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Laulimalide and Paclitaxel: A Comparison of Their Effects on Tubulin Assembly and Their Synergistic Action When Present Simultaneously

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ABSTRACT

Previous work has shown that laulimalide, a sponge-derived natural product, resembles paclitaxel in enhancing tubulin assembly and in its effects on cellular microtubules. The two compounds, however, seem to have distinct binding sites on tubulin polymer. Nearly equimolar amounts of tubulin, laulimalide, and paclitaxel are recovered from microtubules formed with both drugs. In the present study, we searched for differences between laulimalide and paclitaxel in their interactions with tubulin polymer. Laulimalide was compared with paclitaxel and epothilone A, a natural product that competes with paclitaxel in binding to microtubules, for assembly properties at different temperatures and for effects of GTP and microtubule-associated proteins on assembly. Although minor differences were observed among the three drugs, their overall effects were highly similar, except that aberrant assembly products were observed more frequently with paclitaxel and that the polymers formed with laulimalide and epothilone A were more stable at 0°C. The most dramatic difference observed between laulimalide and epothilone A was that only laulimalide was able to enhance assembly synergistically with paclitaxel, as would be predicted if the two drugs bound at different sites in polymer. Because stoichiometric amounts of laulimalide and paclitaxel can cause extensive tubulin assembly, maximum synergy was observed at lower temperatures under reaction conditions in which each drug alone is relatively inactive. Laulimalide-induced assembly, like paclitaxel-induced assembly, was inhibited by drugs that inhibit tubulin assembly by binding at either the colchicine- or vinblastine-binding site. When radiolabeled GTP is present in a reaction mixture with either laulimalide or paclitaxel, nucleotide hydrolysis occurs with incorporation of radiolabeled GDP into polymer.

The microtubule system of eucaryotic cells is readily disrupted by numerous compounds, including an ever-increasing number of agents, for the most part natural products, that cause the hyperassembly of tubulin both in biochemical systems and in cultured cells. The first group of these compounds to be studied in detail was paclitaxel and structurally related taxoids (Schiff et al., 1979; Guéritte, 2001), and interest in the taxoids became more intense as it became clear that they had good activity against human neoplasms (Cortes and Pazdur, 1995; Rowinsky and Donehower, 1995). A number of more recently described drugs with a taxoid-like mechanism of action seem to bind in the same or, perhaps, an overlapping site on tubulin polymers as paclitaxel, based on their acting as competitive inhibitors of the binding of paclitaxel to tubulin (Bollag et al., 1995; Kowalski et al., 1997a,b; Long et al., 1998; Hamel et al., 1999). An exception to this generalization is the compound laulimalide (Pryor et al., 2002), the structure of which is shown in Fig. 1.

Laulimalide was isolated from a variety of sponges in Pacific waters (Corley et al., 1988; Quiñoà et al., 1988; Jefford et al., 1996; Mooberry et al., 1999), and it has been totally synthesized by many investigators (Enev et al., 2001; Ghosh et al., 2001; Mulzer and Ohler, 2001; Paterson et al., 2001; Crimmins et al., 2002; Nelson et al., 2002; Wender et al., 2002; Williams et al., 2002; Gallagher et al., 2004). Mooberry et al. (1999) initially reported that laulimalide induced tubulin assembly, caused cells to accumulate at the G_2/M phase of the cell cycle, and caused paclitaxel-like microtubule bundles to appear in cultured cells. Using synthetic laulimalide, we recently reported that the compound was unable to inhibit the binding of radiolabeled paclitaxel to tubulin polymer or displace a fluorescent paclitaxel analog (a much more sensitive assay for weaker inhibitors) from tubulin polymer. Moreover, when both paclitaxel and laulimalide were mixed in stoichiometric amounts with tubulin, a polymer pellet was isolated that contained tubulin, paclitaxel, and laulimalide in near-equimolar amounts (Pryor et al., 2002). We had also found that laulimalide and paclitaxel had nearly identical activity in a tubulin assembly reaction containing both GTP and heat-treated MAPs across a temperature spectrum.

ABBREVIATIONS: MAP, microtubule-associated protein; MES, 4-morpholineethanesulfonate; TLC, thin layer chromatography.

The present studies were undertaken to determine whether binding at different sites in tubulin polymer resulted in significant differences between polymer formed with laulimalide versus polymer formed with paclitaxel or with epothilone A. Epothilone A was included in the comparison because it had previously been shown to have activities almost identical with those observed with paclitaxel (Bollag et al., 1995; Kowalski et al., 1997b). We found that GTP and MAPs had similar effects on assembly reactions with the three drugs, including hydrolysis of GTP during laulimalide-induced assembly. Our most striking finding was that laulimalide and paclitaxel, but not epothilone A and paclitaxel, could act synergistically in stimulating tubulin assembly, as would be implied by their binding at apparently distinct sites in tubulin polymer.

Materials and Methods

Materials. Laulimalide was synthesized as described previously (Ghosh et al., 2001). Tubulin freed of MAPs and heat-treated MAPs were prepared from bovine brain tissue (Hamel and Lin, 1984), including gel filtration chromatography of the tubulin to remove unbound nucleotide (Grover and Hamel, 1994) as described previously. Paclitaxel and epothilone A were generously provided, respectively, by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Rockville, MD) and by Merck Research Laboratories (Rahway, NJ). GTP (Sigma, St. Louis, MO) and [8-^{14}C]GTP (Moravek Biochemicals, Brea, CA) were repurified to >99% purity by triethylammonium bicarbonate gradient chromatography on DEAE-Sephacel.

Methods. Tubulin assembly was followed turbidimetrically in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. All components except drug were added to cuvettes held at 0°C, and baselines were established at 350 nm. Drugs were then added, and assembly was followed sequentially at 0°C and the indicated higher temperatures. Reaction mixtures (final volumes, 0.25 ml) contained the components indicated in the individual experiments.

Centrifugal analysis of polymer formation was performed with reaction mixtures at 30, 10, and 0°C. For experiments in which turbidity development was correlated with polymer mass, aliquots of reaction mixtures were taken from cuvettes and centrifuged for 15 min at 30,000 rpm in a TLA 100 rotor in an Optima TLX centrifuge (Beckman-Coulter, Fullerton, CA). Centrifugation was either at 30°C in a prewarmed rotor or at 2°C in a prechilled rotor. Protein concentrations of the supernatant and the total reaction mixture were obtained by the Lowry assay, and polymer formation (size of pellet) was determined by subtraction of the supernatant protein concentration from the total protein concentration.

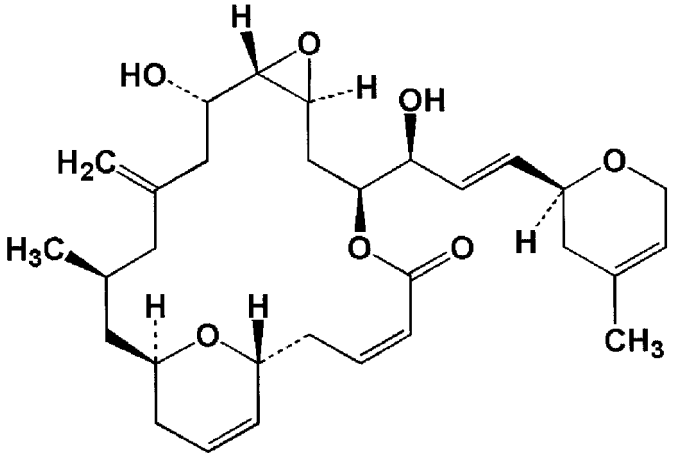

Fig. 1. Structural diagram of laulimalide.

For experiments in which potential synergy of laulimalide and paclitaxel on polymer assembly at 10°C was examined, 100-μl reaction mixtures were prepared containing 1.0 mg/ml (10 μM) tubulin, 4% (v/v) dimethyl sulfoxide, 0.1 M MES, pH 6.9, and varying drug concentrations. Samples were incubated for 45 min at 10°C and centrifuged for 10 min at 10°C at 30,000 rpm in a TLA 100 rotor in an Optima TLX centrifuge. Protein concentrations were determined and calculation of amount of polymer formed was as described above. In a series of eight experiments, in the absence of drug, the pellets formed at 10°C contained an average of 5.2 $\pm$ 1% (S.D.) of the total tubulin, and all data were corrected for the protein pellet formed in the absence of drug. Combination index analysis with the assumption of mutually nonexclusive drug binding sites was performed by the method of Chou and Talalay (1984), using the program CalcuSyn obtained from Biosoft (Ferguson, MO).

Electron micrographs were prepared from samples whose assembly status was monitored turbidimetrically. An aliquot (5–10 μl) was placed on a 200-mesh, carbon-coasted, formvar-treated copper grid, followed by 5 to 10 drops of 1% (w/v) uranyl acetate. Excess stain was removed from the grid by wicking with torn filter paper, and the grids were examined in a Zeiss model 10CA electron microscope (Carl Zeiss GmbH, Jena, Germany). For polymer length determination, areas of the grid were sought where most polymers were well separated. Lengths were determined from digital scans of the micrograph negatives using the program MIPAV (Medical Imaging, Processing, Analysis, and Visualization), developed at the Center for Information Technology, National Institutes of Health, and generously provided by Dr. M. McAuliffe. Details can be obtained at the MIPAV web site (http://mipav.cit.nih.gov).

Identification of exchangeable site nucleotide in polymer induced by laulimalide or paclitaxel was determined by recovery of polymer by ultracentrifugation, extraction of the pellet with 8 M urea, and identification of radiolabeled nucleotides by TLC on polyethylenimine cellulose and autoradiography. Reaction mixtures (1.0 ml) contained tubulin at 1.0 mg/ml, heat-treated MAPs at 0.5 mg/ml, 0.1 M MES, pH 6.6, either 0.5 mM $MgCl_2$ or 0.1 mM $BeSO_4$, drug, if present, at 25 μM, 2.5% dimethyl sulfoxide, and 50 μM [8-^{14}C]GTP. Incubation was for 5 min at 37°C. The reaction mixtures were centrifuged at 47,000 rpm for 30 min at 37°C in a TLA 55 rotor in an Optima TLX centrifuge. The supernatants were discarded, and the pellets were washed three times with a 37°C solution containing 0.1 M MES, pH 6.6, and either 0.5 mM $MgCl_2$ or 0.1 mM $BeSO_4$, as appropriate. The washed pellets were each dissolved in 50 μl of 8 M urea. The resulting solutions were counted, and about 30,000 cpm from each solution was spotted on a full-length sheet of polyethyleneimine cellulose, together with [8-^{14}C]GDP and [8-^{14}C]GTP, spotted separately, as standards. TLC was performed with 1.0 M KH_2PO_4 (pH unadjusted). The dried TLC sheet was exposed to Kodak X-ray film overnight.

Results

Tubulin Assembly with MAPs or GTP Compared with Assembly with Both MAPs and GTP

Turbidimetric Evaluation. Fig. 2 presents a comparative study with laulimalide, paclitaxel, and epothilone A, as well as without drug, with the temperature increased in stages to 30°C, followed by a final 20 min at 0°C to assess the stability of the polymers formed to the low temperature. Figure 2A shows a study with both MAPs and GTP ("complete system") in the reaction mixtures, whereas B and C present experiments in which either GTP (B) or MAPs (C) were omitted.

The results obtained in the complete system (Fig. 2A) were

similar to those described previously (Pryor et al., 2002). Sluggish reactions at 0°C with the drugs were followed by similar turbidity development at 10°C. At 20°C, there was little further increase in turbidity with laulimalide or with epothilone A, but there was a marked further increase with paclitaxel. Without drug there was slight turbidity development at 20°C, followed by brisk assembly at 30°C. With the drugs, the transition from 20 to 30°C had little effect. The polymers formed with laulimalide and epothilone A seemed highly stable at 0°C, whereas the polymer formed without drug rapidly disassembled (no residual polymer was observed by electron microscopic evaluation of samples prepared from such reaction mixtures without drug at 0°C). With paclitaxel, by contrast, turbidity fell slowly, to about half the final reading at 30°C.

Without GTP, but with MAPs (Fig. 2B), the reaction mixture without drug showed an increase in turbidity at 10 to 30°C but no change at 0°C. This probably represents either formation of an ill-defined tubulin-MAP oligomer or tubulin denaturation, for no morphologically distinct structures were observed by electron microscopy without drug. The drugs had negligible effects at 0°C, caused sluggish reactions at 10°C, and caused brisk reactions at 20°C. Proportionally greater effects were observed than in the complete system at 30°C with all three drugs; again, however, the largest increase in turbidity occurred with paclitaxel. The final turbidity readings with all three drugs were similar under this reaction condition. In contrast to the complete system, even with paclitaxel, there was little loss of turbidity when the temperature was reduced from 30 to 0°C in this MAPs-only system.

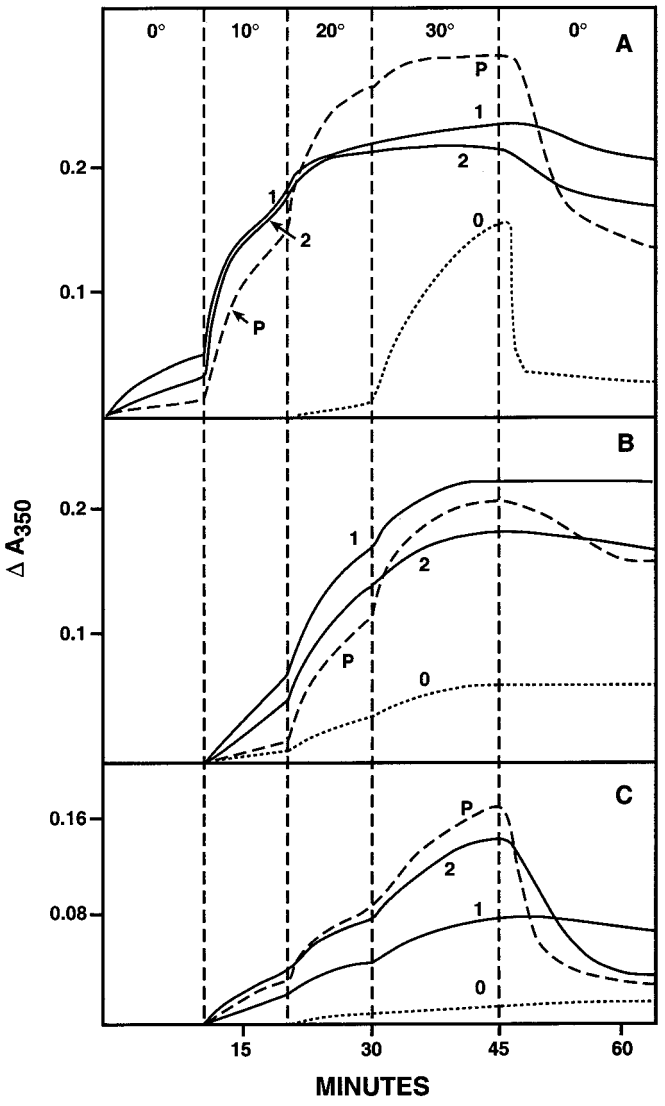

Fig. 2. Tubulin assembly induced by laulimalide, paclitaxel, and epothilone A in the presence of heat-treated MAPs and GTP (A), heat-treated MAPs only (B), or GTP only (C). Each 0.25-ml reaction mixture contained 1.0 mg/ml (10 μM) tubulin, 0.1 M MES, pH 6.9, 1% (v/v) dimethyl sulfoxide, and, as indicated, heat-treated MAPs at 0.75 mg/ml, 0.1 mM GTP, and 10 μM drug. Temperature changes as indicated. Drugs as indicated, as follows: curves 0, no drug; curves P, paclitaxel; curves 1, laulimalide; curves 2, epothilone A.

With GTP, but without MAPs (Fig. 2C), there was little turbidity development even at 30°C without drug; electron microscopic examination again confirmed the complete absence of polymer. With the three drugs, no change in turbidity occurred at 0°C, but there were nearly equivalent step increases in turbidity at the three higher temperatures. The final readings at 30°C, as well as at the lower temperatures, were significantly higher with both paclitaxel and epothilone A than with laulimalide. Returning the temperature to 0°C caused a substantial drop in turbidity with both paclitaxel and epothilone A but not with laulimalide.

In summary, aside from an apparently greater stability of laulimalide polymer compared with paclitaxel polymer to extended incubation at 0°C and the higher turbidity readings obtained with paclitaxel in the complete system and the GTP-only system, there was remarkably little difference between the polymerization reactions induced with laulimalide and with paclitaxel. It should be emphasized, however, that there are significant differences in polymer morphology under different reaction conditions, as described below. Therefore differences in turbidity readings do not necessarily correspond linearly to differences in polymer mass, for polymer morphology probably affects light scattering properties.

Morphologic Evaluation. Samples from reaction mixtures equivalent to those used in the turbidimetry studies shown in Fig. 2 were evaluated by electron microscopy (Fig. 3). Aliquots from reaction mixtures at different temperatures were applied to grids, which were negatively stained with 1% uranyl acetate. Under no reaction condition did we observe marked morphological differences (other than polymer length, see below) as a function of reaction temperature. In particular, samples taken at 30°C had an appearance similar to that of samples taken from the same cuvettes when the temperature was subsequently reduced to 0°C. In Fig. 3, selected polymers with a microtubule morphology are indicated by open arrows, whereas selected polymers with structurally aberrant morphology are indicated by closed arrows.

Figure 3, A and B, shows complete system polymers induced with laulimalide and paclitaxel, respectively. With paclitaxel, the polymer formed was a mixture of microtubules and a variety of open forms (sheets and ribbons), as well as thin structures that consist of very few protofilaments, perhaps even a single protofilament. A single continuous polymer often seemed to have variable morphologies throughout its length. With laulimalide, although a few sheet polymers were observed, a higher proportion of the observed polymers had a microtubule morphology. This probably accounts for the lower turbidity readings with laulimalide and epothilone

A[1] compared with paclitaxel, in that sheets and ribbons probably scatter light to a greater extent than do microtubules.

Figure 3, C and D, shows MAPs-only system polymers induced with laulimalide and paclitaxel, respectively. With paclitaxel, as in the complete system, aberrant sheet polymers were relatively common. With laulimalide, under this reaction condition, almost no sheet polymers were observed, the polymer consisting almost entirely of microtubules. It should be noted that the difference in morphologic appearance between the laulimalide- and paclitaxel-induced polymers seems to be inconsistent with the similar turbidity readings obtained with the two drugs (Fig. 2B), especially because nearly identical amounts of protein were recovered by centrifugation (data not presented).

Figure 3, E and F, shows GTP-only system polymers induced with laulimalide and paclitaxel, respectively. With paclitaxel, the polymer was again a mixture of microtubules and sheets, whereas the laulimalide polymer, as in the complete system, consisted primarily of microtubules mixed with scattered polymers of aberrant morphology. In the GTP-only system, the electron micrographic data are consistent with the greater turbidity observed with paclitaxel compared with laulimalide.

In summary, under all reaction conditions, more aberrant forms were observed with paclitaxel than with laulimalide. With laulimalide, addition of GTP to the reaction mixture seems to increase the proportion of polymer with aberrant morphology (also see below).

Previously, we have speculated that in the complete sys-

[1] With epothilone A, we have observed previously that the polymer consists primarily of microtubules with very few structures of aberrant morphology (Kowalski et al., 1997b) relative to the polymer induced with paclitaxel.

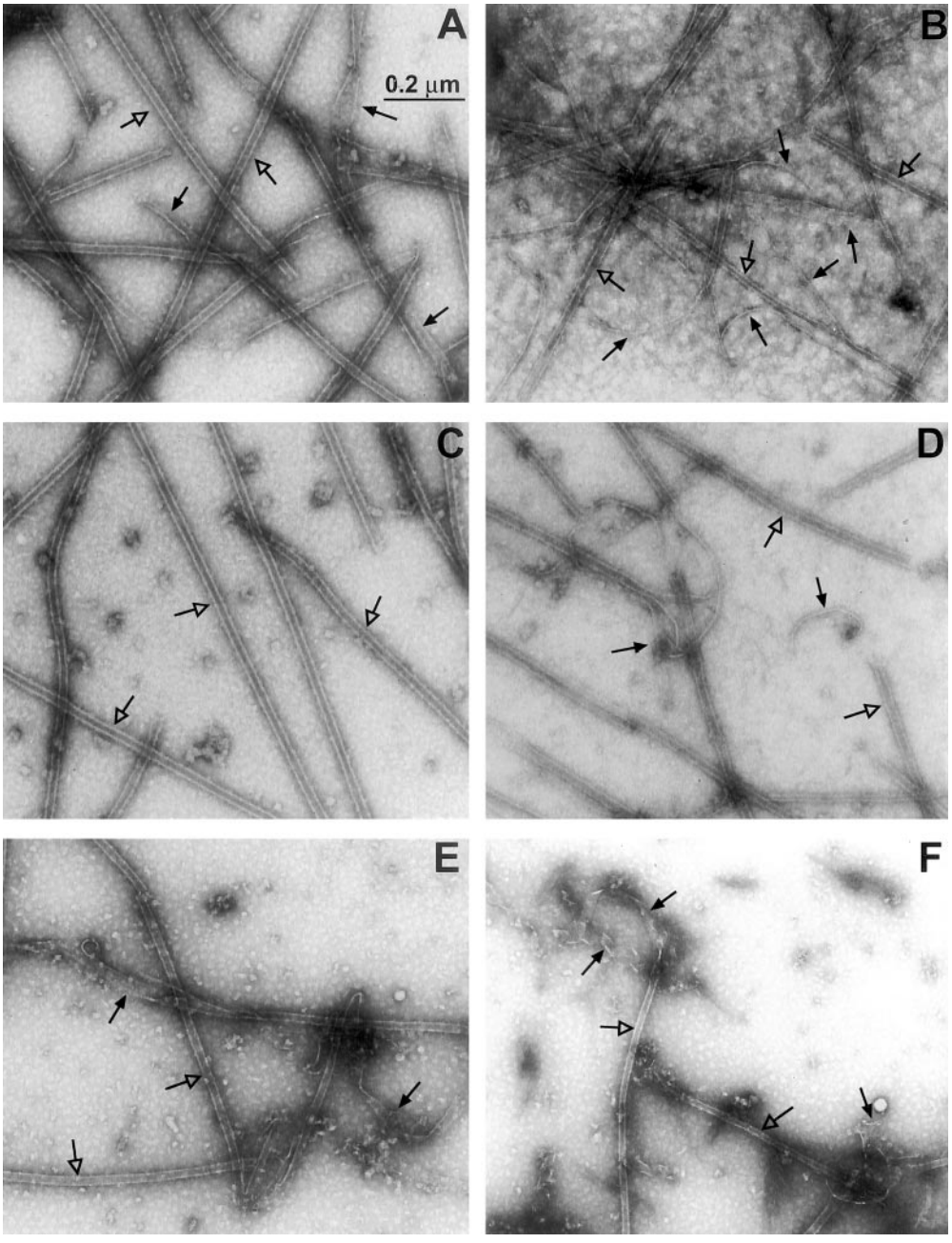

Fig. 3. Morphological appearance of tubulin polymers formed with either laulimalide (A, C, and E) or paclitaxel (B, D, and F) in reaction mixtures containing both heat-treated MAPs and GTP (A and B), heat-treated MAPs only (C and D), or GTP only (E and F). Reaction mixtures contained the components and were followed turbidimetrically as described in the legend for Fig. 2. After about 15 min at 30°C, grids were prepared as described in the text. Reaction mixtures were then chilled to 0°C for 30 min, and grids were prepared. We observed no significant difference between polymer morphology at the two temperatures in two independent experiments, and micrographs prepared from samples at 30°C are shown in the figure. All micrographs were taken at the same magnification; a scale bar is shown in A. Open arrows indicate selected polymers with a microtubule morphology, and closed arrows indicate selected polymers with aberrant morphology.

tem, the greater turbidity loss observed with the paclitaxel-induced polymer compared with polymer formed with epothilone A (Kowalski et al., 1997b), eleutherobin (Hamel et al., 1999), or laulimalide (Pryor et al., 2002) might be caused by preferential disassembly of the morphologically aberrant polymers as opposed to microtubules. The electron microscopic evaluation performed here did not support this idea, so we examined both microtubule lengths and polymer mass, based on recovery of protein by ultracentrifugation. A number of experiments were performed, comparable with those shown in Fig. 2A, with aliquots taken for electron microscopy and centrifugation at the end of the 30°C incubation and again after reduction of the reaction temperature to 0°C for about 30 min. With paclitaxel, there was 30% disassembly based on protein recovery by centrifugation. The average polymer lengths were 1.9 ± 0.9 μm (S.D.) (n = 419) at 30°C and 1.2 ± 0.6 μm (S.D.) (n = 788) at 0°C, or a 37% reduction in length. With laulimalide, there was 14% disassembly based on protein recovery by centrifugation. The average polymer lengths were 1.4 ± 0.7 μm (S.D.) (n = 270) at 30°C and 1.3 ± 0.7 μm (S.D.) (n = 343) at 0°C, or only a 7% reduction in length. Without drug, the microtubules were on average longer, but variability was much greater: average polymer length was 3.5 ± 4 μm (S.D.) (n = 402) at 30°C. We thus conclude that the 0°C decrease in turbidity observed for paclitaxel-induced polymer in the complete system results primarily from endwise disassembly of polymer of both microtubule and sheet morphology. The length data also indicate that in the complete system laulimalide was somewhat more effective than paclitaxel at nucleating assembly, consistent with its modestly greater activity at 0°C.

Tubulin Assembly with Neither MAPS nor GTP—Turbidimetric and Morphologic Evaluation

When 10 μM tubulin was incubated with 10 μM laulimalide, paclitaxel, or epothilone A in 0.1 M MES without either GTP or MAPs in the reaction mixture, there was relatively little increase in turbidity compared with the reaction mixture without drug (Fig. 4A). The reaction became more extensive at higher tubulin concentrations, such as 40 μM (Fig. 4B). Turbidity development occurred primarily at 20 and 30°C with all drugs. The turbidity readings were highest with paclitaxel, and the paclitaxel-induced turbidity showed the greatest reversibility at 0°C. Nevertheless, when laulimalide and paclitaxel were compared across a tubulin concentration range, the two drugs yielded similar critical concentrations for tubulin (Fig. 4C), about 0.2 to 0.4 mg/ml (2–4 μM).

The higher turbidity readings again seem to have been caused primarily by morphological differences between the polymers. With laulimalide, the polymer was almost totally microtubules (Fig. 5A), with sheet polymers very difficult to find, as was the case in the MAPs-only system described above. With paclitaxel, most of the polymers formed displayed aberrant morphology (Fig. 5B). As in Fig. 3, microtubules are indicated by open arrows and aberrant polymers by closed arrows.

Apparent Synergistic Effects of Laulimalide and Paclitaxel, but Not Paclitaxel and Epothilone A, on Tubulin Assembly

The incorporation of stoichiometric and equal amounts of laulimalide and paclitaxel into tubulin polymer can most readily be explained by each drug having a distinct binding site on the $\alpha\beta$-tubulin heterodimer (Pryor et al., 2002). This explanation raises the question of whether the two drugs can act synergistically in inducing tubulin assembly. Because at higher temperatures each drug alone has a maximal effect on assembly, whereas at low temperatures they are relatively inactive (Figs. 2 and 4), we examined the drugs alone and in combination at 0 and 10°C. Synergistic effects were observed under all reaction conditions described here but were most dramatic in the reaction condition without MAPs and without GTP (Fig. 6). In this series of experiments, drug was

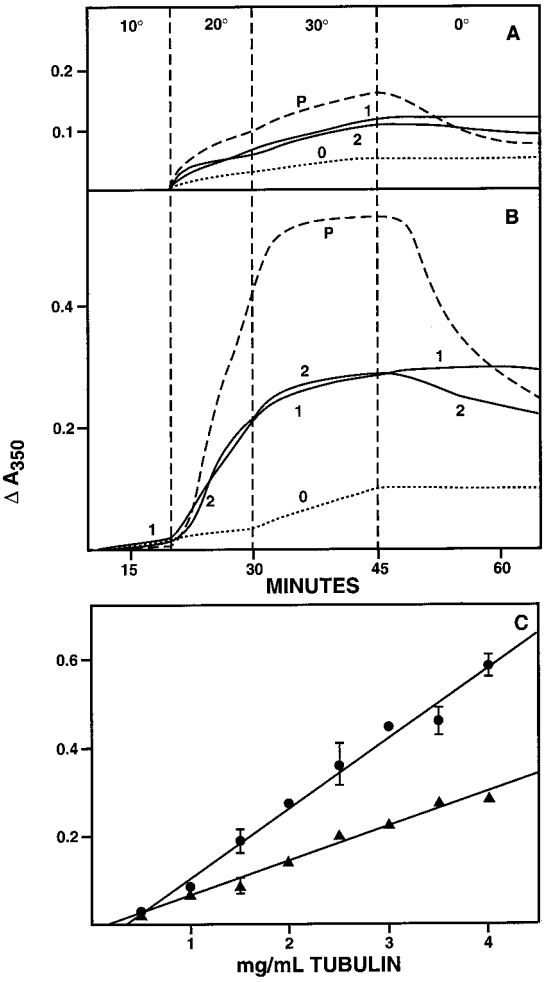

Fig. 4. Assembly of tubulin induced by laulimalide, paclitaxel, or epothilone A when neither heat-treated MAPs nor GTP was added to the reaction mixture. In all experiments, 0.25-ml reaction mixtures contained 10 μM drug, if present, 1% dimethyl sulfoxide, and 0.1 M MES, pH 6.9. A, tubulin concentration was 1.0 mg/ml (10 μM). Curve 0, no drug; curve P, paclitaxel; curve 1, laulimalide; curve 2, epothilone A. B, tubulin concentration was 4.0 mg/ml. Curve 0, no drug; curve P, paclitaxel; curve 1, laulimalide; curve 2, epothilone A. C, turbidity as a function of tubulin concentration. Reaction mixtures contained the indicated tubulin concentrations. ●, paclitaxel; ▲, laulimalide. For A and B, an initial 10-min incubation at 0°C is not shown, because there was no turbidity change in any sample. For C, samples were initially incubated for 2 min at 0°C. The reaction temperature was then jumped to 30°C for an additional 30 min incubation, at which point final turbidity readings were recorded. For the data points in C, standard deviations are shown when they exceeded the size of the symbol (minimum of three values obtained at each tubulin concentration). For C, parallel data were also obtained with no drug in the reaction mixture. This reaction was assumed to represent turbidity caused by nonspecific aggregation, and the values obtained at each tubulin concentration were subtracted from the values obtained with the drugs.

added to tubulin in a reaction mixture held at 0°C, and no turbidity change was observed until the temperature was jumped to 10°C. As shown in Fig. 6, a slight rise in turbidity occurred with 40 μM laulimalide and a larger increase in turbidity occurred with 40 μM paclitaxel (compare Fig. 4). A mixture of 10 μM laulimalide and 10 μM paclitaxel resulted in a higher turbidity reading than occurred with either drug alone at 40 μM. When both drugs were present at 20 μM in the reaction mixture, the turbidity reading was much greater than with either drug alone at 40 μM and equivalent to the turbidity observed at 30°C in the complete system (compare Figure 2A).

In contrast, no synergistic effect was observed when epothilone A and paclitaxel were mixed (Fig. 6, inset). In fact, the turbidity reading obtained with the two drugs at either 10 or 20 μM was below that obtained with 40 μM paclitaxel or 40 μM epothilone A.

We examined other drug mixtures as well, and laulimalide acted synergistically with the following taxoid site compounds: epothilone A, discodermolide (Kowalski et al., 1997a), eleutherobin (Hamel et al., 1999), and sarcodictyin A (Hamel et al., 1999). No synergy was observed with a variety of combinations of paclitaxel, docetaxel, epothilone A, eleutherobin, sarcodictyin A, and discodermolide.

The synergistic effects of laulimalide and paclitaxel in this 10°C reaction system were examined more extensively in a centrifugal assay, with a correction made for the tubulin that pelleted in the absence of either drug (about 5%). Up to about 20 μM, neither laulimalide nor paclitaxel caused a significant increase in the amount of tubulin removed from the reaction mixtures by centrifugation, whereas at 30 and 40 μM, an additional 10% of the tubulin was pelleted (Fig. 7). When both drugs were added to reaction mixtures in equimolar amounts, as little as 2.5 μM laulimalide + 2.5 μM paclitaxel resulted in enhanced tubulin polymerization, with a concentration-dependent increase in the amount of polymer formed. With both drugs present at 20 μM, the polymer formed was almost 60% of the total tubulin. When the data shown in Fig. 7 were analyzed by the combination index method of Chou and Talalay (1984) assuming mutually nonexclusive drug binding sites (Fig. 7, inset), combination indices in the 0.1 to 0.2 range were obtained. These values confirm the qualitative conclusion that the two drugs act synergistically in promoting the assembly of purified tubulin under the reaction conditions used in the studies summarized in Figs. 6 and 7.

We also wondered what effect varying the ratio of the two drugs would have on their synergistic effect on assembly, and a series of experiments with different ratios of the two compounds is summarized in Table 1. Although optimal synergy was observed with equimolar concentrations of the two drugs, significant assembly occurred when either drug was present at as little as one-eighth the concentration of the other. At the two most extreme ratios of 4- and 8-fold differences between the drugs, the low paclitaxel/high laulimalide combination was more effective than the high paclitaxel/low laulimalide combination. This could indicate that paclitaxel is the more important component in the synergistic combination or might simply result from the well known limited solubility of the taxoid. It is also worth considering details of the reaction mixtures in these experiments. With laulimalide/paclitaxel = 1:8, the polymer pellet contained the equivalent of about 2.4 μM tubulin from the original reaction mixture, which also contained 4.4 μM laulimalide + 35.6 μM paclitaxel. Thus, it is possible that the polymer contained stoichiometric amounts of both drugs.

When morphology of polymer formed with equimolar laulimalide and paclitaxel was evaluated, we observed more microtubules than with paclitaxel alone and more sheet polymers than with laulimalide alone (Fig. 5C). Thus, neither

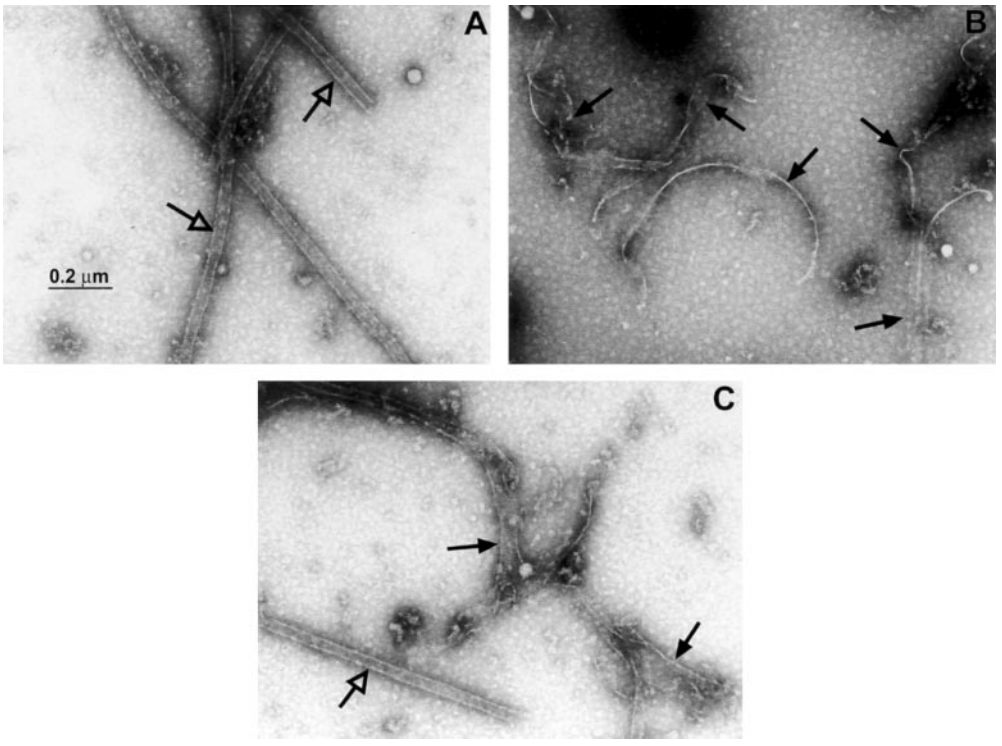

Fig. 5. Morphological appearance of polymer formed either with laulimalide (A), paclitaxel (B), or both compounds (C) in reaction mixtures containing neither heat-treated MAPs nor GTP. Reaction mixtures contained the components and were followed turbidimetrically as described in the legend for Fig. 4A, except that the micrograph shown in C was prepared from a reaction mixture containing both laulimalide and paclitaxel and the reaction at 10°C was followed for 30 min. The drugs were at 10 μM. After about 30 min at 10°C, grids were prepared as described in the text. Reaction mixtures were then incubated at higher temperatures, and grids were prepared at 30°C. Reaction mixtures were then chilled to 0°C for 30 min, and additional grids were prepared. We observed no significant difference between polymer morphology at the different temperatures in two independent experiments, and micrographs from samples at 10°C are shown in the figure. All micrographs were taken at the same magnification; a scale bar is shown in A. Open arrows indicate selected polymers with a microtubule morphology, and closed arrows indicate selected polymers with aberrant morphology.

drug seemed dominant in affecting morphology. Although we did not perform a quantitative comparison of morphology differences under the three drug conditions presented in Fig. 5, two independent experiments were performed, with grids prepared at three successive temperatures (10, 30, and 0°C). Both times many fields were observed, and the examples shown in Fig. 5 are typical.

Inhibitors of Tubulin Assembly Inhibit Laulimalide-Induced Polymerization

It has long been known that tubulin assembly induced by paclitaxel or docetaxel can be inhibited by drugs that inhibit tubulin polymerization. Similar inhibition has been described for assembly induced by epothilone B and discodermolide (Dabydeen et al., 2004). We wondered whether laulimalide-induced assembly might be different and examined the effects of several inhibitory drugs on assembly with laulimalide. Although a detailed quantitation of these effects was not performed, we observed that the laulimalide reaction could be inhibited with maytansine, combretastatin A-4, nocodazole, podophyllotoxin, and halichondrin B. The same drugs also inhibit paclitaxel-induced tubulin assembly (Dabydeen et al., 2004).

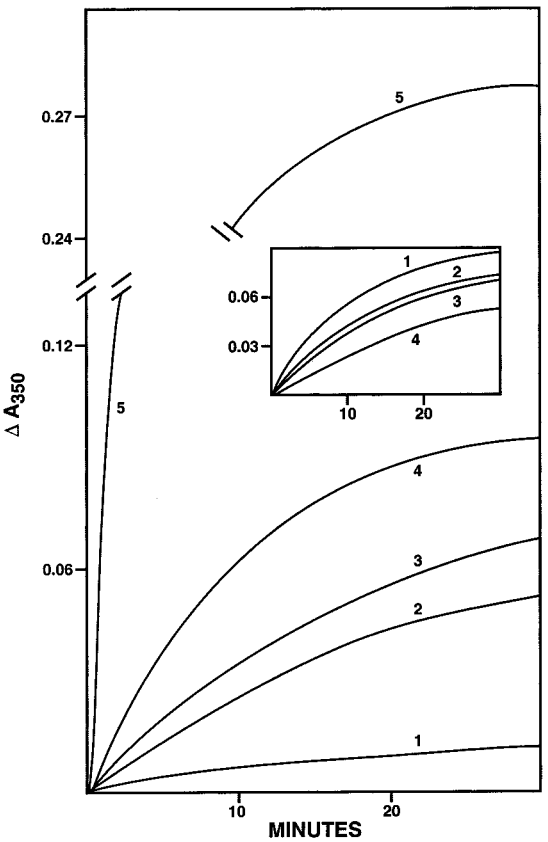

Fig. 6. Laulimalide and paclitaxel, but not paclitaxel and epothilone A (inset), act synergistically in inducing tubulin assembly at 10°C. Reaction mixtures contained 1.0 mg/ml tubulin, 4% dimethyl sulfoxide, 0.1 M MES, pH 6.9, and drugs as indicated. At zero time the temperature was jumped from 0 to 10°C. Curve 1, 40 μM laulimalide; curve 2, 20 μM paclitaxel; curve 3, 40 μM paclitaxel; curve 4, 10 μM laulimalide and 10 μM paclitaxel; curve 5, 20 μM laulimalide and 20 μM paclitaxel. Inset, curve 1, 40 μM paclitaxel; curve 2, 40 μM epothilone A; curve 3, 20 μM paclitaxel and 20 μM epothilone A; curve 4, 10 μM paclitaxel and 10 μM epothilone A.

GTP Hydrolysis during Laulimalide-Induced Assembly

Paclitaxel-induced assembly is accompanied by GTP hydrolysis when the nucleotide is included in the reaction mixture, and the amount of hydrolysis exceeds the amount of tubulin incorporated into polymer (Hamel et al., 1981). In initial experiments, we found that laulimalide-induced assembly was also accompanied by excess GTP hydrolysis. To measure the extent of hydrolysis of exogenously added GTP that was incorporated into polymer, microtubules formed in the presence of [8-^{14}C]GTP were harvested by centrifugation and dissolved in 8 M urea. The nucleotide content of the pellets was then evaluated, with the use of TLC, on polyethylenimine cellulose and autoradiography. The results are

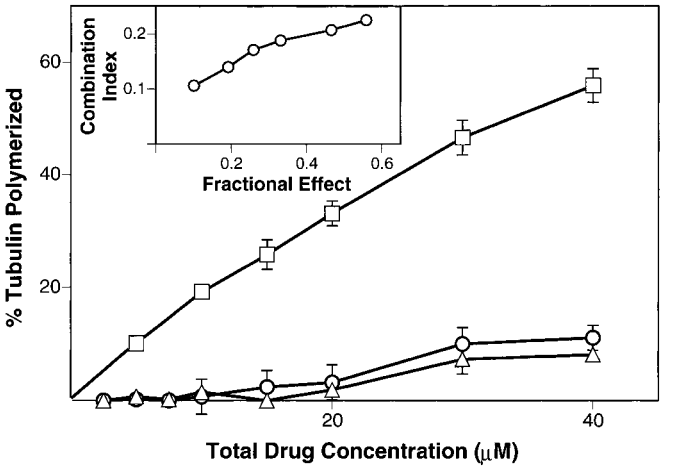

Fig. 7. Effects of laulimalide and paclitaxel, alone or in a 1:1 combination, on tubulin assembly at 10°C as measured by centrifugation. Reaction mixtures contained 1.0 mg/ml tubulin, 4% dimethyl sulfoxide, 0.1 M MES, pH 6.9, and drugs as indicated: ○, paclitaxel only; △, laulimalide only; □, equimolar laulimalide and paclitaxel. Reaction mixtures were incubated at 10°C for 45 min and centrifuged as described in the text. Pellet formation was calculated from total and supernatant protein, with correction for the amount of pellet formed (average, 5%) in the absence of both laulimalide and paclitaxel. No drug pellet was observed in any sample. Each data point represents the average of three independent experiments, and standard errors, unless smaller than the symbol, are indicated. Inset, data analyzed by the combination index method of Chou and Talalay (1984), with the assumption made that the drugs bind at mutually nonexclusive sites.

TABLE 1

Effect of laulimalide-paclitaxel ratio on the amount of tubulin polymer formed at 10°C

Reaction mixtures contained 1.0 mg/ml tubulin, 4% dimethyl sulfoxide, 0.1 M MES, pH 6.9, and drugs as indicated. Total drug was always 40 μM. Reaction mixtures were incubated at 10°C for 45 min and centrifuged as described in the text. Pellet formation was calculated from total and supernatant protein, with correction for the amount of pellet formed (average, 5%) in the absence of both laulimalide and paclitaxel. No drug pellet was observed in any sample. Each value represents the average obtained in three independent experiments, and standard errors are shown. The ratio 1:0 represents 40 μM laulimalide; the ratio 0:1 represents 40 μM paclitaxel.

Laulimalide:Paclitaxel	Tubulin Polymerized
	%
1:0	8.1 ± 0.1
0:1	11 ± 4
1:1	56 ± 3
2:1	44 ± 3
1:2	42 ± 2
4:1	44 ± 4
1:4	34 ± 3
8:1	34 ± 3
1:8	24 ± 1

shown in Fig. 8. Nucleotide extracted from a laulimalide-induced pellet is compared with nucleotide extracted from a paclitaxel-induced pellet, and in both cases, almost all the recovered radiolabeled nucleotide was in the form of GDP (99% GDP with both laulimalide and paclitaxel). In addition, we examined pellets formed without drug in the presence of Be^{2+} cation, as a control for detection of unhydrolyzed GTP in polymer. In previous studies, we had observed that with Mg^{2+}, radiolabeled nucleotide in the pellet was primarily in the form of GDP, whereas with Be^{2+} relatively little GTP was hydrolyzed during assembly (Hamel et al., 1992). This observation with Be^{2+} was confirmed (Fig. 8) (99% of the radiolabeled nucleotide was GTP), whereas with Mg^{2+} and no drug, the pellet also contained primarily GDP (data not shown).

Discussion

Previous studies have shown that laulimalide and paclitaxel probably bind at different sites on tubulin polymer (Pryor et al., 2002). Laulimalide was unable to inhibit the binding of [^{3}H]paclitaxel to polymer or to displace a fluorescent taxoid from polymer. The latter assay is able to detect even weak binding to the taxoid site (Andreu and Barasoain, 2001). Moreover, near-equimolar amounts of tubulin, laulimalide, and paclitaxel were recovered from polymer formed in a reaction mixture containing equimolar amounts of the protein and each of the drugs.

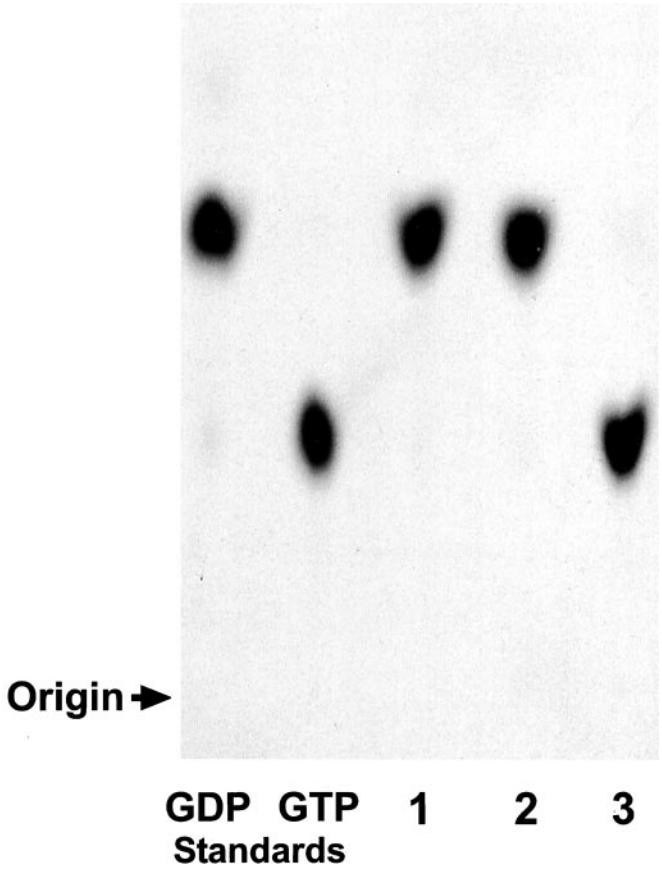

Fig. 8. Autoradiogram of polymer-bound, exchangeable site nucleotide after assembly with laulimalide or paclitaxel. Preparation of reaction mixtures and isolation and extraction of polymer-bound, radiolabeled nucleotide was described in the text. Origin and [8-^{14}C]GDP and [8-^{14}C]GTP standards as indicated. Sample 1, the reaction mixture contained paclitaxel. Sample 2, the reaction mixture contained laulimalide. Sample 3, the reaction mixture contained 0.1 mM $BeSO_4$ but no drug.

We have shown here that laulimalide and paclitaxel act synergistically in inducing tubulin assembly. When optimal reaction conditions are used, stoichiometric amounts of drugs that induce tubulin polymerization can induce nearly complete assembly of tubulin. Therefore, when we attempted to demonstrate synergistic assembly by combining laulimalide and paclitaxel, it was necessary to use suboptimal reaction conditions. The most dramatic results were obtained when both MAPs and GTP were excluded from reaction mixtures and when assembly was followed at 10°C. Furthermore, we could demonstrate similar apparent synergy by combining laulimalide with other drugs that inhibit paclitaxel binding to tubulin (e.g., laulimalide + epothilone A and laulimalide + discodermolide). In contrast, besides the results shown here with paclitaxel and epothilone A, we found no evidence of synergy in combinations of a wide variety of drugs that inhibit paclitaxel binding to tubulin (e.g., docetaxel + eleutherobin and epothilone A + discodermolide).

The synergistic effects of two drugs binding at different sites on tubulin polymer raises the question of whether the two drugs will act synergistically in cells. This is especially pertinent in view of the report that discodermolide and paclitaxel show synergistic cytotoxicity (Martello et al., 2000), despite discodermolide's strong binding at the taxoid site on tubulin polymer (Kowalski et al., 1997a). Although initial experiments failed to demonstrate significant synergistic cytotoxicity between laulimalide and paclitaxel (Pryor et al., 2002), this possibility should be explored more extensively. Many combinations of antitubulin agents do cause synergistic cytotoxicity. Besides the paclitaxel/discodermolide combination (Martello et al., 2000), synergy has been reported with taxoid/estramustine (Speicher et al., 1992; Kreis et al., 1997), taxoid/vinca alkaloid (Photiou et al., 1997; Roch et al., 1997; Carles et al., 1998; Giannakakou et al., 1998; Aoe et al., 1999; Culine et al., 1999; Budman et al., 2000; Budman and Calabro, 2002; Budman et al., 2002), taxoid/dicoumarol (Madari et al., 2003), and estramustine/vinca alkaloid (Batra et al., 1996; Kreis et al., 1997) combinations in a wide variety of cell types. Moreover, a paclitaxel/vinorelbine combination has a dramatic, synergistic effect in vivo in the treatment of murine P388 leukemia (Knick et al., 1995).

Except for its ability to act synergistically with taxoid site drugs and to bind independently to tubulin polymer, laulimalide behaves remarkably like paclitaxel and epothilone A (as well as eleutherobin; see Hamel et al., 1999) in its interactions with tubulin. It shows similar temperature dependence for assembly, and GTP and MAPs have similar effects on the overall assembly reaction. Consistent with these qualitative properties, we found that the critical concentration of tubulin for assembly with both drugs was similar in the absence of both MAPs and GTP.

Laulimalide-induced assembly, however, did differ from paclitaxel-induced assembly in that the morphology of the polymer was largely microtubules under all reaction conditions. The frequency of aberrant structures was much lower with laulimalide than with paclitaxel but this does not seem to derive from binding to a different site. Using similar reaction conditions, we have found that epothilone A and eleuth-

erobin also induce formation of polymers that largely display microtubule morphology (Hamel et al., 1999).

We also examined how occupancy of the "laulimalide site" might affect the interaction of tubulin with other ligands. As with paclitaxel, the basic properties of these other sites were unaltered when laulimalide was present in the reaction mixture. Polymerization with laulimalide, as well as assembly with taxoid site drugs, is subject to inhibition by drugs that bind at the colchicine site and the vinca domain. Moreover, neither laulimalide nor paclitaxel seems to impinge functionally on the exchangeable nucleotide site. Although GTP is no longer an absolute requirement for assembly, polymerization is enhanced by addition of GTP to the reaction mixture, and the nucleotide is hydrolyzed to GDP during the assembly process.

In summary, the interaction of laulimalide with tubulin and tubulin polymer is almost indistinguishable from the interaction of several taxoid site drugs with tubulin. There are two major differences. Laulimalide alone has no ability to inhibit paclitaxel binding to tubulin polymer. Laulimalide alone is able to act synergistically with paclitaxel in inducing tubulin assembly at colder temperatures.

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References

Andreu JM and Barasoain I (2001) The interaction of baccatin III with the taxol binding site of microtubules determined by a homogeneous assay with fluorescent taxoid. *Biochemistry* **40:**11975–11984.

Aoe K, Kiura K, Ueoka H, Tabata M, Matsumura T, Chikamori M, Matsushita A, Kohara H, and Harada M (1999) Effect of docetaxel with cisplatin or vinorelbine on lung cancer cell lines. *Anticancer Res* **19:**291–299.

Batra S, Karlsson R, and Witt L (1996) Potentiation by estramustine of the cytotoxic effect of vinblastine and doxorubicin in prostatic tumor cells. *Int J Cancer* **68:**644–649.

Bollag DM, McQueney PA, Zhu J, Hensens O, Koupal L, Liesch J, Goetz M, Lazarides E, and Woods C (1995) Epothilones, a new class of microtubule stabilizing agents with a taxol-like mechanism of action. *Cancer Res* **55:**2325–2333.

Budman DR and Calabro A (2002) In vitro search for synergy and antagonism: evaluation of docetaxel combinations in breast cancer cell lines. *Breast Cancer Res Treat* **74:**41–46.

Budman DR, Calabro A, and Kreis W (2002) Synergistic and antagonistic combinations of drugs in human prostate cancer cell lines in vitro. *Anticancer Drugs* **13:**1011–1016.

Budman DR, Calabro A, Wang LG, Liu XM, Stiel L, Adams LM, and Kreis W (2000) Synergism of cytotoxic effects of vinorelbine and paclitaxel in vitro. *Cancer Investig* **18:**695–701.

Carles G, Braguer D, Sabeur G, and Briand C (1998) The effect of combining antitubulin agents on differentiated and undifferentiated human colon cancer cells. *Anticancer Drugs* **9:**209–221.

Chou T-C and Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22:**27–55.

Corley DG, Herb R, Moore RE, Scheuer PJ, and Paul VJ (1988) Laulimalides: new potent cytotoxic macrolides from a marine sponge and a nudibranch predator. *J Org Chem* **53:**3644–3646.

Cortes JE and Pazdur R (1995) Docetaxel. *J Clin Oncol* **13:**2643–2655.

Crimmins MT, Stanton MG, and Allwein SP (2002) Asymmetric total synthesis of (−)-laulimalide: exploiting the asymmetric glycolate alkylation reaction. *J Am Chem Soc* **124:**5958–5959.

Culine S, Roch I, Pinguet F, Romieu G, and Bressolle F (1999) Combination paclitaxel and vinorelbine therapy: in vitro cytotoxic interactions and dose-escalation study in breast cancer patients previously exposed to anthracyclines. *Int J Oncol* **14:**999–1006.

Dabydeen DA, Florence GJ, Paterson I, and Hamel E (2004) A quantitative evaluation of the effects of inhibitors of tubulin assembly on polymerization induced by discodermolide, epothilone B and paclitaxel. *Cancer Chemother Pharmacol* **53:**397–403.

Enev VS, Kaehlig H, and Mulzer J (2001) Macrocyclization via allyl transfer: total synthesis of laulimalide. *J Am Chem Soc* **123:**10764–10765.

Gallagher BM Jr, Fang FG, Johannes CW, Pesant M, Tremblay MR, Zhao H, Akasaka K, Li X, Liu J and Littlefield BA (2004) Synthesis and biological evaluation of (−)-laulimalide analogues. *Bioorg Med Chem Lett* **14:**575–579.

Ghosh AK, Wang Y, and Kim JT (2001) Total synthesis of microtubule-stabilizing agent (−)-laulimalide. *J Org Chem* **66:**8973–8982.

Giannakakou P, Villalba L, Li H, Poruchynsky M, and Fojo T (1998) Combinations of paclitaxel and vinblastine and their effects on tubulin polymerization and cellular cytotoxicity: characterization of a synergistic schedule. *Int J Cancer* **75:**57–63.

Grover S and Hamel E (1994) The magnesium-GTP interaction in microtubule assembly. *Eur J Biochem* **222:**163–172.

Guéritte F (2001) General and recent aspects of the chemistry and structure-activity relationships of taxoids. *Curr Pharmaceut Design* **7:**1229–1249.

Hamel E and Lin CM (1984) Separation of active tubulin and microtubule-associated proteins by ultracentrifugation and isolation of a component causing the formation of microtubule bundles. *Biochemistry* **23:**4173–4184.

Hamel E, del Campo AA, Lowe MC, and Lin CM (1981) Interactions of Taxol, microtubule-associated proteins, and guanine nucleotides in tubulin polymerization. *J Biol Chem* **256:**11887–11894.

Hamel E, Lin CM, Kenney S, Skehan P, and Vaughns J (1992) Modulation of tubulin-nucleotide interactions by metal ions: comparison of beryllium with magnesium and initial studies with other cations. *Arch Biochem Biophys* **295:**327–339.

Hamel E, Sackett DL, Vourloumis D, and Nicolaou KC (1999) The coral-derived natural products eleutherobin and sarcodictyins A and B: effects on the assembly of purified tubulin with and without microtubule-associated proteins and binding at the polymer taxoid site. *Biochemistry* **38:**5490–5498.

Jefford CW, Bernardinelli G, Tanaka J, and Higa T (1996) Structures and absolute configurations of the marine toxins, latrunculin A and laulimalide. *Tetrahedron Lett* **37:**159–162.

Knick V, Eberwein D, and Miller CG (1995) Vinorelbine tartrate and paclitaxel combinations: enhanced activity against in vivo P388 murine leukemia cells. *J Natl Cancer Inst* **87:**1072–1077.

Kowalski RJ, Giannakakou P, Gunasekera SP, Longley RE, Day BW, and Hamel E (1997a) The microtubule-stabilizing agent discodermolide competitively inhibits the binding of paclitaxel (Taxol) to tubulin polymers, enhances tubulin nucleation reactions more potently than paclitaxel, and inhibits the growth of paclitaxel-resistant cells. *Mol Pharmacol* **52:**613–622.

Kowalski RJ, Giannakakou P, and Hamel E (1997b) Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol). *J Biol Chem* **272:**2534–2541.

Kreis W, Budman DR, and Calabro A (1997) Unique synergism or antagonism of combinations of chemotherapeutic and hormonal agents in human prostate cancer cell lines. *Br J Urol* **79:**196–202.

Long BH, Carboni JM, Wasserman AJ, Cornell LA, Casazza AM, Jensen PR, Lindel T, Fenical W, and Fairchild CR (1998) Eleutherobin, a novel cytotoxic agent that induces tubulin polymerization, is similar to paclitaxel (Taxol). *Cancer Res* **58:**1111–1115.

Madari H, Panda D, Wilson L, and Jacobs RS (2003) Dicoumarol: a unique microtubule stabilizing natural product that is synergistic with Taxol. *Cancer Res* **63:**1214–1220.

Martello LA, McDaid HM, Regl DL, Yang CPH, Meng DF, Pettus TRR, Kaufman MD, Arimoto H, Danishefsky SJ, Smith AB, et al. (2000) Taxol and discodermolide represent a synergistic drug combination in human carcinoma cell lines. *Clin Cancer Res* **6:**1978–1987.

Mooberry SL, Tien G, Hernandez AH, Plubrukarn A, and Davidson BS (1999) Laulimalide and isolaulimalide, new paclitaxel-like microtubule-stabilizing agents. *Cancer Res* **59:**653–660.

Mulzer J and Ohler E (2001) An intramolecular case of Sharpless kinetic resolution: total synthesis of laulimalide. *Angew Chem Int Ed Engl* **40:**3842–3846.

Nelson SG, Cheung WS, Kassick AJ, and Hilfiker MA (2002) A de novo enantioselective total synthesis of (−)-laulimalide. *J Am Chem Soc* **124:**13654–13655.

Paterson I, De Savi C, and Tudge M (2001) Total synthesis of the microtubule-stabilizing agent (−)-laulimalide. *Org Lett* **3:**3149–3152.

Photiou A, Shah P, Leong JK, Moss J, and Retsas S (1997) In vitro synergy of paclitaxel (Taxol) and vinorelbine (Navelbine) against human melanoma cell lines. *Eur J Cancer* **33:**463–470.

Pryor DE, O'Brate A, Bilcer G, Díaz JF, Wang Y, Wang Y, Kabaki M, Jung MK, Andreu JM, Ghosh AK, et al. (2002) The microtubule stabilizing agent laulimalide does not bind in the taxoid site, kills cells resistant to paclitaxel and epothilones and may not require its epoxide moiety for activity. *Biochemistry* **41:**9109–9115.

Quiñoà E, Kakou Y, and Crews P (1988) Fijianolides, polyketide heterocyclics from a marine sponge. *J Org Chem* **53:**3642–3644.

Roch I, Bressolle F, and Pinguet F (1997) In vitro schedule-dependent interaction between paclitaxel and vinorelbine in A2780 parental and multidrug-resistant human ovarian cancer cell lines. *Int J Oncol* **11:**1379–1385.

Rowinsky EK and Donehower RC (1995) Paclitaxel (Taxol). *N Engl J Med* **332:**1004–1014.

Schiff PB, Fant J, and Horwitz SB (1979) Promotion of microtubule assembly in vitro by Taxol. *Nature (Lond)* **277:**665–667.

Speicher LA, Barone L, and Tew KD (1992) Combined antimicrotubule activity of estramustine and Taxol in human prostatic carcinoma cell lines. *Cancer Res* **52:**4433–4440.

Wender PA, Hegde SG, Hubbard RD, and Zhang L (2002) Total synthesis of (−)-laulimalide. *J Am Chem Soc* **124:**4956–4957.

Williams DR, Mi L, Mullins RJ, and Stites RE (2002) Synthesis of (−)-laulimalide: an agent for microtubule stabilization. *Tetrahedron Lett* **43:**4841–4844.

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