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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†

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ABSTRACT: Laulimalide is a cytotoxic natural product that stabilizes microtubules. The compound enhances tubulin assembly, and laulimalide is quantitatively comparable to paclitaxel in its effects on the reaction. Laulimalide is also active in P-glycoprotein overexpressing cells, while isolaulimalide, a congener without the drug's epoxide moiety, was reported to have negligible cytotoxic and biochemical activity [Mooberry et al. (1999) *Cancer Res. 59*, 653−660]. We report here that laulimalide binds at a site on tubulin polymer that is distinct from the taxoid site. We found that laulimalide, while as active as paclitaxel, epothilone A, and eleutherobin in promoting the assembly of cold-stable microtubules, was unable to inhibit the binding of radiolabeled paclitaxel or of 7-*O*-[*N*-(2,7-difluoro-4'-fluoresceincarbonyl)-L-alanyl]paclitaxel, a fluorescent paclitaxel derivative, to tubulin. Confirming this observation, we demonstrated that microtubules formed in the presence of both laulimalide and paclitaxel contained near-molar quantities, relative to tubulin, of both drugs. Laulimalide was active against cell lines resistant to paclitaxel or epothilones A and B on the basis of mutations in the M40 human β-tubulin gene. We also report that a laulimalide analogue lacking the epoxide moiety, while less active than laulimalide in biochemical and cellular systems, is probably more active than isolaulimalide. Further exploration of the role of the epoxide in the interaction of laulimalide with tubulin is therefore justified.

The growing clinical usefulness of the taxoids (*1*) and the discovery of new natural products [epothilones (*2*), discodermolide (*3*), eleutherobin (*4*), and laulimalide (*5*)] with the same mechanism of action have occurred nearly simultaneously. The key feature of this mechanism is the binding of these compounds to microtubules, resulting in stabilization of these polymers (*6*) and quenching of their dynamic properties (*7*). Cells exposed to such drugs are unable to form a normal mitotic spindle, cannot divide after DNA replication, and soon undergo apoptosis. The epothilones, discodermolide, and eleutherobin inhibit the binding of [^{3}H]-paclitaxel to tubulin polymer in a competitive manner (*8*−*10*), suggesting that they bind to the same or overlapping sites on the protein.

We report here that laulimalide (*5*) (Figure 1) is totally unable to inhibit the binding of [^{3}H]paclitaxel or Flutax-2,[1] a fluorescent paclitaxel derivative, to tubulin polymer. Moreover, we have formed microtubules that contain near-stoichiometric amounts of both paclitaxel and laulimalide relative to their tubulin content. These findings strongly support the existence of a drug binding site on microtubules distinct from that occupied by taxoids. Laulimalide would be the first example of a ligand for this site, assuming it does not bind in a site thought to exist primarily in unpolymerized tubulin, such as the colchicine site, or in structurally aberrant polymers, such as the vinca site.

We have also examined the effects of laulimalide on the growth of a series of drug-resistant ovarian cancer cell lines.

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[1] Abbreviations: Flutax-2, 7-*O*-[*N*-(2,7-difluoro-4'-fluoresceincarbonyl)-L-alanyl]paclitaxel; [^{3}H]paclitaxel, [3"-^{3}H]paclitaxel; *trans*-desoxylaulimalide, (2*Z*,5*R*,6*Z*,9*R*,11*S*,15*S*,16*E*,19*S*)-15-hydroxy-19-[(1*S*,2*E*)-1-hydroxy-3-[(2*S*,4*Z*)-4-methyl-3,6-dihydro-2*H*-pyran-2-yl]-prop-2-en-1-yl]-11-methyl-13-methylene-20,21-dioxa-bicyclo[15.3.1]heneicosa-2,6,16-triene-1-one; MAPs, microtubule-associated proteins; 2-ethoxyestradiol analogue, 3,17β-diacetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-triene; HPLC, high-performance liquid chromatography.

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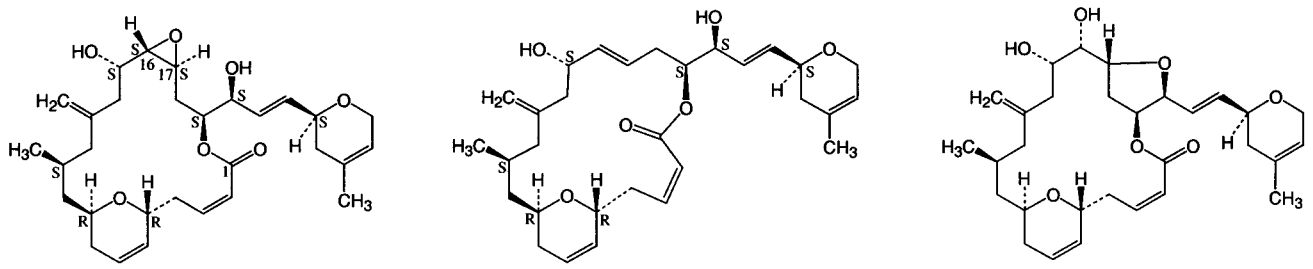

FIGURE 1: Structures of laulimalide, *trans*-desoxylaulimalide, and isolaulimalide. Note that equivalent chiral centers have the same configurations in laulimalide and *trans*-desoxylaulimalide.

We confirmed that laulimalide was active in multidrug-resistant cells overexpressing P-glycoprotein (*5*). We also found that laulimalide was active in epothilone- (*11*) and paclitaxel-resistant (*12*) cell lines bearing taxoid site mutations in the M40 β-tubulin gene.

In addition, synthetic efforts to define structure−activity relationships for laulimalide have yielded *trans*-desoxylaulimalide (structure in Figure 1), our most active analogue to date. While less active than laulimalide, *trans*-desoxylaulimalide, which lacks the epoxide moiety, was significantly more active than isolaulimalide (*5*) (structure in Figure 1), which also has no epoxide group, implying that this functionality may not be essential for the interaction of laulimalide with tubulin.

EXPERIMENTAL PROCEDURES

Materials. Synthetic laulimalide (*13*) and heat-treated MAPs from bovine brain (*14*) were prepared as described previously. Two preparations of bovine brain tubulin, with no significant MAPs content, were used in our experiments. The turbidimetry studies and [^{3}H]paclitaxel binding studies (see below, Figure 2 and Table 1, respectively) were performed with tubulin prepared as described in ref *14*. The Flutax-2 binding studies and the studies analyzing polymer-bound drugs (Figures 3 and 4, respectively) were performed with tubulin prepared as described in ref *15*. Paclitaxel, baccatin III, and [^{3}H]paclitaxel were provided by the Drug Synthesis & Chemistry Branch, National Cancer Institute, and natural epothilones A and B by Merck Research Laboratories. Docetaxel was obtained from Aventis Pharmaceuticals. Synthetic eleutherobin (*16*) and sarcodictyin A (*17*) were generous gifts of Dr. K. C. Nicolaou, Scripps Research Institute. The 2-ethoxyestradiol analogue (*18*) and Flutax-2 (*19*) were generous gifts from Dr. M. Cushman, Purdue University, and Dr. F. Amat-Guerri, Consejo Superior de Investigaciones Cientificas, respectively. The synthesis of *trans*-desoxylaulimalide will be described elsewhere. The nuclear magnetic and mass spectroscopic data and elemental analysis of *trans*-desoxylaulimalide were in agreement with the assigned structure.

Methods. Details of the assays for microtubule assembly (*10*) and for isolation of the β-tubulin mutants (*11*, *12*) were described previously. Cytotoxicity assays on MCF-7 and ovarian cell lines were performed as described previously (*12*). The ovarian cells were grown for 72 h and the MCF-7 line for 48 h following drug addition in 96-well microtiter plates before measurement of cell protein with sulforhodamine B.

The assay for inhibition of binding of [^{3}H]paclitaxel to tubulin polymer was described in detail previously (*10*). In this assay, 2.5 μM tubulin is initially assembled with 25 μM 2′,3′-dideoxyguanosine 5′-triphosphate, a potent inducer of microtubule nucleation, in 0.75 M monosodium glutamate. Mixtures of [^{3}H]paclitaxel and potential inhibitors were added to the polymer for a final tubulin concentration of 2 μM and the desired drug concentrations. In developing this assay, we showed that over 90% of the tubulin was polymerized in the first phase of the incubation and that with 2.0 μM paclitaxel and no inhibitor, 71% of the added [^{3}H]paclitaxel bound to the polymer in the second phase of the incubation. (Binding was stoichiometric with 4.0 μM [^{3}H]paclitaxel.) The polymer was harvested by centrifugation at 14 000 rpm for 20 min in 1.5 mL microfuge tubes in an Eppendorf model 5417C centrifuge. The amount of radiolabel in both the total reaction mixture and the supernatant was determined in a scintillation counter, and pellet radiolabel was obtained by subtraction.

Inhibition of binding of Flutax-2 to microtubules was measured in microtiter plates by fluorescence polarization anisotropy as described previously (*20*), except that the glutaraldehyde-stabilized microtubules were stored frozen in liquid nitrogen and drug binding was at 37 °C. The microtubules (50 nM in terms of taxoid binding sites; the stoichiometry of binding of the fluorescent derivative relative to the tubulin content was about 90%) and 50 nM Flutax-2 were mixed prior to addition of varying concentrations of potential inhibitor. For analysis of the experiments presented here, we used the K_a obtained previously for the binding of Flutax-2 to microtubules. This was 2.2×10^7 M^{-1} (*21*). The glutaraldehyde-fixed microtubules methodology was originally developed to permit more accurate measurement of the binding parameters of taxoids to tubulin polymer (*21*) using fluorescence techniques, since the binding affinities of more active members of the taxoid class is so high. Without fixation, microtubules with picomoles of binding sites per milliliter would rapidly disassemble, since the concentration of tubulin would be so far below the critical concentration. The methodology also made it possible to obtain quantitative measures of the binding of low-affinity ligands for the taxoid site, since high ratios of ligand to fluorescent probe (i.e., Flutax-2) could be used (*20*). In the experiments presented here, 50 nM Flutax-2 bound to an equivalent number of taxoid binding sites with a stoichiometry of about 0.4. To

obtain higher stoichiometries, the sensitivity of the assay for weaker ligands would be greatly reduced. With 175 nM Flutax-2, saturation of the taxoid sites is about 75%, and 90% saturation is reached with 0.5 μM Flutax-2.

The cobinding of laulimalide and paclitaxel to microtubules was evaluated by isolating polymer by centrifugation, extracting the pellet, and analyzing the extracted bound ligands by HPLC. Calf brain tubulin (3.5 mg, final concentration, 35 μM) was mixed into reaction mixtures (1.0 mL) containing 3.4 M glycerol, 10 mM phosphate (pH 6.5), 6 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, and 37.5 μM laulimalide and/or 37.5 μM paclitaxel. The reaction mixtures were warmed to 37 °C for 30 min and centrifuged for 10 min at 50 000 rpm in a prewarmed Beckman TL120.2 rotor. The microtubule pellets were resuspended in 1.0 mL of 10 mM phosphate buffer (pH 7.0), and the tubulin concentration was determined spectrofluorometrically (*22*). The pellets, as well as the supernatants, were extracted 3 times with 1.0 mL of CH_2Cl_2. This procedure quantitatively separates the drugs, which enter the organic phase, from the tubulin, which precipitates at the organic−aqueous interface. After evaporation of the CH_2Cl_2, the residues were each dissolved in 100 μL of 70% (v/v) methanol. Standard solutions of paclitaxel and laulimalide were processed analogously. HPLC analysis was performed on a C-18 column (Supercosil, LC18 DB, 250 × 4.6 mm, 5 mm bead size) developed isocratically with 70% methanol at a flow rate of 1.0 mL/min. Drug concentrations in the pellet were determined by integration of eluted peaks in comparison to the areas produced by known quantities of the drugs from the standard solutions.

RESULTS AND DISCUSSION

Tubulin Assembly. In initial experiments, we confirmed the report of Mooberry et al. (*5*) that laulimalide appeared to be quantitatively similar to paclitaxel in its ability to induce microtubule assembly. Originally, we had planned to compare laulimalide with epothilone A because of the structural similarities between the two compounds (epoxide moiety, bulky side chain, lactone macrocycle) and because the effect of epothilone A on tubulin assembly is also quantitatively similar to that of paclitaxel (*2*, *8*). When we discovered that laulimalide had no apparent ability to inhibit taxoid binding to tubulin (see below), it became important to include in the comparative studies, as controls, drugs less potent than laulimalide as stimulators of assembly that nonetheless were able to inhibit taxoid binding to tubulin.

The polymerization studies presented in Figure 2 were performed with a reaction system containing 10 μM tubulin, the drug usually at 10 μM, heat-treated MAPs, and GTP, with polymerization followed turbidimetrically. Temperature in these experiments was increased stepwise until relatively rapid assembly had occurred in each reaction mixture. Following assembly, the temperature was returned to 0 °C to evaluate polymer stability. In panel A, the reaction without drug (curve 0) is compared to reactions with 10 μM laulimalide, paclitaxel, or epothilone A (curves 1, P, and 2, respectively) and with 10 or 40 μM *trans*-desoxylaulimalide (curves 3L and 3H, respectively). The chief differences between the reactions induced by paclitaxel and laulimalide or epothilone A were that a greater turbidity change occurred at warmer temperatures with paclitaxel and that the paclitaxel-induced polymer seemed less cold stable. The higher turbidity plateau obtained with paclitaxel probably occurs because a higher proportion of tubulin sheets was formed with paclitaxel than with the other drugs under our reaction conditions (*10*). These sheets may also account for the partial loss of turbidity observed with paclitaxel. The laulimalide analogue was distinctly less active than the parent compound, but its effect on assembly was enhanced by increasing the drug concentration.

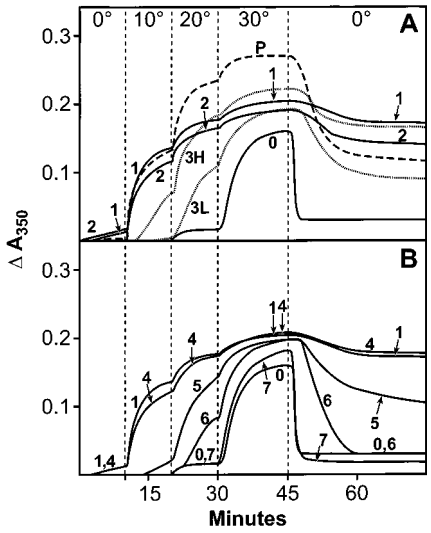

FIGURE 2: Drug-induced tubulin assembly in the presence of MAPs and GTP. Reaction mixtures (final volume, 0.25 mL) containing all components except the drug were placed in cuvettes held at 0 °C in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. Baselines were established at 350 nm, and the drug was added in dimethyl sulfoxide (final concentration, 1%). Turbidity changes were followed, with the indicated temperature changes made at the time points indicated by the dashed lines to the left of the temperature. Reaction mixtures contained 0.1 M 4-morpholineethanesulfonate (pH 6.9 with NaOH in 1 M stock solution), 10 μM (1.0 mg/mL) tubulin, 0.75 mg/mL heat-treated MAPs, and 0.1 mM GTP. (A) Curve 0, no drug; P, 10 μM paclitaxel; 1, 10 μM laulimalide; 2, 10 μM epothilone A; 3L, 10 μM *trans*-desoxylaulimalide; 3H, 40 μM *trans*-desoxylaulimalide. (B) Curve 0, no drug; 1, 10 μM laulimalide; 4, 10 μM eleutherobin; 5, 10 μM sarcodictyin A; 6, 10 μM 2-ethoxyestradiol analogue; 7, 10 μM baccatin III.

In panel B (Figure 2), assembly data is shown for three compounds clearly less active than laulimalide as inducers of tubulin assembly. These compounds, which had all been found to have relatively weak activity as inhibitors of the binding of [^{3}H]paclitaxel to tubulin polymer (*10*,*18*,*20*), were sarcodictyin A (curve 5), the 2-ethoxyestradiol analogue (curve 6), and baccatin III (curve 7). Assembly induction by baccatin III is greater when higher concentrations of the compound are used (data not shown). Assembly induced by eleutherobin, a more active congener of sarcodictyin A (*10*), is represented by curve 4, and assembly with laulimalide by curve 1.

Failure of Laulimalide to Inhibit Binding of Either [^{3}H]-Paclitaxel or Flutax-2 to Tubulin Polymer. In previous studies (*8*−*10*, *18*, *20*), we had found that discodermolide, docetaxel, epothilones A and B, eleutherobin, and, to a lesser extent, sarcodictyins A and B, baccatin III, and the 2-ethoxyestradiol analogue had inhibited the binding of [^{3}H]-paclitaxel to tubulin polymer. The inhibition patterns obtained with the more potent inhibitors all were of the competitive

Table 1: Laulimalide and *trans*-Desoxylaulimalide Are Unable to Inhibit the Binding of [^{3}H]Paclitaxel to Tubulin Polymer[a]

drug added	% inhibition ± SE
30 μM laulimalide	0
30 μM *trans*-desoxylaulimalide	0
30 μM sarcodictyin A	41 ± 9
5 μM eleutherobin	75 ± 2
30 μM eleutherobin	89 ± 6
5 μM epothilone A	67 ± 3
30 μM epothilone A	91 ± 4

[a] The potential inhibitor and [^{3}H]paclitaxel, in amounts sufficient to yield the indicated concentrations of the potential inhibitors and 2 μM paclitaxel, were mixed in a microfuge tube. Tubulin polymer (final tubulin concentration, 2 μM; at least 90% of the tubulin in polymer) was added to each tube. Following a 30 min incubation at 37 °C, the reaction mixtures were centrifuged, and the amount of radiolabel present in an aliquot of each supernatant was determined in a liquid scintillation counter. The radiolabel content of uncentrifuged reaction mixtures was also determined. The pellet was assumed to contain the difference between the total radiolabel and the supernatant radiolabel. In the absence of an effective inhibitor, about 25% of the added radiolabel was recovered in the supernatant. The data presented are results from all determinations with each compound at the indicated concentrations. At least 20 experiments were performed with laulimalide and 3 with *trans*-desoxylaulimalide, and no inhibitory effect was ever observed. The inhibitory control drugs were each examined at least three times. For further details, see text and ref *10*. SE, standard error.

type, suggesting that they bind to the same or overlapping sites on tubulin. We examined laulimalide for its effect on this reaction, and neither laulimalide nor *trans*-desoxylaulimalide inhibited [^{3}H]paclitaxel binding (Table 1). In at least 20 variations of this experiment, with laulimalide concentrations as high as 50 μM with 2 μM tubulin, this same negative result was obtained. Laulimalide failed to inhibit [^{3}H]-paclitaxel binding even when laulimalide and tubulin polymer were incubated prior to addition of the paclitaxel, excluding slow binding of laulimalide relative to paclitaxel, or when polymer was generated in the presence of MAPs, excluding a binding site generated by the interaction of tubulin with MAPs, as might have occurred in the assembly reaction of Figure 2 (data not presented). In some experiments, in fact, laulimalide led to an increase in the amount of [^{3}H]paclitaxel bound to polymer, possibly indicating that the combination of laulimalide and paclitaxel caused an increase in the amount of polymer formed, and thus an increase in the number of available sites for paclitaxel. In the experiments presented in Table 1, weak inhibition was obtained with sarcodictyin A, while both epothilone A and eleutherobin potently inhibited the binding of [^{3}H]paclitaxel to the tubulin polymer.

Because this result with laulimalide was so unexpected, we confirmed it with Flutax-2 (*20*) (Figure 3). The strength of the fluorescence polarization anisotropy signal permits the use of nanomolar concentrations of Flutax-2 and tubulin and consequently a much higher inhibitor-to-taxoid ratio than is usually possible with [^{3}H]paclitaxel binding assays. In this assay, too, laulimalide was noninhibitory (Figure 3, solid circles), in contrast to the potent inhibition obtained with paclitaxel (open squares) or docetaxel (open circles). In these experiments, the maximum ratio of laulimalide to Flutax-2 was 400:1, versus 25:1 in the [^{3}H]paclitaxel experiments.

We had previously used this assay, with the high ratio of inhibitor to taxoid that it makes possible, to document convincingly the ability of baccatin III to bind in the taxoid site (*20*) despite the much weaker effect of baccatin III as

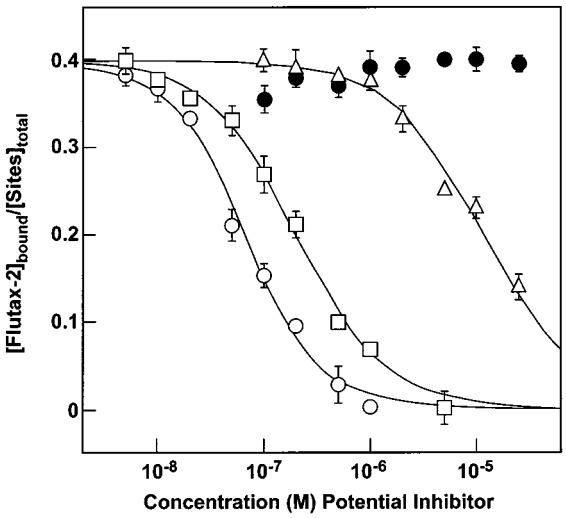

FIGURE 3: Inhibition of binding of Flutax-2 to tubulin polymer by paclitaxel, docetaxel, and the 2-ethoxyestradiol analogue but not by laulimalide. The glutaraldehyde-stabilized microtubules were 40% saturated with Flutax-2 in reaction mixtures without inhibitor, based on the concentrations of Flutax-2 and taxoid binding sites, which were both 50 nM, and the binding constant of Flutax-2 to the site at 37 °C, which is 2.2×10^7 M^{-1} (*21*). Besides the components described in the text, reaction mixtures contained the indicated concentrations of laulimalide (●), paclitaxel (□), docetaxel (○), or the 2-ethoxyestradiol analogue (△). The values shown are averages obtained in at least four independent experiments. The curves connecting the latter three sets of data points were generated to provide the best fit value of the apparent equilibrium binding constants (in competition with Flutax-2), assuming one to one binding in the same site, using an unpublished computer program (J. F. Díaz). These values are $2.2 \pm 0.2 \times 10^5$ M^{-1} for the 2-ethoxyestradiol analogue, $1.5 \pm 0.4 \times 10^7$ M^{-1} for paclitaxel, and $6.7 \pm 1.2 \times 10^7$ M^{-1} for docetaxel. Standard errors for the data points are shown in the Figure, unless smaller than the symbol, and are also indicated for the apparent equilibrium binding constants. In the previous study (*20*), at 25 °C, the average apparent binding constants obtained for paclitaxel, docetaxel, and baccatin III were, respectively, 3.7×10^7, 6.0×10^7, and 1.5×10^5 M^{-1}.

compared with laulimalide on tubulin assembly (Figure 2B). As a further control, for contrast to the inability of laulimalide to inhibit Flutax-2 binding, we examined the effect of another weak inducer of assembly, the 2-ethoxyestradiol analogue, to inhibit Flutax-2 binding, and we found the steroid derivative to have an effect on Flutax-2 binding similar to that observed previously with baccatin III (Figure 3, open triangles). Preliminary studies have also indicated that epothilone A and eleutherobin are similar to paclitaxel in their inhibitory effects on the binding of Flutax-2 to microtubules (data not presented).

Simultaneous Binding of Laulimalide and Paclitaxel to Microtubules. We next sought positive evidence that laulimalide and paclitaxel could bind to microtubules simultaneously by examining the ligand content of microtubule pellets by HPLC. We have previously shown by this technique that docetaxel and paclitaxel (*22*) or that baccatin III and paclitaxel (*20*) mutually excluded each other from binding to microtubules, consistent with the three compounds binding to the same site on polymer. Figure 4 demonstrates that the opposite occurs with paclitaxel and laulimalide. When 35 μM tubulin was polymerized in the presence of 37.5 μM paclitaxel, 92% of the tubulin was recovered in the polymer pellet, as well as paclitaxel in an amount

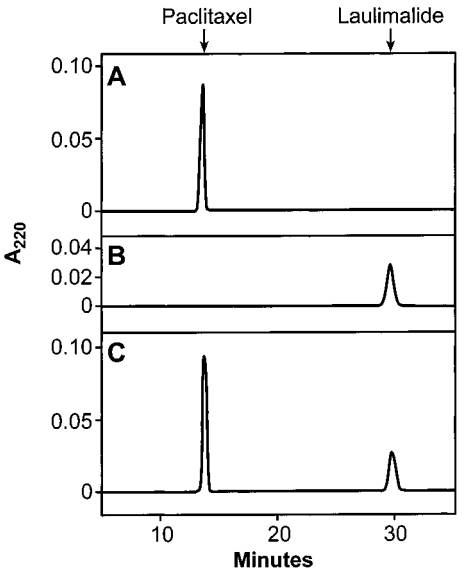

FIGURE 4: Binding of paclitaxel (A), laulimalide (B), or both laulimalide and paclitaxel to microtubules. Tubulin was polymerized, the polymer was harvested by centrifugation, the tubulin content of each pellet was determined, and bound ligand was extracted from the pellet protein and analyzed by HPLC as described in the text. Each injection contained equivalent amounts of extract in terms of tubulin content of the pellets. (A) HPLC analysis of extract from microtubules formed in the presence of paclitaxel. (B) HPLC analysis of extract from microtubules formed in the presence of laulimalide. (C) HPLC analysis of extract from microtubules formed in the presence of both laulimalide and paclitaxel.

equivalent to 1.04 mol/mol tubulin (Figure 4A). When 37.5 μM laulimalide was used instead, 96% of the tubulin was in the polymer, along with an amount of laulimalide equivalent to 0.93 mol/mol tubulin (Figure 4B). When both drugs at 37.5 μM were used to induce assembly, 91% of the tubulin was in polymer with near stoichiometric amounts of both drugs: 0.96 mol paclitaxel/mol tubulin and 0.83 mol laulimalide/mol tubulin. Finally, when laulimalide was added in excess to tubulin, in the absence or presence of paclitaxel or docetaxel, only a stoichiometric amount of laulimalide relative to tubulin was found in the microtubule pellet (data not shown).

This result confirms the observations with [^{3}H]paclitaxel and Flutax-2 that laulimalide does not interfere with the binding of taxoids to tubulin. The most straightforward interpretation of these data is that laulimalide does not bind in the taxoid site on β-tubulin. Instead, it must bind at an alternate location in the $\alpha\beta$-tubulin dimer. In control experiments, we excluded the trivial possibility that the drugs are not soluble under the reaction condition evaluated. Without tubulin, less than 1% of added drug is removed by centrifugation, and in the case of paclitaxel, little drug is found in the polymer pellet in the presence of excess docetaxel, indicating minimal nonspecific entrapment. However, it is difficult to eliminate the possibilities of nonspecific but high affinity binding of drug to tubulin (for example, analogous to what occurs with albumin) or that a new drug binding site on tubulin polymer might be generated by the binding of either paclitaxel or laulimalide to the microtubules.

Effects of Laulimalide on Drug-Resistant Cell Growth. The above biochemical findings made it particularly interesting to evaluate the effects of laulimalide on the growth of cells resistant to paclitaxel and the epothilones. We therefore examined a series of drug-resistant human ovarian carcinoma cell lines for their sensitivity to laulimalide in comparison with paclitaxel and epothilones A and B (Table 2). A2780/AD10 is a multidrug-resistant line that overexpresses P-glycoprotein. PTX10 and PTX22 were selected for resistance to paclitaxel (*12*) and A8 and B10 for resistance to epothilones A and B (*11*), respectively. The latter four lines were all found to have mutations in the M40 β-tubulin gene at amino acids near the binding site for taxoids (Figure 5), as derived from the electron crystallographic tubulin polymer model (*23*). As previously noted, laulimalide (*5*), like epothilones A and B (*2*), is not a good substrate for P-glycoprotein, and the A2780/AD10 cell line retained sensitivity to laulimalide. All four lines with drug resistance based on mutations in the β-tubulin gene also retained their sensitivity to laulimalide. It is particularly interesting to note that the relative resistance values of the mutant lines relative to the parental line observed for laulimalide with the paclitaxel-resistant lines, especially PTX10, are lower than those obtained for either epothilone A or B; similarly, with the epothilone resistant lines, the relative resistance values for laulimalide are lower than those obtained for paclitaxel. This could derive from laulimalide binding to a distinct site on tubulin polymer, as strongly indicated by the biochemical evidence. At the same time, however, the paclitaxel-resistant lines (PTX10 and PTX22) retain significant sensitivity to the epothilones (*8*, Table 2) and discodermolide (*9*), and the epothilone-resistant cells retain partial sensitivity to paclitaxel (*11*, Table 2).

It should be noted, moreover, that there is a significant difference in the β-tubulin isotype composition of the ovarian carcinoma cells and of the bovine brain tubulin used in the biochemical studies. The major isotype of the carcinoma cells, that coded by the M40 gene, is β_{I} (*12*). In contrast, the major isotype of bovine brain tubulin is β_{II}, with β_{I} representing less than 5% of the total (*24*). Nevertheless, the sequence identity between β_{I} and β_{II} is 96.6% (almost 99% if residues in the carboxy terminus, not resolved in the electron crystallographic model, are excluded). Of the four residues mutated in the paclitaxel- and epothilone-resistant lines, Phe_{270}, Thr_{274}, and Arg_{282} are conserved among all human, indeed all vertebrate, β-tubulin isotypes. The amino acid residue at position 364 is alanine in β_{I} and serine in the other isotypes. Moreover, the β-tubulin residues proposed by Nogales et al. (*25*) to be directly involved in paclitaxel binding are conserved entirely between the β_{I} and β_{II} isotypes.

The apparent binding of laulimalide at a new site on tubulin polymer raised the interesting possibility that this agent might have synergistic cytotoxic activity with compounds that bind at the taxoid site. This possibility particularly merited exploration in view of recent findings with discodermolide. Even though the kinetic evidence showed that discodermolide competitively inhibited the binding of [^{3}H]paclitaxel to tubulin polymer (*9*), Martello et al. (*26*) found that paclitaxel and discodermolide had synergistic cytotoxic activity against several cancer cell lines. This finding could indicate that discodermolide and paclitaxel may bind at overlapping rather than identical sites on tubulin polymer. With the parental 1A9 cells, we were able to demonstrate synergistic cytotoxicity between paclitaxel and discodermolide at selected drug concentrations. Thus far,

Table 2: Human Ovarian Carcinoma Cells Resistant to Paclitaxel or Epothilones Retain Sensitivity to Laulimalide

	cell lines[a]					
	IC_{50} (nM) ± SE[b]					
	(relative resistance[c])					
compound	1A9	PTX10	PTX22	A8	B10	A2780/AD10
laulimalide	3.9 ± 0.4	6.0 ± 1 (1.5)	6.3 ± 1 (1.6)	9.2 ± 2 (2.4)	15 ± 0.2 (3.8)	31 ± 0.6 (7.9)
epothilone A	1.7 ± 0.3	18 ± 7 (11)	4.3 ± 1 (2.5)	93 ± 30 (55)	125 ± 25 (74)	16 ± 0.6 (9.4)
epothilone B	0.17 ± 0.08	0.70 ± 0.4 (4.1)	0.32 ± 0.2 (1.9)	6.4 ± 4 (38)	9.0 ± 5 (53)	2.6 ± 2 (15)
paclitaxel	1.7 ± 0.3	50 ± 11 (29)	34 ± 3 (20)	13 ± 2 (7.6)	16 ± 4 (9.4)	4000 ± 900 (2400)

[a] The parental cell line 1A9, a clone of line A2780, was used to select paclitaxel-resistant lines PTX10 and PTX22 and epothilone-resistant lines A8 and B10. These lines have four different mutations in the M40 β-tubulin gene (see legend of Figure 5 for details). The multidrug-resistant, P-glycoprotein overexpressing line A2780/AD10, selected in the presence of adriamycin, was also derived from A2780. [b] SE, standard error. IC_{50} values were determined at least 5 times. [c] The IC_{50} value of the resistant line divided by the IC_{50} value of the 1A9 line.

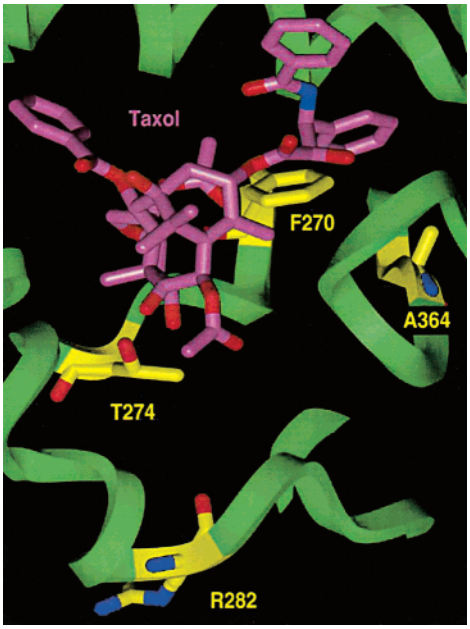

FIGURE 5: Location of amino acids altered in β-tubulin in paclitaxel- and epothilone-resistant cell lines and their relationship to bound paclitaxel (Taxol). The β-tubulin backbone is shown as a green ribbon, except for the locations of the four pertinent amino acids. For these amino acids, carbon atoms are indicated in yellow. For paclitaxel, carbon atoms are shown in magenta. In both the four amino acids and paclitaxel, blue indicates nitrogen atoms and red oxygen atoms. In the PTX10 line, Phe_{270} (F270) is mutated to valine; in PTX22, Ala_{364} (A364) to threonine; in A8, Thr_{274} (T274) to isoleucine; and in B10, Arg_{282} (R282) to glutamine. From the electron crystallographic tubulin model (*23*, *25*).

however, we have found no combination of laulimalide with paclitaxel, epothilone A or B, or discodermolide that indicates synergistic activity.

Cytotoxic Activity of trans-Desoxylaulimalide. Finally, we performed an initial cytotoxicity study with *trans*-desoxylaulimalide, comparing it with paclitaxel and laulimalide for effects on the growth of human MCF-7 breast cancer cells. Consistent with its partial activity in the tubulin assembly assay, *trans*-desoxylaulimalide had reduced potency as an inhibitor of cell growth. IC_{50} values of 360, 7.0, and 2.4 nM were obtained for *trans*-desoxylaulimalide, laulimalide, and paclitaxel, respectively. Thus *trans*-desoxylaulimalide is about 1/50 as active as laulimalide in the MCF-7 cells. This compares favorably with the 340-fold lower activity in MDA-MB-435 breast cancer cells observed for isolaulimalide (*5*), which also lacks the epoxide moiety.

Conclusions. In summary, while laulimalide has microtubule assembly promoting activity comparable to the activities of paclitaxel, epothilone A, and eleutherobin and much greater than the activities of sarcodictyin A, the 2-ethoxyestradiol analogue, and baccatin III, it is unable to inhibit the binding of either [^{3}H]paclitaxel or Flutax-2 to tubulin polymer. In contrast, the relatively weak inducers of assembly sarcodictyin A, the 2-ethoxyestradiol analogue, and baccatin III all are able to interfere with taxoid binding to polymer. Even more compelling, when tubulin polymer was formed in the presence of both laulimalide and paclitaxel, it contained nearly molar equivalents of both drugs relative to the tubulin content. Laulimalide thus appears to bind at a previously unknown drug binding site on microtubules. The results with the paclitaxel- and epothilone-resistant ovarian cancer lines bearing mutated β-tubulin genes further support this conclusion from the biochemical experiments. Although the paclitaxel-resistant mutants remain sensitive to the epothilones and the epothilone-resistant mutants remain partially sensitive to paclitaxel, the relative resistance values for laulimalide are the lowest observed in all cases. It should be possible to locate the binding site of laulimalide on the $\alpha\beta$-tubulin dimer by the electron crystallographic technique (*23*, *25*), provided that the drug binds to zinc-induced sheets and remains bound during sample preparation. We have also shown here that the epoxide moiety of laulimalide is probably not an essential feature for activity of this new drug family. Although less active than laulimalide, *trans*-desoxylaulimalide did stimulate the tubulin assembly reaction and inhibit the growth of MCF-7 cells. This partial activity of *trans*-desoxylaulimalide occurred even though the C16−C17 olefin bond is in the *trans*-configuration, while configuration of the macrocycle at the C16−C17 epoxide in laulimalide is in the *cis*-configuration. Further synthetic efforts to clarify structure−activity relationships in this exciting new drug class are fully justified, and these efforts may lead to the creation of more active molecules. Clearly, an initial target for such synthetic work should be a C16−C17 olefin analogue with the *cis*-configuration at the double bond.

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