic leads and agents used for the treatment of cancer target microtubule dynamics. Paclitaxel is an exceptional example that is currently used for treating a wide range of tumors. New, non-taxane microtubule stabilizers, including several epothilones, are advancing through clinical trials. Laulimalide is a potent microtubule stabilizer that binds to tubulin at a site that does not overlap the taxane-binding site. It is active against paclitaxel-resistant cancer cells. Notwithstanding its therapeutic potential, laulimalide is relatively unstable, rearranging to a more stable but less active isomer. The goal of this study was to evaluate the ability of laulimalide and two designed laulimalide analogues, C_{16}-C_{17}-des-epoxy laulimalide (LA1) and C_{20}-methoxy laulimalide (LA2), to inhibit cell proliferation in combination with other tubulin-binding and non-tubulin-binding antiproliferative antimitotic agents. The synthetic laulimalide analogues retain the mechanism of action of the natural compound but do not share its instability. We studied the ability of the laulimalides to act synergistically with paclitaxel, 2-methoxyestradiol, and monastrol, an Eg5 kinesin inhibitor. The results show that all three of the laulimalides acted synergistically with paclitaxel and 2-methoxyestradiol to inhibit proliferation with the analogues exhibiting significantly larger synergistic effects. The combination of laulimalide and monastrol was not synergistic and provided only additive effects. The laulimalide analogues LA1 and LA2 had a greater degree of synergy with both paclitaxel and 2-methoxyestradiol than was observed with laulimalide. Our results show that the laulimalides together with other tubulin-binding antimitotic agents provide synergistic antiproliferative actions. The data are consistent with the previously reported ability of laulimalide and paclitaxel to act synergistically to polymerize tubulin in vitro. These important findings suggest that specific combinations of microtubule-targeting agents should be considered for clinical utilities as they have excellent potential to improve clinical response.

Keywords: Laulimalide; paclitaxel; 2-methoxyestradiol; microtubule stabilizers; drug synergism

Introduction

Drugs that target microtubules are some of the most important drugs used in the treatment of cancer. Paclitaxel, the first microtubule stabilizer identified, is the most effective microtubule-targeting drug used clinically. The clinical success of paclitaxel led to the search for other microtubule stabilizers that would share the efficacy of the taxanes, paclitaxel and docetaxel, and yet would retain activity against taxane-resistant tumors. A number of other microtubule stabilizers have been discovered including the epothilones, discodermolide, the laulimalides, peloruside A, and dictyostatin. Reports suggest that the epothilones are clinically active and, importantly, that they have activity against taxane-resistant tumors. Laulimalide and peloruside A are attracting

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10.1021/mp060016h CCC: $33.50
Published on Web 06/30/2006

attention because of the special advantages they could offer as therapeutics in potentially circumventing many different taxane resistance mechanisms.

Tubulin-targeting antimitotics are often separated into two groups: those that increase the density of cellular microtubules and promote tubulin polymerization (microtubule stabilizers) and those that cause a loss of cellular microtubules and inhibit tubulin polymerization (microtubule depolymerizers). The vinca alkaloids, colchicine, dolastatin 10, and 2-methoxyestradiol (2ME2) represent microtubule-depolymerizing compounds, and several microtubule stabilizing agents are listed above. Relatively high concentrations of all of these drugs are needed to induce dramatic changes in cellular microtubules. A large body of evidence shows that at the lowest effective concentrations microtubule stabilizers and microtubule depolymerizers inhibit microtubule dynamics that are critically important for normal mitotic progression, thus causing mitotic arrest and initiation of apoptosis.[1,2] While all share a common mechanism of action, slight differences are observed between the microtubule stabilizers paclitaxel and discodermolide. Interestingly, discodermolide and paclitaxel have synergistic antiproliferative actions and the synergistic effects were specific for this combination. The combination of paclitaxel with epothilone B resulted in only additive effects.[3] Although both discodermolide and paclitaxel markedly inhibited microtubule dynamics, slight mechanistic differences were observed.[4] Detailed evaluations of the mechanisms by which discodermolide inhibits microtubule dynamics showed that it differs from paclitaxel and epothilone B in its ability to increase the frequency of catastrophe rescue.[4] Importantly, the combination of discodermolide and paclitaxel caused synergistic suppression of microtubule dynamics.[5] The ability of these two microtubule stabilizers to act in a synergistic manner might be related to their slightly different mechanisms of inhibiting microtubule dynamics.[4]

Laulimalide is a structurally distinct microtubule-stabilizing agent that was originally isolated from the marine sponge *Cacospongia mycofijiensis*.[6] It is a potent inhibitor of proliferation, and it has the ability to circumvent P-glycoprotein (Pgp)-mediated drug resistance and resistance due to mutations in the paclitaxel binding site.[6,7] Laulimalide causes the formation of aberrant mitotic spindles and G_2/M arrest, leading to apoptosis.[6] Similar to other microtubule stabilizers, laulimalide at high concentrations increases interphase microtubule density and causes the formation of thick microtubule bundles.[6] Laulimalide was the first microtubule stabilizer identified that binds to tubulin at a site that does not overlap the taxane-binding site.[7] Laulimalide does not displace radiolabeled paclitaxel, and the incubation of paclitaxel and laulimalide with tubulin resulted in equimolar associations of these two drugs bound to the tubulin polymer, consistent with discreet, nonoverlapping binding sites.[7] Studies of the nature of the interaction of laulimalide and tubulin show that laulimalide and paclitaxel have almost identical effects on purified tubulin.[8] Interestingly, paclitaxel and laulimalide synergistically stimulated tubulin assembly reactions at cold temperatures; such suboptimal polymerization conditions increase the likelihood of detecting synergistic actions.[8]

The total chemical synthesis of laulimalide has been accomplished by several groups,[9−14] and a number of laulimalide analogues have been synthesized.[7,15,16] Two

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laulimalide analogues, C_{16}-C_{17}-des-epoxy laulimalide (LA1) and C_{20}-methoxy laulimalide (LA2), were designed to minimize the intrinsic chemical instability of laulimalide. These two analogues retain the microtubule-stabilizing activity of the parent compound and the ability to evade Pgp-mediated drug resistance and resistance due to mutations in the taxane-binding site.[15,16]

Combinations of drugs can have antagonistic, additive, or synergistic effects when used together. Additive effects are observed when each drug in the combination generates an effect that is merely the sum of their individual effects. When two drugs used together create an effect greater than the sum of their individual effects, they are said to be synergistic or superadditive. However, some drugs seem to compete with one another and reduce their individual potencies. In this case the combination is said to be antagonistic, or subadditive. We evaluated the effects of laulimalide and laulimalide analogues in combination with other antimitotic compounds to identify the nature of their interactions. Our data suggest that the laulimalides in combination with other tubulin-targeting antimitotic agents provide significant synergistic actions. Interestingly, the laulimalide analogues were substantially more synergistic than the natural product. In contrast, laulimalide and monastrol, an inhibitor of the mitotic kinesin Eg5,[17,18] provide only additive antiproliferative effects.

Materials and Methods

Laulimalide and Laulimalide Analogues. Laulimalide was purified from the marine sponge *C. mycofijiensis* as previously described.[6] Laulimalide was solubilized in ethanol, and the solution was stored under argon at −20 °C or −80 °C. The laulimalide analogues C_{16}-C_{17}-des-epoxy laulimalide (LA1) and C_{20}-methoxy laulimalide (LA2) were designed for enhanced chemical stability and were synthesized as reported previously.[15] The analogues were also solubilized in ethanol and stored at −20 °C or −80 °C.

Other Materials. Paclitaxel, 2-ME2, and monastrol were purchased from Sigma (St. Louis, MO).

Cell Culture. A549 human lung carcinoma cells were purchased from American Type Culture Collection (Manassas, VA), and they were maintained in RPMI 1640 (Biosource, Camarillo, CA) containing 50 μg/mL gentamycin and 10% fetal bovine serum (FBS). MDA-MB-435 breast cancer cells were obtained from the Lombardi Cancer Center (Georgetown University, Washington, DC). MDA-MB-435 cells were maintained in IMEM (Biosource, Camarillo, CA) with 10% FBS and 25 μg/mL gentamycin.

Sulforhodamine B Assay. The sulforhodamine B (SRB) assay was used to determine inhibition of proliferation and cytotoxicity of the various agents in the different cell lines.[19,20] Cells were plated into 96-well plates at predetermined densities (30 000 cells per well for A549 cells and 50 000 cells per well for MDA-MB-435 cells), and allowed to adhere and grow for 24 h. The cells were exposed to the drugs simultaneously for 48 h. After treatment, the cells were fixed and cellular protein was stained with SRB. The absorbance was read at 560 nm. Cytotoxicity is indicated by absorbance values less than the absorbance read at time 0, the time of drug addition.[20]

Analysis of Drug Synergism. The antiproliferative effects of the laulimalides were evaluated alone and in combination with paclitaxel, 2ME2, or monastrol. Detailed dose−response relationships were defined for each of the compounds singly and in combination using the SRB assay. The linear portions of the log dose−response curves were fit and the equations of the lines determined. The linear equations derived from combination treatments allowed for the calculation of the concentration of a drug combination required for a specific inhibitory effect. The individual dose−response curves were used to calculate singular potencies as well as the drug ratio, or relative potency of each drug used in combination to achieve a specific effect.[21−23] The drug ratio accounts for the different potencies of each drug. The ratio determines the relative proportion each drug comprises in the combination.[21−23] However, the drug dose−response curves for various drugs are not always parallel. When the drugs are used together, the relative potency might not be constant over the full-range drug response. Because of this potential variability, drug ratios were calculated separately for each data point used in the analysis. In the end, for a single data point such as the IC_{50}, the individual concentrations of each drug, the concentration of the drug pair, and the dose ratio were calculated and averaged and the standard error was determined. These values were then used to generate isobolograms and the combination indices (CIs).

Combination Index. The CI analyses were performed for the combined effects of the laulimalides and paclitaxel or 2ME2 according to the methods of Chou and Talalay.[24] The CI measures the degree of enhancement or reduction in the

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potency of a combination. CI values were calculated using the mutually exclusive assumption which assumes that the two drugs have a similar mechanism of action. The CI = $(D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where D_1 and D_2 are the two different drugs. $(D)_1$ and $(D)_2$ represent the concentration of drug D_1 or D_2 required in combination to produce a specific effect. $(Dx)_1$ and $(Dx)_2$ are the concentrations of the same drug, either D_1 or D_2, required to produce the same effect singularly. $(D)_1$ and $(D)_2$ were calculated from the combination dose response equation. $(Dx)_1$ and $(Dx)_2$ were calculated from the individual dose response equations. The CI values were calculated over a range of effects from the IC_{10} to IC_{90} to demonstrate the combination's characteristics over the range of inhibitory effects. A CI value of less than 1.0 is considered synergistic while a CI value greater than 1.0 is considered to be antagonistic, and a CI value approximately equal to 1.0 represents an additive interaction.[24]

Flow Cytometry. The effects of the antimitotic compounds on cell cycle distribution were evaluated singly and in combination by flow cytometry using standard methods.[16] A 24 h time point was evaluated, and the percentage of the cells in each phase of the cell cycle was determined by integrating the area under the peaks.

Results

Effects of Laulimalide in Combination with Paclitaxel, 2ME2, or Monastrol. To begin to determine the nature of the responses of cells to laulimalide and paclitaxel in combination, a simple set of experiments was conducted in A549 and MDA-MB-435 cells. Cells were treated with concentrations of laulimalide or paclitaxel that caused modest antiproliferative effects, in the range of 5−30% inhibition. Because inhibition greater than 100% cannot be measured, synergistic interactions are best identified using low antiproliferative concentrations. The antiproliferative activities of compounds were evaluated singly, and in combination. Bar graphs representing one set of data points in A549 (Figure 1A) and in MDA-MB-435 cells (Figure 1D) are shown. The inhibitory effects of each drug alone, the mathematical sum of the two drugs in combination, and the actual experimentally observed combination effect are plotted. Values representing the difference between the predicted additive percent inhibition value and the experimentally derived percent inhibition value, defined as the synergistic difference, are shown for each drug combination. In both the A549 and MDA-MB-435 cell lines paclitaxel and laulimalide were found to act synergistically: the resultant inhibition of proliferation was greater than the sum of their individual effects. The synergistic difference provided by laulimalide and paclitaxel in A549 cells was 15.5 (Figure 1A), and in MDA-MB-435 cells the synergistic difference was 22.2 (Figure 1D). The results suggest that laulimalide and paclitaxel have synergistic antiproliferative actions in these two cell lines.

The effects of the combination of 2ME2, a tubulin-targeting microtubule-depolymerizing agent, with laulimalide were also evaluated. The combination of 2ME2 and laulimalide showed synergism in both cell lines. In the A549 cell line the synergistic difference was 31.2 (Figure 1B), and in the MDA-MB-435 cell line the pair were also synergistic, with a synergistic difference of 11.9 (Figure 1E).

The antiproliferative effects of monastrol and laulimalide were also tested. Monastrol was selected for these studies because, like the other drugs used, it is an antimitotic agent with antiproliferative effects. Unlike the other compounds used in this study, monastrol is not a tubulin-binding antimitotic agent, it is an inhibitor of the mitotic kinesin Eg5. The combination of laulimalide and monastrol caused additive effects, with a calculated synergistic difference of 3.6 in A549 cells (Figure 1C) and 7.3 in MDA-MB-435 cells (Figure 1F). These data suggest that monastrol and laulimalide do not provide synergistic antiproliferative actions and probably interact in an additive manner.

Effects of the Laulimalide Analogues When Combined with Paclitaxel or 2ME2. Because laulimalide was synergistic with both paclitaxel and 2ME2, we tested the effects of the laulimalide analogues LA1 and LA2 to determine whether synergistic antiproliferative actions were shared among the laulimalides. LA1 and LA2 were evaluated singly and in combination with paclitaxel or 2ME2 in A549 cells. The effects of LA1 and paclitaxel when used singly and in combination are shown in Figure 2A. Each compound alone caused modest antiproliferative effects at the concentration tested. When added together the predicted additive effect was 19% inhibition of proliferation. The actual measured inhibition was 53.1%, yielding a synergistic difference of 34.1. The results show that LA1 was synergistic with paclitaxel. The combination of LA1 and 2ME2 was evaluated, and the combination provided more inhibition of proliferation than was anticipated from an additive relationship (Figure 2B) and a synergistic difference of 35.4 was obtained. The effects of LA2 were evaluated in combination with paclitaxel or 2ME2. The results show that this laulimalide is also synergistic in combination with paclitaxel, providing a synergistic difference of 43.4 for one dose pair (Figure 2C). LA2 was also found to be synergistic with 2ME2, and this combination yielded a synergistic difference of 27.7 (Figure 2D). Consistent with the effects of laulimalide, the laulimalide analogues LA1 and LA2 were synergistic with both paclitaxel and 2ME2. It is interesting to note that the degree of synergism appeared to be greater with the synthetic analogues than with the natural compound and this effect was more pronounced with the laulimalides in combination with paclitaxel than in combination with 2ME2.

Isobologram Analyses of Laulimalide in Combination with Paclitaxel or 2ME2. Isobolograms provide a useful mechanism to visualize the effects of dose pairings to distinguish effects that derivate from simple additive interactions.[21−23] Isobolograms were constructed for multiple drug pairs in A549 cells, and the results of several dose pairs are shown in Figure 3. These data represent dose pairs

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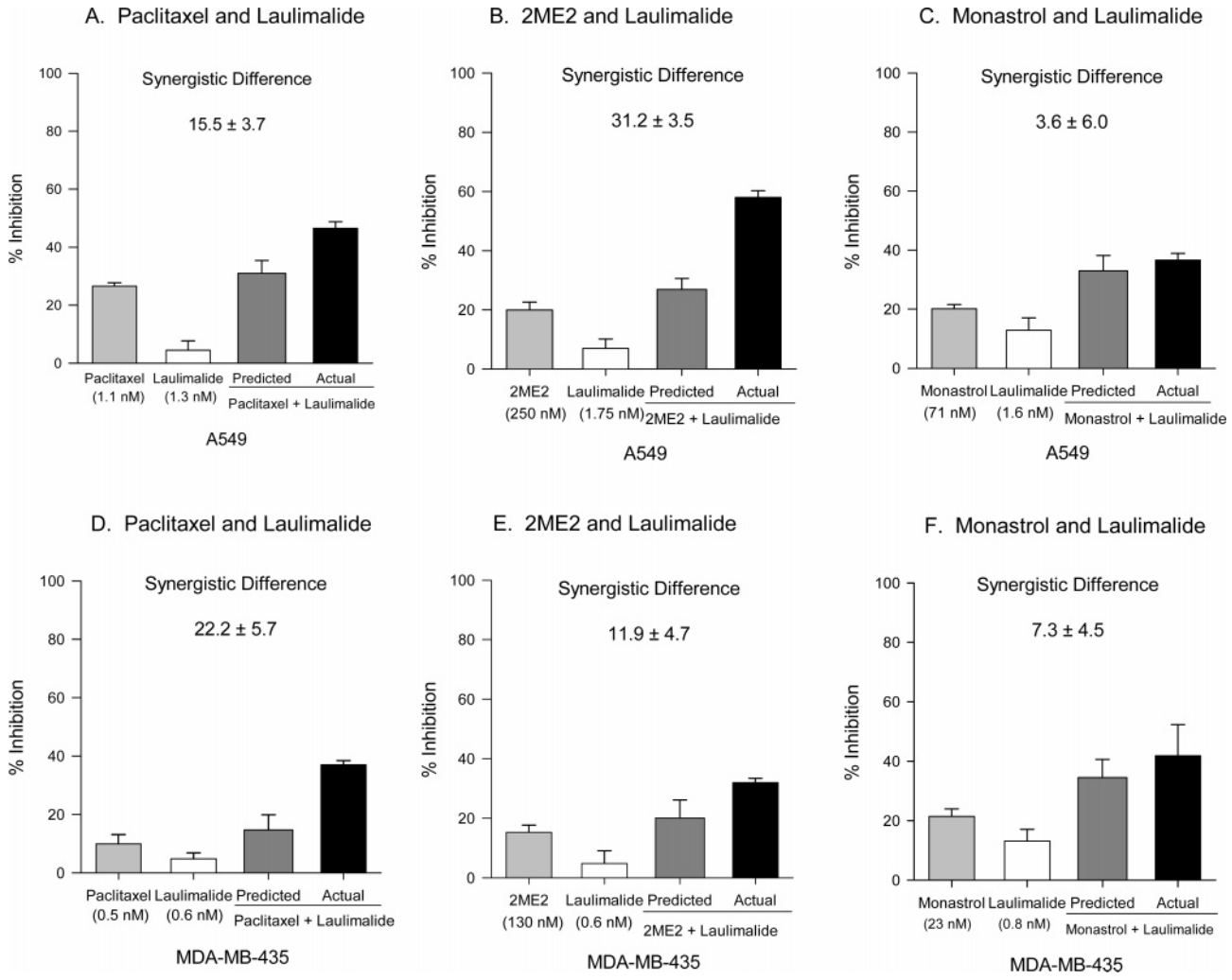

Figure 1. Antiproliferative effects of combinations of laulimalide and other antimitotic agents. A549 or MDA-MB-435 cells were treated with low inhibitory concentrations of the two agents alone and in combination. Inhibition of proliferation was measured using the SRB assay. The effects of each compound singly and in combination are shown. The predicted value is the sum of the individual effects of each drug in the pair. The actual value is the experimental result obtained from the combination. The synergistic difference is defined as the numerical difference between the predicted and measured antiproliferative effects of the agents used in combination. $n = 3 \pm$ SE.

(isoboles) that cause 20% inhibition of proliferation. This level of effect was selected for sensitive detection of synergistic activity. All of the individual drug concentrations were plotted as axial points in the Cartesian plot and are the averages obtained from three experiments. The line connecting the two points, the line of additivity, represents all dose pairs that would produce additive effects, i.e., the x and y values correspond to concentrations of each drug that, when combined, would produce 20% growth inhibition. The solitary point within the plot represents the experimental concentrations of a combination treatment that produced 20% inhibition. A point below the line of additivity represents synergism, and a point above the line of additivity is subadditive. Points close to the line of additivity suggest additive interactions.[21]

The effects of each of the laulimalides in combination with paclitaxel were evaluated in A549 cells (Figure 3A−C). All three isobolograms graphically depict synergistic interactions between these two drug classes. The level of synergism was greater for the laulimalide analogues LA1 and LA2 than was observed for laulimalide, and these results are consistent with the results obtained in simple additive experiments (Figures 1 and 2). Interestingly, these data suggest that there are differences in the responses of cells to the combination of laulimalide or the laulimalide analogues with paclitaxel, yet there is little difference between LA1 and LA2 in their interactions with paclitaxel.

The effects of 2ME2 and the laulimalides in combination are presented in Figure 3E,F. The isobolograms suggest that 2ME2 and the laulimalides have synergistic antiproliferative effects as the isoboles fall below the line of additivity. The magnitude of synergism was similar among the three laulimalides when each was used in combination with 2ME2,

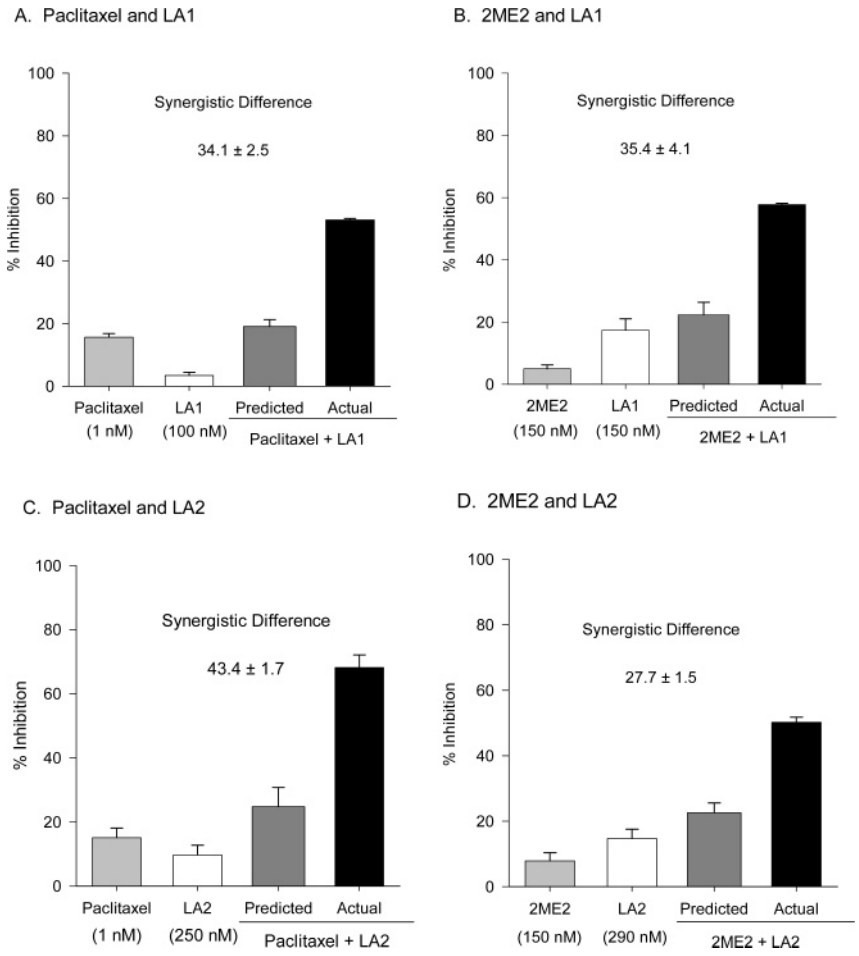

Figure 2. Antiproliferative effects of laulimalide analogues in combination with paclitaxel or 2ME2. A549 cells were treated with low inhibitory concentrations of two compounds singly and in combination. The predicted value is the sum of the effects of each agent used alone. The actual value is the experimentally measured value obtained when the agents were used in combination. The synergistic difference is the numerical difference between the predicted sum and the experimental value. $n = 3 \pm$ SE.

in contrast to the results obtained with paclitaxel. Similar results were seen with MDA-MB-435 cells (data not shown).

The Combination Index. The CI provides another mechanism to evaluate the synergistic actions of two drugs used together.[3,5,8] The CI values were individually calculated according to the methods of Chou and Talalay for nine different effect levels from 10% to 90% inhibition of proliferation for each of the drug combinations in A549 cells.[24] The CI values calculated for the combination of laulimalide and paclitaxel are less than 1, indicating synergism, for all the points below 50% inhibition of proliferation (Figure 4A). The CI values obtained for the combinations of LA1 and paclitaxel and LA2 and paclitaxel indicate synergistic interactions over a large range, from 10% to 70% inhibition of proliferation (Figure 4B,C). The combination of 2ME2 and laulimalide was also synergistic below 50% inhibition of proliferation (Figure 4D), similar to the effects that were obtained with the combination of laulimalide and paclitaxel. Over the range from 60% to 90% inhibition of proliferation, the combinations of laulimalide with either paclitaxel or 2ME2 were subadditive (Figure 4A,D). The CI values calculated for the laulimalide analogues LA1 or LA2 with 2ME2 (Figure 4E,F) indicate that these combinations are synergistic over a large effect range, 10−90% inhibition of proliferation with LA1, and slightly less for LA2, 10−60% inhibition of proliferation. These analyses are consistent with the results obtained with the isobolograms which indicated superadditive effects of the combination of the laulimalide analogues with either paclitaxel or 2ME2. The CI analyses indicate that any of the laulimalides together with paclitaxel or 2ME2 provide synergistic antiproliferative actions and the degree of synergism is greater with the laulimalide analogues LA1 and LA2 than is observed with the parent compound.

Shift in the Dose Response Curves. When describing the ability of discodermolide to act synergistically with paclitaxel, Martello and colleagues[3] evaluated the effects of adding a constant low concentration of paclitaxel to a range of concentrations of discodermolide. They plotted the discodermolide dose−response curves, and noted the shift in IC_{50} values for each concentration of paclitaxel. These experiments were useful as an alternate mechanism to show the superadditive effects of this drug combination. These same types of experiments were conducted with the laulimalides.

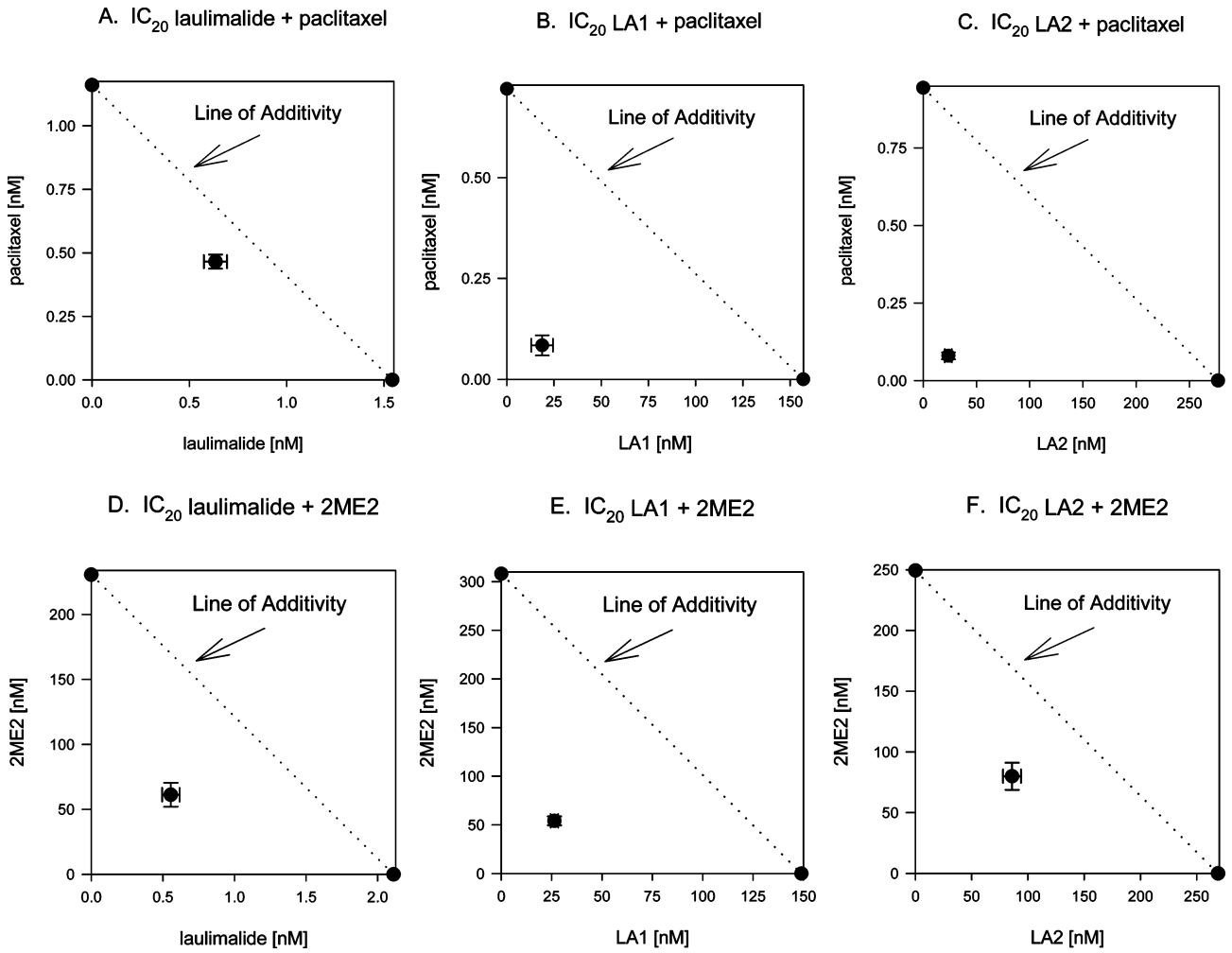

Figure 3. Isobolographic analyses of the effects of the laulimalides in combination with paclitaxel or 2ME2 in A549 cells. Isobolograms were generated using dose−response curves and represent an IC_{20} concentration for individual and combination treatments. $n = 3 \pm$ SE.

A full dose−response curve for each laulimalide was constructed along with a second dose−response curve in which a low concentration of either paclitaxel or 2ME2 was added with the laulimalides. The antiproliferative effects of that single concentration of 2ME2 or paclitaxel alone were also measured. This allowed construction of theoretical, additive dose−response curves that were compared with actual dose−response curves obtained experimentally with the combination. A shift to the left of the actual dose−response curve, as compared to the theoretical curve, and a lowering of the IC_{50} value from that anticipated from additive effects would indicate synergistic antiproliferative activities.

The effects of paclitaxel added to LA1 are shown in Figure 5 as an example of this type of analysis. The effects of LA1 alone as well as the actual dose response curve obtained by adding 1.2 nM paclitaxel, which alone causes 21.8% inhibition, to each point are shown on the LA1 dose−response curve (Figure 5). The third curve in Figure 5 represents a theoretical dose−response curve that was generated by assuming that the antiproliferative effects of the two drugs in combination at each point would be equal to the mathematical sum for each drug used singly. IC_{50} values were calculated from the actual and theoretical dose−response curves. Experiments were conducted for each of the laulimalides in combination with paclitaxel or 2ME2 and the effects on dose−response curves and the IC_{50} values calculated for each combination. The results of the experiments are presented in Table 1. The anticipated and actual IC_{50} values are shown for each combination. The fold difference was calculated to demonstrate the magnitude of each dose−response curve shift. These data are consistent with the isobologram and CI analyses that show that the combination of laulimalide, LA1, or LA2 together with paclitaxel or 2ME2 resulted in synergistic antiproliferative responses.

Effects of Combinations of Antimitotic Agents on G_2/M Accumulation and Cytotoxicity. The ability of tubulin-binding antimitotic agents to inhibit proliferation is linked with their ability to interrupt normal mitotic events ultimately leading to initiation of apoptosis.[1] Studies were conducted to identify whether the combinations of laulimalide and paclitaxel or laulimalide and 2ME2 had synergistic effects

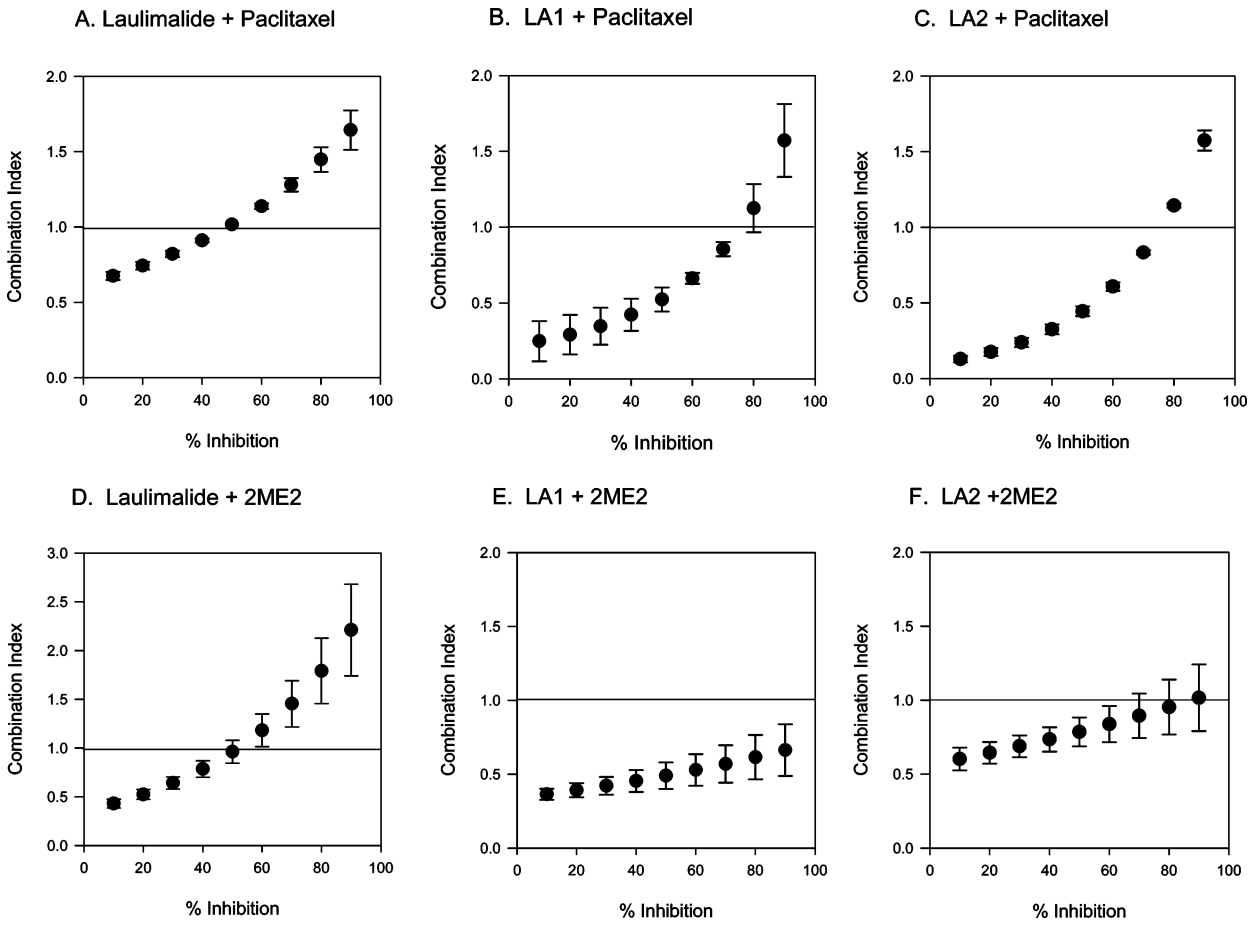

Figure 4. CI analyses of the effects of the laulimalides in combination with paclitaxel or 2ME2 in A549 cells. CI values were calculated for each point shown as described in Materials and Methods. The CI values are the average of 3 experiments and are shown ± SE. The CI values were plotted as a function of the particular inhibitory effect. CI values below 1 represent a synergistic combination, CI values equal to 1 are additive, and CI values above 1 represent subadditive combinations.

on mitotic accumulation. A range of concentrations of each compound were evaluated for the ability to initiate G_2/M accumulation at 24 h. Laulimalide, paclitaxel, and 2ME2 each caused a concentration dependent increase in the number of cells in G_2/M. The effects of various combinations were evaluated, and the results (Figure 6) show that the combination of laulimalide and paclitaxel causes more G_2/M accumulation than is predicted from the effects of each compound used singly (Figure 6A). The predicted additive effects for the experiment shown were 28.4%, yet experimentally 38.6% of the cells were in the G_2/M phase of the cell cycle. Similar effects were obtained with the combination of 2ME2 and laulimalide (Figure 6B). The predicted additive effect was 36.1%, and experimentally 57.5% of the cells were in G_2/M. These data provide mechanistic insights that link synergistic mitotic accumulation with inhibition of proliferation.

Individually, the laulimalides, paclitaxel, and 2ME2 all cause antiproliferative effects, and at slightly higher concentrations they are all cytotoxic in the MDA-MB-435 and A549 cells lines. The ability of combinations of laulimalide with paclitaxel or 2ME2 to exhibit synergistic cytotoxic effects was also evaluated using the SRB assay. Laulimalide together with paclitaxel or 2ME2 provided synergistic cytotoxicity (data not shown), presumably through an apoptotic process as each of these agents initiates apoptosis when used as a single agent.

Discussion

We evaluated the effects of laulimalide and synthetic laulimalide analogues in combination with other antimitotic agents to determine whether they would have additive or synergistic antiproliferative actions. Several methods were used to analyze synergism including isobolograms and CI values. The results are consistent, and indicate that the laulimalides in combination with paclitaxel or 2ME2 caused significant synergistic effects. To identify whether these effects were related to the interactions of the agents with their intracellular target, tubulin, the ability of a non-tubulin-targeting antimitotic, monastrol, was also evaluated in combination with laulimalide. The studies indicated that monastrol and laulimalide together provided only additive antiproliferative effects. The nature of the synergistic interaction of the drugs examined in this study appears to be related to their tubulin dependent antimitotic actions. It is interesting that the synergistic combinations identified in this study

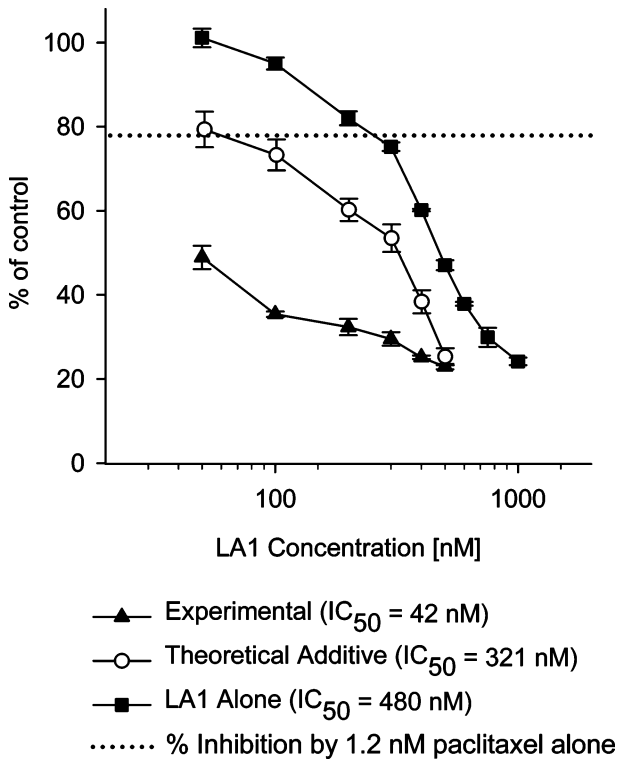

Figure 5. Effects of paclitaxel on the LA1 dose−response relationship. The theoretical dose−response curve was calculated by adding the percent inhibition of each of the drugs when used singly in A549 cells. The experimental dose−response curve was obtained by adding a fixed concentration of paclitaxel, 1.2 nM, which singly caused 21.8% inhibition to each concentration of LA1. $n = 3 \pm$ SE.

Table 1. Theoretical and Actual IC_{50} Values of Combination Treatments[a]

	IC_{50}, nM		
combination	predicted additive	experimental combination	fold difference
laulimalide and paclitaxel	7.2 ± 0.3	3.1 ± 0.1	2.3
laulimalide and 2ME2	7.6 ± 0.1	5.6 ± 0.1	1.4
LA1 and paclitaxel	321 ± 18	42 ± 0.7	7.6
LA1 and 2ME2	505 ± 3	175 ± 3	2.9
LA2 and paclitaxel	484 ± 2.5	167 ± 19	2.9
LA2 and 2ME2	859 ± 31	394 ± 4	2.3

[a] Inhibition of proliferation was determined using the SRB assay. $n = 3 \pm$ SE.

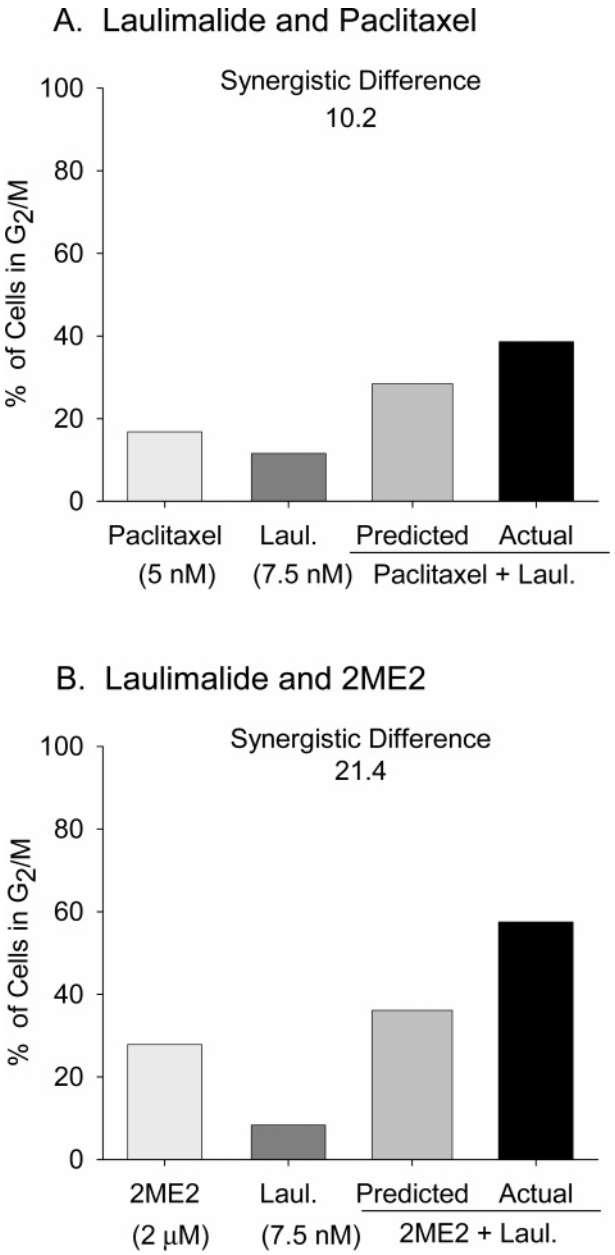

Figure 6. Effects of laulimalide and paclitaxel or 2ME2 on mitotic accumulation. Cells were treated for 24 h with laulimalide (7.5 nM), paclitaxel (5 nM), or 2ME2 (2 μM) or a combination of paclitaxel and laulimalide or laulimalide and 2ME2. The predicted value is the additive sum of the effects of each agent used alone. The actual value is the experimentally measured value obtained when the compounds were used in combination. The synergistic difference is the difference between the predicted sum and the experimental value. The data are from 1 of 2 representative experiments.

involve combinations of drugs that bind to microtubule/tubulin on nonoverlapping sites. Whether this is required for the synergistic actions is not yet known.

Although all the laulimalides we evaluated provided synergistic actions with both paclitaxel and 2ME2, the laulimalide analogues LA1 and LA2 achieved a higher level of synergism than was obtained with laulimalide. The isobolographic data and CI values indicate that the laulimalide analogues and paclitaxel provided a greater degree of synergism than was obtained with paclitaxel and laulimalide. The CI analyses indicate that LA1 in combination with 2ME2 provided lower CI values than were obtained with the combination of 2ME2 and laulimalide, indicative of a higher degree of synergism. The laulimalide analogues in combination with either paclitaxel or 2ME2 yielded CI values less than 1 over a wider efficacy range than was obtained with laulimalide. It is possible that this might indicate slight mechanistic differences between these ana-

logues and the natural product. Earlier mechanistic studies indicated that LA1 and LA2 were indistinguishable from laulimalide;[15] however, these new data might suggest subtle differences. The synergistic antiproliferative actions of laulimalide and paclitaxel are consistent with the ability of laulimalide and paclitaxel to act synergistically to polymerize tubulin in vitro.[8] Although all the data available suggest that laulimalide binds to a distinct binding site on microtubules, laulimalide and paclitaxel have almost identical effects on purified tubulin.[8] Two slight differences were noted, however. Aberrant tubulin polymers in the form of sheets and ribbons were more common in polymers assembled in the presence of paclitaxel, while most of the polymers formed with laulimalide had microtubule-like structures.[8] The laulimalide-initiated tubulin polymers were also more stable during prolonged incubation at 0 °C as compared to paclitaxel-induced tubulin polymers. The ability of paclitaxel and laulimalide to synergistically stimulate tubulin assembly suggests that they might act in a slightly different manner which leads to synergistic and not additive interactions. The synergistic effects of laulimalide in combination with paclitaxel were not unique. The combination of laulimalide together with epothilone A, discodermolide, eleutherobin, or sarcodicyin A also initiated synergistic tubulin assembly.[8] The synergistic effects of laulimalide in combination with other microtubule stabilizers were unique. Multiple combinations of paclitaxel with other microtubule stabilizers that bind to tubulin within the taxane-binding site did not yield superadditive assembly of tubulin polymer.[8]

Whether the synergistic effects of paclitaxel and laulimalide on tubulin assembly would translate into synergistic antiproliferative actions was the next important question. Our data suggest that laulimalide or the laulimalide analogues together with paclitaxel provide synergistic antiproliferative actions. However, the data showing that the laulimalides are also synergistic with the microtubule depolymerizer 2ME2 suggests that the effects of these drugs on tubulin assembly reactions might not be the underlying mechanism for their antiproliferative effects. Laulimalide-induced tubulin assembly in vitro was inhibited by a variety of microtubule-depolymerizing agents, including agents such as 2ME2, that bind to tubulin at the colchicine binding site.[8] If the effects on tubulin assembly in vitro were related to the ability of these drugs to inhibit cellular proliferation, then the combination of 2ME2 and laulimalide would be expected to be subadditive or antagonistic. In contrast, our data suggest a synergistic interaction between 2ME2 and the laulimalides. In addition, the laulimalide analogues were synergistic with 2ME2 over a slightly wider range of concentrations than was obtained with these compounds in combination with paclitaxel. The synergistic actions of the laulimalides with both a microtubule stabilizer and a microtubule depolymerizer can be resolved by the capacity of both classes of tubulin-interacting agents, at their lowest antiproliferative concentrations, to inhibit microtubule dynamics leading to mitotic arrest.[1,2] This hypothesis is supported by the ability of laulimalide and 2ME2 to act synergistically to cause G_2/M accumulation. Further studies are needed to determine the specific mechanistic interactions of these various compounds that lead to synergistic antimitotic actions.

The ability of two tubulin-binding antiproliferative drugs to have synergistic effects is not unprecedented. The combination of discodermolide and paclitaxel is synergistic for inhibition of proliferation and for the ability to inhibit microtubule dynamics.[3,5] A number of other combinations of tubulin-binding drugs are also reported to have synergistic actions, including the combinations of a taxane with a colchicine site binding agent,[25] paclitaxel and vinorelbine,[26−28] paclitaxel and vinblastine,[29] and paclitaxel and estramustine.[30] Not all combinations of tubulin-targeting antimitotic agents provide synergistic actions. Paclitaxel in combination with either epothilone B or eleutherobin provided only additive antiproliferative effects.[3] Cryptophycin 1 and vinblastine together produced only additive antiproliferative actions.[31] These studies suggest that drugs which bind to different binding sites have a higher propensity of acting synergistically, and this is consistent with our results. Mechanistically, it is not known exactly how this occurs, but it might involve synergistic actions on microtubule dynamics. The synergistic antiproliferative effects of discodermolide and paclitaxel are linked with the ability of this pair of drugs to synergistically inhibit microtubule dynamics.[5] The slightly different effects of discodermolide and paclitaxel on dynamic instability might provide the basis for the synergistic actions on microtubule dynamics and on inhibition of proliferation. It is interesting to speculate that the laulimalides' effects on microtubule dynamics might differ somewhat from the action of other tubulin-targeting antimitotic drugs and that this provides the opportunity for synergistic inhibition of microtubule dynamics. Additional studies are needed to test this hypothesis.

Tubulin-targeting antimitotic agents have important utilities in the clinical setting for the treatment of cancer. Although all these drugs have a similar mechanism of action, they are

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not identical. The microtubule stabilizers paclitaxel, epothilone B, eleutherobin, and discodermolide all bind within the taxane-binding site on microtubules. All of these compounds, except discodermolide, can support the growth of a paclitaxel dependent cell line.[3] The ability of discodermolide to act synergistically with paclitaxel provided clues for the possibility of slight mechanistic differences. The synergism data presented here indicate that the laulimalides, like discodermolide, might differ slightly in mechanism as compared to other microtubule stabilizers and microtubule depolymerizers. Elucidation of the specific molecular mechanisms of action of these chemically diverse microtubule interacting agents will provide important information for rational combinations that might increase antitumor efficacy and reduce toxic side effects.

Efforts to design more stable analogues of laulimalide have resulted in two lead compounds that provide even more impressive synergism with both paclitaxel and 2ME2 than the parent compound. In advancing these agents toward clinical trials it will be important to evaluate them for in vivo synergistic antitumor efficacy with other tubulin-binding antimitotic agents including paclitaxel and 2ME2.

Acknowledgment. We thank the Natural Products branch of the National Cancer Institute for the supplying *Cacospongia mycofijiensis* and Veena Satitpatipan for isolating laulimalide from the sponge. We thank Sayee G. Hegde, Robert D. Hubbard, and Lei Zhang for their efforts in the design and synthesis of LA1 and LA2. This work was supported by grants from the National Cancer Institute (CA31841 to P.A.W. and CA81388 to B.S.D.), the Joe and Jessie Crump Foundation (S.L.M.), and the Amon Carter Foundation (S.L.M.).

MP060016H