Microtubule-stabilizing agents based on designed laulimalide analogues

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Laulimalide is a potent, structurally unique microtubule-stabilizing agent originally isolated from the marine sponge *Cacospongia mycofijiensis*. Laulimalide exhibits an activity profile different from other microtubule-binding agents, notably including effectiveness against paclitaxel-resistant cells, but it is intrinsically unstable. Five analogues of laulimalide were designed to exhibit enhanced chemical stability yet retain its exceptional biological activities. Evaluations of these analogues showed that all are effective inhibitors of cancer-cell proliferation yet differ substantially in potency with an IC_{50} range of 0.12–16.5 μM. Although all of the analogues initiated cellular changes similar to laulimalide, including increased density of interphase microtubules, aberrant mitotic spindles, and ultimately apoptosis, differences among the analogues were apparent. The two most potent analogues, C_{16}-C_{17}-des-epoxy laulimalide and C_{20}-methoxy laulimalide, appear to have a mechanism of action identical to laulimalide. The C_{16}-C_{17}-des-epoxy, C_{20}-methoxy laulimalide derivative, which incorporates both chemical changes of the most potent analogues, was significantly less potent and initiated the formation of unique interphase microtubules unlike the parent compound and other analogues. Two C_2-C_3-alkynoate derivatives had lower potency, and they initiated abnormal microtubule structures but did not cause micronucleation or extensive G_2/M accumulation. Significantly, paclitaxel- and epothilone-resistant cell lines were less resistant to the laulimalide analogues. In summary, analogues of laulimalide designed to minimize or eliminate its intrinsic instability have been synthesized, and some have been found to retain the unique biological activities of laulimalide.

antimitotics | synthetic chemistry

Paclitaxel (Taxol, Bristol-Myers Squibb), the first microtubule stabilizer identified, has proved to be of great value for the treatment of many types of cancer (1). The clinical successes of paclitaxel led to the development of a second-generation taxane, docetaxel (Taxotere, Aventis, Bridgewater, NJ), and initiated the intense search for other compounds with a similar mechanism of action. Several classes of structurally diverse microtubule-stabilizing compounds have been identified. The first nontaxane stabilizers identified, the epothilones and discodermolide, had excellent preclinical activities and are being evaluated in clinical trials as anticancer agents.

Laulimalide is a potent microtubule stabilizer that was originally isolated from the sponge *Cacospongia mycofijiensis* (2). Similar to the effects of other microtubule stabilizers, laulimalide increases the density of interphase microtubules and causes the formation of microtubule bundles and abnormal mitotic spindles, effects that culminate in mitotic arrest and initiation of apoptosis. Laulimalide stimulates the polymerization of bovine brain tubulin consistent with a direct interaction between the compound and tubulin (2, 3). Unlike the other stabilizers identified, laulimalide does not displace [^{3}H]paclitaxel or Flutax-2, a fluorescent paclitaxel derivative, and cell lines with mutations in the paclitaxel-binding site are not cross-resistant to laulimalide. These data suggest that laulimalide is the first stabilizer identified that does not bind to tubulin within the paclitaxel-binding site (3). In addition laulimalide has advantages over the taxanes in that it is a poor substrate for transport by P-glycoprotein (Pgp) (2, 3).

Laulimalide is a structurally unique 20-membered macrolide (4, 5). The unusual structure and interesting biological activities of laulimalide led to its total synthesis by several groups using diverse approaches (reviewed in ref. 6; see also refs. 7–15). However, laulimalide is intrinsically unstable and, under mildly acidic conditions, it is converted to isolaulimalide, a significantly less potent isomer, via ring opening of the sensitive C_{16}-C_{17}-epoxide by the C_{20}-hydroxyl group. Herein, we describe the biological activities of five laulimalide analogues that were designed to circumvent the degradation pathway of the parent compound through modification or removal of the chemically reactive structural moieties. Significantly, all designed analogues retain the ability to stabilize microtubules, form abnormal mitotic spindles, and initiate apoptosis. Subtle differences were noted among the analogues, providing key information on the structural basis of laulimalide's activities, as required for the design of superior therapeutic candidates.

Materials and Methods

Chemical Synthesis of Laulimalide Analogues. Laulimalide analogues were designed and synthesized as reported in refs. 12 and 16.

Cell Culture. A-10, HeLa, and MDA-MB-435 cells were maintained as described in ref. 17. The parental 1A9 and the paclitaxel- and epothilone A-resistant PTX10, PTX22, and A8 cell lines were provided by Paraskevi Giannakakou (18, 19) and maintained as described in ref. 17.

Sulforhodamine B Assay. The sulforhodamine B assay was used to measure inhibition of proliferation and cytotoxicity as described in ref. 17.

Indirect Immunofluorescence. A-10 and HeLa cells were used to evaluate the effects of the analogues on interphase and mitotic microtubules. After an 18-h incubation, the microtubule, centrosomal, and nuclear structures were evaluated by indirect immunofluorescence as described in refs. 2 and 17. Centrosomes were visualized by using antibodies for centrin and γ-tubulin. Digital photographs were taken, and selected images were deconvoluted, colorized, and compiled by using METAMORPH (Universal Imaging, Media, PA) and AUTOQUANT (AutoQuant Imaging, Watervliet, NY) software.

Abbreviations: Pgp, P-glycoprotein; LA1, C_{16}-C_{17}-des-epoxy laulimalide; LA2, C_{20}-methoxy laulimalide; LA3, C_2-C_3-alkynoate laulimalide; LA4, C_{16}-C_{17}-des-epoxy, C_{20}-methoxy laulimalide; LA5, C_2-C_3-alkynoate, C_{16}-C_{17}-des-epoxy laulimalide.

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Cellular Tubulin Polymerization. The effects of paclitaxel and laulimalide analogues on cellular tubulin polymerization were evaluated (17–19). MDA-MB-435 cells were exposed to the test compounds or vehicle for 1 h at 37°C followed by lysis in hypotonic buffer as described in ref. 19. Cellular constituents were then separated by centrifugation. The supernatants containing soluble, cytosolic tubulin were removed and the pellets, comprising particulate material including polymerized cytoskeletal tubulin, were resuspended in buffer. The cytosolic, soluble, and particulate fractions were resolved by SDS/PAGE and β-tubulin was detected by Western blotting techniques.

Flow Cytometry. MDA-MB-435 cells were treated with the laulimalide analogues (LA1–LA5) or vehicle for 24 h at the approximate IC_{85} for inhibition of proliferation (500 nM LA1, 1 μM LA2, 6.4 μM LA3, 20 μM LA4, and 20 μM LA5). After incubation, the cells were stained with Krishan's reagent (20), and the DNA content was analyzed by using a Becton Dickinson FACScan flow cytometer.

Western Blots. MDA-MB-435 cells were treated with the various analogues at the approximate IC_{85} for inhibition of proliferation for 24 h. After incubation with drugs the cells were harvested and cellular proteins were extracted in modified radioimmunoprecipitation buffer in the presence of protease inhibitors. The protein concentrations of the samples were measured, and cell lysates containing equal amounts of protein were separated by SDS/PAGE, transferred to Immobilon P (Millipore), and probed with specific antibodies. The p85 poly(ADP-ribose) polymerase fragment antibody was purchased from Promega and the Bcl-2 antibody was from Pharmingen.

Results

Laulimalide Analogues. Laulimalide analogues were designed and synthesized to minimize the instability of the parent compound and yet retain microtubule-stabilizing activity and the ability to circumvent Pgp-mediated drug resistance (16). The structures of laulimalide, isolaulimalide, and five laulimalide analogues are presented in Fig. 1. Three structural features that contribute to the undesired conversion of laulimalide to isolaulimalide were targeted individually for modification: the electrophilic C_{16}-C_{17}-epoxide was removed to yield des-epoxide laulimalide (LA1), the nucleophilic C_{20}-hydroxyl was converted to a methyl ether (LA2) to attenuate its reactivity toward the sensitive epoxide, and the C_2-C_3-enoate was converted to an alkynoate (LA3) as a means to change the orientation of the C_{16}-C_{17}-epoxide relative to the C_{20}-hydroxyl. Analogues that combine two functional group conversions (LA4 and LA5) were also examined. At a molecular level these changes would prevent or attenuate the laulimalide to isolaulimalide isomerization.

Inhibition of Proliferation. The laulimalide analogues were evaluated for their ability to inhibit proliferation and cause cytotoxicity in several cancer cell lines by using the sulforhodamine B assay and comparing with the activity of natural laulimalide obtained previously in the MDA-MB-435 cell line (2). As indicated by the dose–response curves in MDA-MB-435 and HeLa cells (Fig. 2), the C_{16}-C_{17}-des-epoxy laulimalide (LA1) is the most potent analogue of this series with an IC_{50} of 120 nM, $\approx$19-fold less potent than laulimalide, which has an IC_{50} of 5.7 nM in the MDA-MB-435 cell line (2, 16). C_{20}-methoxy laulimalide (LA2) is the second most potent of this series with an IC_{50} of 240 nM in MDA-MB-435 cells. The C_2-C_3-alkynoate laulimalide (LA3), C_{16}-C_{17}-des-epoxy, C_{20}-methoxy laulimalide (LA4), and C_2-C_3-alkynoate, des-epoxy laulimalide (LA5), are less potent but still effective in the low micromolar range, with IC_{50} values of 2.5–16.6 μM (16). The dose-response curves indicate that the analogues differ in potency, yet significantly, they are all effective inhibitors of cellular proliferation and exhibit cytotoxicity in these two cancer cell lines.

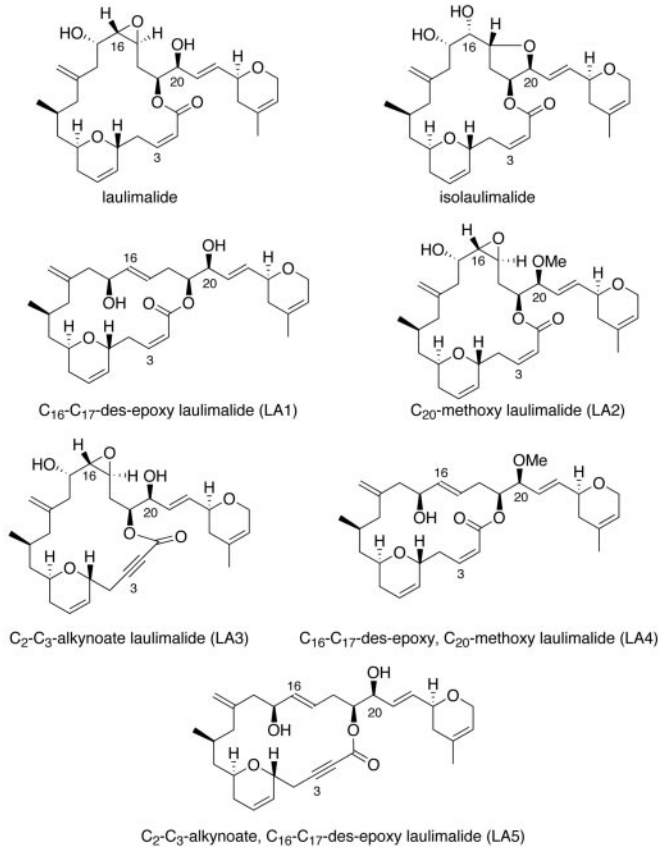

Fig. 1. Chemical structures and nomenclature abbreviations of laulimalide, isolaulimalide, and synthetic laulimalide analogues.

Cellular Effects of the Laulimalide Analogues: Interphase Microtubules. Microtubule stabilizers cause characteristic changes in interphase microtubules. They increase the density of cellular microtubules and, at higher concentrations, cause the formation of abnormally long, thick bundles or short, thick tufts of microtubules. Laulimalide causes an increase in the density of cellular microtubules and the formation of short thick tufts of microtubules in the cell periphery (2). The ability of the laulimalide analogues to initiate these characteristic changes in interphase cells was evaluated by using the A-10 and HeLa cell lines. All of the analogues caused an increased density of cellular microtubules. Interestingly, however, differences among the analogues were noted. The normal filamentous array of interphase microtubules with spaces between tubules was observed in vehicle-treated cells (Fig. 3*A*). The laulimalide analogues LA1 and LA2 caused an increase in the density of interphase microtubules beginning at 0.5 μM in A-10 cells and 250 nM in HeLa cells, and a dose-dependent increase in interphase microtubule density occurred with higher concentrations. Microtubule bundling and the formation of thick microtubule tufts that nucleate independent of the microtubule organizing center were apparent with higher concentrations in A-10 cells (Fig. 3*A*), and these effects are consistent with the effects of laulimalide (2). The two alkynoate analogues, LA3 and LA5, caused an increase in the density of cellular microtubules (Fig. 3*A*). However, at the concentrations where bundling was expected to occur, 50 and 100 μM, overt toxicity of the A-10 cells was observed and few cells remained. This effect is not common among microtubule-stabilizing agents and the alkynoate derivatives, therefore, differ in this regard. The

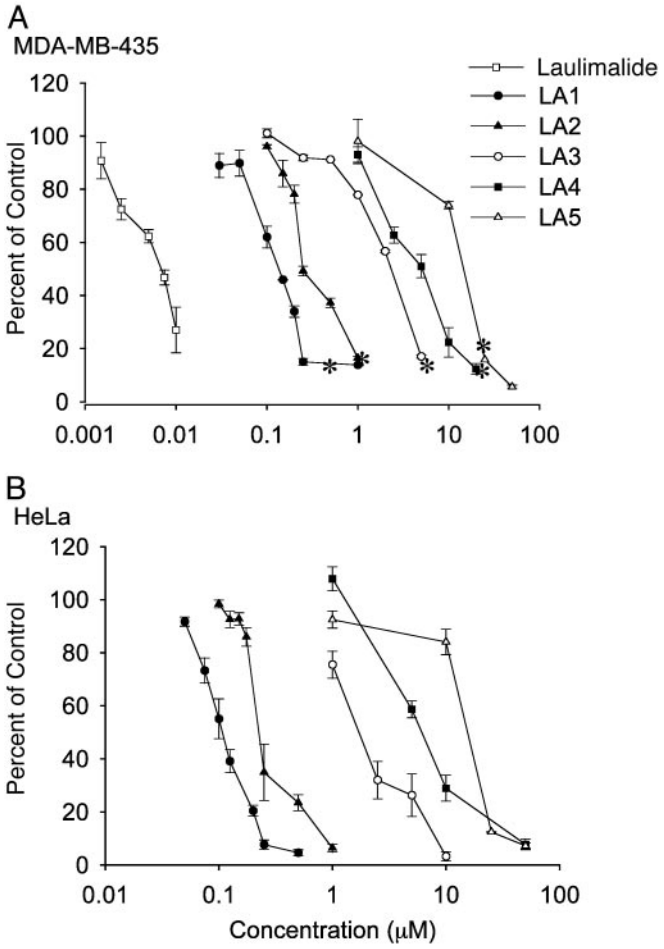

Fig. 2. Dose–response curves for laulimalide and analogues in MDA-MB-435 cells and analogues in HeLa cells ($n = 3 \pm$ SD). Each point shown is from at least two experiments because in some instances different points were used for the various experiments. The 1 μM point for LA1 and 25 μM point for LA5 are from one experiment. Asterisks (*A*) indicate the approximate IC_{85} values for each of the analogues.

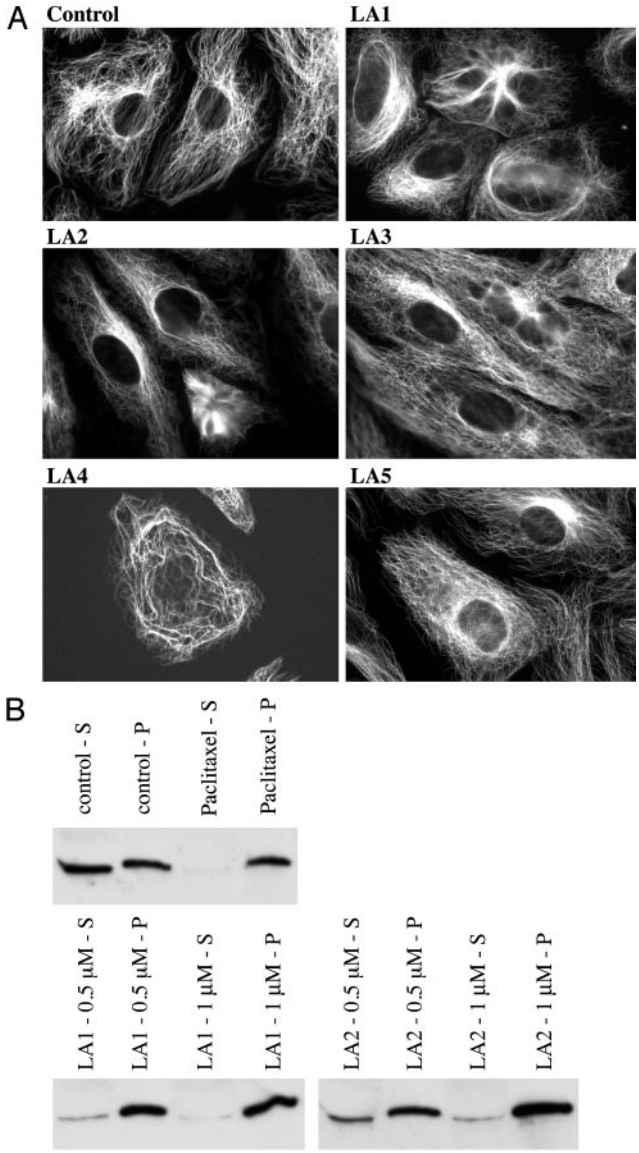

Fig. 3. Microtubule-stabilizing effects of laulimalide analogues. (*A*) The effects of the analogues on interphase microtubules. Cells were treated with the laulimalides for 18 h with vehicle, 10 μM LA1, 10 μM LA2, 10 μM LA3, 100 μM LA4, and 25 μM LA5. (*B*) Cellular tubulin polymerization was evaluated by treating intact cells with the concentrations of compounds indicated for 1 h, separating soluble (S) and polymerized (P) tubulin, and quantifying by Western blotting with β-tubulin antibody. Controls were cells treated with vehicle or paclitaxel (10 nM).

long thick "ropy" microtubule structures observed in cells treated with 100 μM LA4 (Fig. 3*A*) are unique to this analogue and have not been observed with other stabilizers.

Nuclear Structure. The formation of micronuclei is a hallmark of microtubule stabilizers and particularly laulimalide (2). We examined the ability of the analogues to initiate micronucleation. LA1 and LA2 caused extensive micronucleation in A-10 cells, and modest micronucleation was observed in LA4-treated cells (data not shown). Micronucleation was not observed in cells treated with a range of concentrations of LA3 and LA5, the two alkynoate derivatives.

Effects of Analogues on Cellular Tubulin Polymerization. A cellular tubulin polymerization assay was used to confirm that the stabilizer-induced increased density of cellular microtubules observed microscopically in cells represents a quantitative shift in the cellular equilibrium between soluble and polymerized tubulin. Intact cells were treated with paclitaxel, the two most potent analogues LA1 or LA2, or vehicle for 1 h, and soluble and polymerized tubulin were quantitated by Western blotting techniques. A concentration-dependent increase in tubulin polymer was observed in response to LA1 and LA2 (Fig. 3*B*). The increase in polymerized tubulin was accompanied by a decrease in soluble tubulin. Similar to the effects of paclitaxel, LA1 and LA2 cause a shift in cellular tubulin equilibrium toward increased polymer, a common characteristic of microtubule stabilizers.

Mitotic Spindles. Mitotic spindles are highly dynamic structures that are very sensitive to the effects of antimitotics. Abnormal mitotic spindles occur with concentrations of antimitotic drugs that do not affect interphase microtubules. Laulimalide has unique effects on mitotic spindles in that the majority of spindles formed in the presence of laulimalide are circular (2). The effects of the laulimalide analogues on mitotic spindles were evaluated in A-10 cells and HeLa cells. All five analogues caused the formation of aberrant mitotic spindles in both cell lines. Several

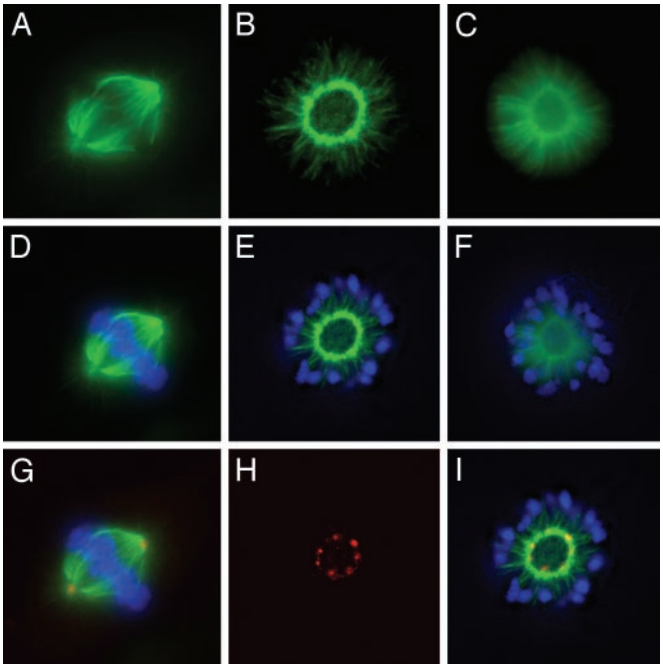

Fig. 4. Effects of the laulimalide analogues on mitotic structures. Mitotic spindles were visualized by using a β-tubulin antibody (green), DNA with 4′,6-diamidino-2-phenylindole (blue), and centrosomal structures with a γ-tubulin antibody (red). A-10 cells were treated with vehicle (*A, D*, and *G*) 0.5 μM LA1 (*B, E, H*, and *I*), or 2 μM LA2 (*C* and *F*). Normal mitotic spindles are bipolar (*A*), whereas the laulimalide analogue-induced mitotic spindles are predominantly circular (*B* and *C*). Chromosomal alignment in the metaphase plate is evident in vehicle-treated cells (*D*) and although highly abnormal, the laulimalide-analogue-induced circular mitotic spindle structures also appear to orient the DNA toward the periphery of the spindles (*E* and *F*). Normal bipolar spindles radiate from two centrosomes (*G*). Multiple centrosomes were detected in the cell treated with LA1 and LA2. LA1-induced centrosomal amplification is shown (*H*), with seven centrosomes in a ring. The orientations of the DNA, mitotic spindles, and centrosomes in the LA1-treated mitotic cells are shown (*I*).

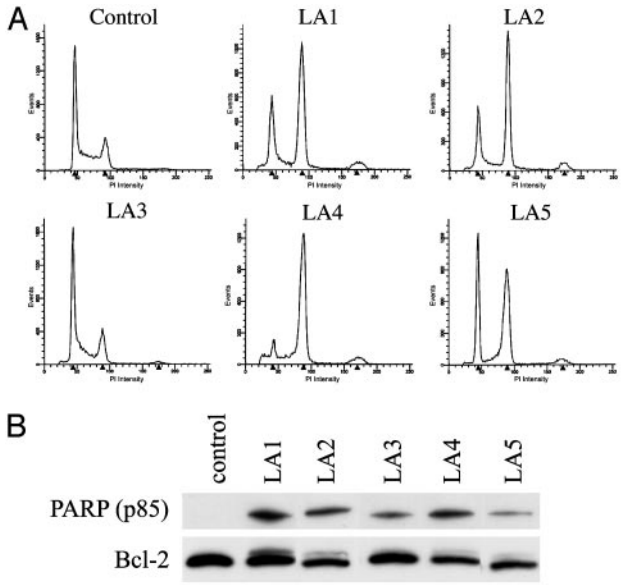

Fig. 5. Cellular effects of laulimalide analogues. (*A*) Effects of laulimalide analogues on cell-cycle distribution. MDA-MB-435 cells were treated with the IC_{85} of the analogues (500 nM LA1, 1 μM LA2, 6.4 μM LA3, 20 μM LA4, and 20 μM LA5), and effects on cell cycle were evaluated at 24 h. The number of events detected by flow cytometry is plotted as a function of propidium iodide fluorescence intensity. (*B*) Effects of analogues on poly(ADP-ribose) polymerase (PARP) cleavage and Bcl-2 phosphorylation. The effects of the analogues in MDA-MB-435 cells were evaluated after a 24-h incubation with the IC_{85} of the various analogues.

different abnormal mitotic structures were observed, including circular, multipolar, and other spindles with little apparent organization.

The effects of LA1 and LA2 on mitotic spindles were studied in detail. In both cell lines the laulimalide analogues caused the formation of multipolar (data not shown) or circular mitotic spindles (Fig. 4 *B* and *C*). A concentration-dependent increase in circular spindles occurred with both analogues. A normal, bipolar spindle (Fig. 4*A*) orients the DNA in the metaphase plate (Fig. 4*D*). The laulimalide-analogue-induced circular mitotic spindles appear to orient the DNA, as it is found in a circular pattern at the periphery of the spindle structures (Fig. 4 *E* and *F*). The kinetochore protein CENP-F is arranged in a circular pattern suggesting spindle microtubule–kinetochore interactions (data not shown) and is consistent with aberrant but ordered arrangement of DNA and mitotic spindles.

Normal mitotic spindles nucleate from two centrosomes (Fig. 4*G*). In A-10 cells the circular mitotic spindle structures formed in the presence of LA1 and LA2 radiate from multiple centrosomes, and often these centrosomes are in a circular arrangement (Fig. 4 *H* and *I*). A concentration-dependent increase in the centrosomes occurs with both LA1 and LA2. Centrosome amplification was confirmed by using antibodies for both γ-tubulin and centrin. Abnormal γ-tubulin structures were also observed in HeLa cells, but the γ-tubulin staining was more diffuse in HeLa. No evidence of monoastral mitotic spindles was observed.

Consistent with the effects of the natural compound, the synthetic analogues cause the formation of abnormal mitotic spindles and specifically circular spindles, suggesting a common mechanism of antimitotic action. Paclitaxel and the epothilones were recently described as causing centrosomal amplification (21). The laulimalide analogues, like paclitaxel and epothilone B, induced centrosomal amplification, and this effect appears to be a common characteristic of microtubule stabilizers and is independent of interaction with the paclitaxel-binding site.

Cell Cycle Progression. Antimitotics cause the formation of nonfunctional mitotic spindles, leading to mitotic dysfunction. The ability of the laulimalide analogues to initiate G_2/M accumulation was studied. The data (Fig. 5*A*) show that all of the analogues, with the exception of LA3, cause G_2/M accumulation within 24 h at the IC_{85}. The effects of LA1 and LA2 are identical, and LA4 caused a higher percentage of cells to accumulate in G_2/M consistent with the effects of laulimalide (2). The alkynoate derivative LA5 caused less G_2/M accumulation than the other analogues, and the other alkynoate derivative LA3 caused no G_2/M accumulation.

Initiation of Apoptosis and Bcl-2 Phosphorylation. The ability of the laulimalide analogues to initiate apoptosis and Bcl-2 phosphorylation was evaluated. Evidence of poly(ADP-ribose) polymerase cleavage, consistent with activation of caspase 3, a late effector caspase, was detected within 24 h in lysates from cells treated with the IC_{85} of the analogues (Fig. 5*B*). Bcl-2 phosphorylation is a common characteristic shared among microtubule stabilizers. All of the analogues, with the exception of LA3, caused Bcl-2 phosphorylation (Fig. 5*B*). The inability of LA3 to initiate Bcl-2 phosphorylation is consistent with its effects on cell-cycle distribution, as it did not cause G_2/M accumulation. Modest but reproducible Bcl-2 phosphorylation occurred with the other alkynoate, LA5.

Table 1. IC_{50} values for inhibition of proliferation

Analogue	IC_{50}, μM*			
	1A9	PTX10	PTX22	A8
LA1	0.189 ± 0.018	0.353 ± 0.053	1.22 ± 0.16	0.625 ± 0.13
des-epoxy-laulimalide		(1.9)	(6.5)	(3.3)
LA2	0.450 ± 0.027	0.862 ± 0.105	1.76 ± 0.09	0.531 ± 0.032
C_{20}-methoxy-laulimalide		(1.9)	(3.9)	(1.2)
LA4	10.9 ± 1.23	10.3 ± 1.65	9.68 ± 2.41	11.6 ± 3.4
des-epoxy, C_{20}-methoxy laulimalide		(0.95)	(0.89)	(1.1)

*IC_{50} values are the mean (±SD) for growth inhibition measured by using the sulforhodamine B assay. For LA1 and LA2, $n = 3$; for LA4, $n = 2$. The *Rr* values are shown in parentheses.

Effects of the Laulimalide Analogues on Paclitaxel-Resistant Cells Lines. Effects of the laulimalide analogues on the paclitaxel- and epothilone-resistant cell lines were examined. Cell lines that are resistant to paclitaxel and the epothilones were developed and the nature of the resistance was identified (18, 19). The PTX10 and PTX22 cell lines were selected for resistance to paclitaxel and the A8 cell line for resistance to epothilone A (18, 19). These cell lines have mutations in the M40β-tubulin gene in amino acids found in the region of the taxane-binding site (18, 19). The effects of laulimalide in these cell lines were evaluated and compared with the effects in the parental 1A9 cell line (3). The relative resistance (*Rr*) values were calculated by dividing the IC_{50} values of the resistant cell lines by the IC_{50} value in the parental cell line. The PTX10 cell line has a $Rr = 29$ for paclitaxel, the PTX22 cell line a $Rr = 20$ for paclitaxel, and the A8 cell line a $Rr = 7.6$ for paclitaxel (3). In contrast, laulimalide has *Rr* values of 1.5–2.4 for these cell lines (3). *Rr* values similar to those of laulimalide were measured for the laulimalide analogues (Table 1). The PTX10 cell lines had *Rr* values for LA1, LA2, and LA4 that were essentially identical to the values obtained with laulimalide. Interestingly, the PTX22 cell line has *Rr* values of 6.5 and 3.9 for LA1 and LA2, respectively, yet the *Rr* value for LA4 was ≈1 (Table 1). The A8 cell line was also more resistant to LA1, yet no resistance to either LA2 or LA4 was measured. One experiment with LA3 and LA5 indicated no cross-resistance of the cell lines to these compounds. These data suggest that there are differences among the analogues with regard to their interaction with the Ala-364 residue of tubulin that is mutated in the PTX22 cell line (18).

Discussion

The goal of this effort was to advance the structural understanding of laulimalide's mode of action, thereby establishing the basis for the design of clinical candidates based on this mechanism. Five laulimalide analogues were designed and synthesized and were found to retain the promising biological activities of the parent compound, including its potent antiproliferative and proapoptotic effects against cancer cells, its microtubule-stabilizing activities, and its ability to circumvent Pgp-mediated drug resistance. A wide range of potencies was achieved in this series of derivatives, and significant structure–activity relationships have emerged.

All of the analogues retain activity against highly drug-resistant cells, suggesting that none of the chemical modifications caused increased recognition for transport by Pgp (16). As new stabilizers are developed it is critically important to maintain the ability to circumvent Pgp-mediated resistance, thereby maintaining advantages over the original taxanes.

Stabilizing laulimalide by removal of the C_{16}-C_{17}-epoxide (LA1) or incorporation of C_{20}-methoxy (LA2) caused a 19- and 41-fold decrease in potency as compared with the natural compound, and this is far superior to the 175-fold lower potency of laulimalide's degradation product isolaulimalide. Significantly, apart from differences in potency, the C_{16}-C_{17}-des-epoxy and C_{20}-methoxy compounds retain the biological effects of laulimalide. LA1 and LA2 cause the characteristic short, thick bundles of interphase microtubules, representing a shift in equilibrium between soluble and polymerized tubulin. The circular mitotic spindles, characteristic of laulimalide, are the predominant aberrant spindles formed in the presence of LA1 and LA2. The spindles are not monoastral, indicating that the laulimalides do not inhibit centrosomal duplication or separation. Rather, the laulimalides appear to initiate centrosomal amplification. With regard to all cellular parameters examined, LA1 and LA2 are identical to laulimalide with the exception of potency.

Our studies add important information about the role of the C_{16}-C_{17}-epoxide moiety in the biological activities of laulimalide. The epoxide is not required for cellular activity but is associated with the excellent potency of the natural compound. Our data are in agreement with the two other groups that have synthesized and conducted biochemical and potency studies on des-epoxy laulimalide (LA1). In bovine brain tubulin assembly reactions, des-epoxy laulimalide stimulated tubulin polymerization, yet it was less potent than laulimalide (3). Both laulimalide and des-epoxy laulimalide were unable to inhibit [^{3}H]paclitaxel binding (3). In cytotoxicity experiments, des-epoxy laulimalide was reported by Pryor *et al.* (3) and Ahmed *et al.* (15) to be 11- to 50-fold less potent, and these values are similar to our 19-fold difference. Stabilization of the laulimalide scaffold by either removal of the C_{16}-C_{17}-epoxide or addition of the C_{20}-methoxy was tolerated without changing the mechanism of action.

The removal of the epoxide and simultaneous addition of the C_{20}-methoxy (LA4) was not advantageous with regard to potency. The combination of these two alterations yielded an analogue derivative that was significantly less potent than either des-epoxy laulimalide (LA1) or C_{20}-methoxy laulimalide (LA2). From a mechanistic perspective, LA4 caused cellular changes similar to laulimalide: abnormal mitotic spindles, mitotic accumulation, and Bcl-2 phosphorylation. However, LA4 initiated changes in the interphase microtubules that were unique. We do not know how LA4 causes the formation of long, curved, thick microtubules, but we speculate that differences might be because of changes in the number of protofilaments that comprise the microtubule. Paclitaxel stabilizes protofilament associations (22), and although the binding site of laulimalide is not yet known, it is reasonable to assume that the laulimalides might also have effects on protofilament associations.

The two alkynoate derivatives LA3 and LA5 were substantially less potent than other analogues and, whereas they retain some of the cellular characteristics of laulimalide, the two alkynoate derivatives differed in other cellular effects. Some abnormal mitotic spindles were noted in cells treated with these analogues, but at the IC_{85}, the concentration where all of the other

analogues and laulimalide caused extensive G_2/M accumulation, these analogues caused no (LA3) or modest (LA5) accumulation. They did not initiate micronucleation and instead caused the A-10 cells to round up and lose adhesion. The results suggest that the alkynoate analogues LA3 and LA5 differ somewhat from the other analogues, and it is possible that the highly reactive alkynoate moiety may initiate nonspecific cytotoxicity.

In summary, the unique and therapeutically promising biological effects of laulimalide can be achieved with designed synthetically accessible analogues that lack the chemically sensitive C_{16}-C_{17} epoxide (LA1) or contain a modified nucleophilic C_{20}-hydroxyl (LA2). These results provide crucial information on the structural basis for laulimalide's mode of action and form the basis for the design of new and stable analogues that could be advanced toward therapeutic applications.

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