Laulimalide and Isolaulimalide, New Paclitaxel-Like Microtubule-Stabilizing Agents

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ABSTRACT

A mechanism-based screening program aimed at the discovery of new antimicrotubule agents from natural products yielded laulimalide and isolaulimalide, two compounds with paclitaxel-like microtubule-stabilizing activity. Treatment of A-10 cells with laulimalide resulted in a dose-dependent reorganization of the cellular microtubule network and the formation of microtubule bundles and abnormal mitotic spindles. Coincident with the microtubule changes, these two compounds induced nuclear convolution and the formation of multiple micronuclei. Laulimalide is a potent inhibitor of cellular proliferation with IC_{50} values in the low nanomolar range, whereas isolaulimalide is much less potent with IC_{50} values in the low micromolar range. In contrast to paclitaxel, both laulimalide and isolaulimalide inhibited the proliferation of SKVLB-1 cells, a P-glycoprotein overexpressing multidrug-resistant cell line, suggesting that they are poor substrates for transport by P-glycoprotein. Incubation of MDA-MB-435 cells with laulimalide resulted in mitotic arrest and activation of the caspase cascade of proteolytic enzymes that accompany apoptotic cell death. Laulimalide stimulated tubulin polymerization and, although less potent than paclitaxel, it was more effective. Laulimalide-induced tubulin polymers resembled paclitaxel-induced polymers, although the laulimalide-induced polymers appeared notably longer. Laulimalide and isolaulimalide represent a new class of microtubule-stabilizing agents with activities that may provide therapeutic utility.

INTRODUCTION

Microtubules play key roles in many cellular processes including intracellular transport, motility, architecture, and division. Normal cell division is dependent on the dynamic instability of microtubules during the course of mitosis and cytokinesis. Interruption of microtubule dynamics has proven to be an effective target for cancer chemotherapy (1). Agents that target microtubules, such as the Vinca alkaloids, have been used in the treatment of cancer for over 30 years. New agents such as paclitaxel (Taxol™) have demonstrated effectiveness against a broad spectrum of tumors including ovarian, breast, and lung carcinomas. Paclitaxel has a mechanism of action unlike any of the tubulin-targeting agents that preceded it to the clinic (2). It promotes tubulin polymerization, stabilizes microtubules, and thereby alters normal microtubule dynamics, leading to the formation of abnormal mitotic spindles, mitotic arrest, and the initiation of apoptosis (1, 2). At high concentrations, paclitaxel causes thick microtubule bundles to form, whereas agents such as the Vinca alkaloids bring about the complete loss of cellular microtubules (2).

The clinical successes of the taxanes, paclitaxel and the semisynthetic derivative docetaxel (Taxotere) prompted the search for new agents with similar mechanisms of action. The search for new paclitaxel-like agents has been vigorous with the goal of providing new agents with advantages over paclitaxel, specifically those that can circumvent transport by drug efflux pumps that confer multidrug resistance (3). Since the discovery of the mechanism of action of paclitaxel, only three other nontaxane chemical classes (epothilones A and B, discodermolide, and eleutherobin) have been identified that possess a similar mode of action. The epothilones A and B were isolated from the myxobacterium Sorangium cellulosum as a result of large-scale screening effort (4). The epothilones have generated significant interest, as they retain activity against drug-resistant cell lines (5, 6). Discodermolide was purified from the marine sponge Discodermia dissoluta as an immunosuppressant and was screened for antimotic activity on the basis of a predictive structure-activity relationship when compared with other tubulin-interacting drugs (6). Discodermolide promotes tubulin assembly more potently than paclitaxel (6, 7), and it is an effective inhibitor of cell growth in paclitaxel-resistant cells (7). Eleutherobin, a potent cytotoxin from the soft coral Eleutherobia sp., promotes tubulin polymerization but exhibits cross-resistance to paclitaxel-resistant cell lines (8, 9). The potential therapeutic usefulness of these new microtubule-stabilizing compounds and whether they will provide advantages over the taxanes have yet to be determined.

In addition to the microtubule-stabilizing agents discodermolide and eleutherobin, there are many other microtubule-targeting marine natural products (reviewed in Ref. 10). Because marine organisms have proven to be a rich source of compounds that target eukaryotic microtubules, we tested extracts from marine invertebrates collected in the Marshall Islands in a mechanism-based screening program aimed at the discovery of new antimicrotubule agents. Strong paclitaxel-like microtubule-stabilizing activity was found in the crude lipophilic extract from the marine sponge Cacospongia mycofijensis. Bioassay-directed purification of the extract yielded the compounds laulimalide and isolaulimalide, 18-membered macrocyclic lactones that had been previously isolated from sponges collected in Indonesia (11), Vanuatu (12), and Okinawa (13). Isolaulimalide was shown to be a laulimalide rearrangement product, formed through the acid-catalyzed attack of the side chain hydroxyl group on the epoxide ring. At the time of isolation, both compounds were known to be cytotoxic, however, the mechanism of action was not known. We report here that laulimalide and isolaulimalide are paclitaxel-like microtubule-stabilizing agents. These two compounds, in contrast to paclitaxel, seem to be poor substrates for the drug efflux pump P-glycoprotein.

MATERIALS AND METHODS

Sponge Collection and Identification. The sponge was collected at depths of 10–30 m on the exposed ocean reef-slopes of Majuro and Arno Atolls, Republic of the Marshall Islands, and kept frozen until use. The sponge is presently named C. mycofijensis (14), but was previously classified as Spungia mycofijensis. Chemical Isolation of Laulimalide and Isolaulimalide. Frozen sponge samples were lyophilized and extracted with methanol. After concentration, the residue was dissolved in 20% aqueous methanol and extracted with chloroform. The remaining aqueous methanol solution was concentrated, and the residue was partitioned between water and extracted with n-butanol. The combined chloroform and butanol fractions were chromatographed over Seph-
adex LH-20 (20% methanol in dichloromethane), followed by reversed-phase high-performance liquid chromatography (octadecyl saline, gradient 20–80% acetonitrile in H2O) and normal-phase high-performance liquid chromatography (silica, 5% methanol in dichloromethane) yielding pure samples of laulimalide and isolaulimalide. From 100 g of dry sponge tissue, 16 mg of laulimalide and 5 mg of isolaulimalide were obtained. The structures of laulimalide and isolaulimalide were confirmed through the comparison of spectral data with published data (15, 16) and by comparison with an authentic sample kindly supplied to us by Drs. J. Tanaka and T. Higa (University of the Ryukyus, Nishinara, Japan).

Laulimalide and isolaulimalide stocks were solubilized in 100% ethanol and stored at −70°C to inhibit the spontaneous conversion of laulimalide into isolaulimalide. Working dilutions were stored at −20°C. Drug stocks were used within 4 months of dilution, and no loss of potency, suggesting conversion, was seen over this time period.

Cell Culture. The A-10 rat aortic smooth muscle cell line and SK-OV-3 ovarian carcinoma cell line were purchased from American Type Culture Collection (Manassas, VA). SKVLB-1 cells were the generous gift of Dr. Victor Ling (British Columbia Cancer Center, Vancouver, British Columbia). The SKVLB-1 line is a P-glycoprotein overexpressing subline of SK-OV-3 that was selected for resistance to vinblastine (15). The MDA-MB-435 cell line, a human breast adenocarcinoma line, was kindly provided by Dr. Mai Higazi (Georgetown University, Washington, DC). SK-OV-3, SKVLB-1, and A-10 cells were cultured in BME supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 50 μg/ml gentamicin. SKVLB-1 cells were grown in the presence of 1 μg/ml vinblastine to maintain selection pressure for the overexpression of P-glycoprotein. MDA-MB-435 cells were maintained in Richters medium (Biofluids, Inc., Rockville, MD) with 10% fetal bovine serum and 50 μg/ml gentamicin.

Indirect Immunofluorescence. A-10 cells were plated onto glass coverslips and grown until 70–85% confluent, then treated with drugs, as described in the figure legends. The cells were fixed with ice-cold methanol for 5 min, blocked for 20 min with 10% calf serum in PBS, and incubated for 90 min with monoclonal β-tubulin antibody (T-4026; Sigma Chemical Co., St. Louis, MO). After a series of washes, the cells were incubated with FITC-conjugated sheep antirabbit IgG (F-3008; Sigma Chemical Co.) for 1 h. The coverslips were washed, stained with 0.1 μg/ml DAPI for 10 min, and mounted. Cellular microtubules and chromatin were visualized and photographed using a Zeiss Axioscan fluorescence microscope with optics for fluorescein and DAPI.

Inhibition of Cell Proliferation. The IC50 for inhibition of cellular proliferation was determined by measuring cell-associated protein after drug treatment using the sulforhodamine B assay (16, 17).

Cell Cycle Analysis. MDA-MB-435 cells were treated with 20 nM laulimalide for 24 h. The cells were stained with propidium iodide, as described previously (18), and DNA content was measured using a Coulter EPICS XL-MCL flow cytometer and plotted as the number of events versus propidium iodide fluorescence intensity.

Tubulin Assembly. The assembly of purified bovine brain tubulin was monitored using the CytoDYNAMIX Screen (Cytoskeleton, Denver, CO). This assay uses a 96-well assay plate format with 200 μg of lyophilized purified tubulin in each well. The tubulin was reconstituted with ice-cold 180 μl G-PGM buffer [80 mM NaPipes (pH 6.8), 1 mM MgCl2, 1 mM EGTA, and 1 mM GTP] containing laulimalide (1–20 μM), paclitaxel (0.5–20 μM), or vehicle control (1% ethanol). The assay was conducted at 37°C in a temperature-controlled microtiter plate reader. Tubulin polymerization was monitored spectrophotometrically by the change in absorbance at 340 nm. The absorbance was measured at 1-min intervals for 60 min.

Immunoblot Analysis. After laulimalide treatment, the cells were harvested and protein extracted in radioimmunoprecipitation buffer in the presence of protease inhibitors, as described previously (18). The protein concentration of each sample was determined (Pierce Reagents, Rockford, IL), and samples containing equal amounts of protein were separated by PAGE with SDS using standard Laemmli techniques. The proteins were transferred onto Immobilon membranes in Tris/glycine buffer. The proteins of interest were probed with specific antibodies and detected by chemiluminescence (ECI, Amersham Corp., Arlington Heights, IL). The PARP antibody was obtained from Boehringer Mannheim (Indianapolis, IN), and the caspase 3 antibody was purchased from PharMingen (San Diego, CA).

Electron Microscopy. Bovine brain tubulin (glycerol-free) was purchased from Cytoskeleton and was used at a concentration of 1 mg/ml in 0.1 M 2-[N-morpholino]ethanesulfonic acid buffer (pH 6.9) with 0.1 mM MgCl2 and 0.1 mM residual GTP from the tubulin supply buffer. Either paclitaxel or laulimalide was added to achieve a final concentration of 10 μM, the temperature was increased from 18°C to 37°C, and absorbance at 350 nm was monitored over time. When maximal polymerization had been achieved, aliquots of tubulin were removed and applied to 300 mesh carbon-coated Formvar-treated copper grids. The tubulin polymers were stained with 1% uranyl acetate and examined and photographed using an electron microscope.

Statistical Analysis. The Kruskal-Wallis test (19) was used to statistically compare the average percentage of cells with micronuclei between the paclitaxel- and laulimalide-treated groups. The means between the laulimalide- and paclitaxel-treated groups were compared at each drug concentration.

RESULTS

Effects of Laulimalide, Isolaulimalide, and Paclitaxel on Cellular Microtubules. Strong paclitaxel-like microtubule-stabilizing activity was found in the crude lipophylic extract from the marine sponge C. mysceoffjijiensis. The extract was cytotoxic, and after treatment the only cell remnants that remained were thick, short microtubule bundles. Bioassay-directed purification of the extract yielded the microtubule-active compounds laulimalide and isolaulimalide (Fig. 1), as well as the microfilament disruptor latrunculin A. Studies with the purified compounds showed that both laulimalide and isolaulimalide caused dramatic reorganization of cellular microtubules.

A-10 cells were treated with laulimalide, isolaulimalide, or paclitaxel for 18 h and the morphological effects on microtubules examined by indirect immunofluorescence techniques. The control cells exhibited normal microtubule arrays with filamentous microtubules radiating from the microtubule organizing center to the cell periphery (Fig. 2A). Treatment of the cells with laulimalide disrupted the normal microtubule array; the microtubules were more numerous and appeared to occupy more of the cytoplasm. A 2-μM concentration of laulimalide caused microtubule bundles to form throughout the cytoplasm (Fig. 2B). Cells treated with 20 μM laulimalide exhibited bundles of short tufts of microtubules (Fig. 2C) that were prevalent in the cell periphery and seemed to be independent of nucleation from...
microtubule organizing centers. Isolaulimalide at concentrations between 2–20 μM caused an increase in the density of cellular microtubules, but no microtubule bundles were present (data not shown). Paclitaxel at concentrations between 1–20 μM initiated the formation of a highly organized array of microtubules, some of which formed thick microtubule bundles. Long thick microtubule bundles often surrounded the nucleus (Fig. 2D). The extensive long microtubule hoops and bundles that formed after treatment with 2 μM paclitaxel (Fig. 2D) were not seen with laulimalide. Cellular microtubules stabilized with paclitaxel, laulimalide or isolaulimalide were resistant to vinblastine-induced depolymerization (data not shown).

Effects on Nuclear Structure. A hallmark of both A-10 and SK-OV-3 cells treated with a wide range of concentrations of laulimalide and isolaulimalide was the formation of multiple micronuclei. The effects of laulimalide on nuclear structure are visible in Fig. 2. The normal rounded shape of the nucleus, which is devoid of microtubules, can be detected (Fig. 2, A and D), whereas in laulimalide-treated cells this distinct microtubule-free area containing the discrete central nucleus was lost and only vesicle-like areas devoid of microtubules remained (Fig. 2, B and C). Nuclear staining of control cells revealed a central compact nucleus (Fig. 3A), whereas laulimalide-treated cells exhibited a dramatic breakdown of the nucleus into micronuclei (Fig. 3B). Similar nuclear changes occurred with isolaulimalide (data not shown). Paclitaxel also initiated the formation of micronuclei, as has been reported by others (20). Analysis of the incidence of micronuclei formation showed that a higher percentage of laulimalide-treated cells contained micronuclei than cells treated with the same concentrations of paclitaxel. The data in Fig. 4 show that in A-10 cells 0.02–2 μM laulimalide caused micronuclei formation in approximately 60% of the cells. Paclitaxel at the same concentrations caused approximately 40% of the cells to exhibit this abnormal restructuring of the nucleus. At all concentrations tested, laulimalide caused a higher percentage of cells to exhibit micronuclei when compared with paclitaxel-treated cells. The difference between the two drugs was statistically significant at all five concentrations tested (P <0.0001).

Effects of Laulimalide on Cell Cycle Progression and Mitotic Spindles. A common characteristic of antimicrotubule agents is their ability to initiate mitotic arrest. Flow cytometric analysis revealed that laulimalide-induced cell cycle arrest in G2-M in MDA-MB-435 breast carcinoma cells within 9 h of treatment (Fig. 5). This is consistent with the effects of other antimicrotubule agents, where disruption of microtubule dynamics prevents normal mitotic progression and leads to mitotic arrest. Abnormal mitotic spindles were seen in both A-10 cells and SK-OV-3 cells after treatment with laulimalide and isolaulimalide. The mitotic cells were rounded, and the spindles formed a circular pattern with spindle microtubules radiating outward from a microtubule-free core in the center of the cell (Fig. 6A). In the mitotic cells, the nuclear membranes were not apparent, and the chromatin was condensed and aligned in a circular pattern. Abnormal mitotic spindles were visible in paclitaxel-treated cells and were typically tri-or tetra-polar (Fig. 6B) and did not exhibit the morphology seen with the laulimalides.

Effects of Laulimalide, Isolaulimalide, and Paclitaxel on Cell Proliferation of Drug-Sensitive and Multidrug-Resistant Cell
The early literature on laulimalide reported that it was a cytotoxin (11, 13). In this study, experiments were conducted to determine the IC50 values for laulimalide and isolaulimalide in two drug-sensitive cell lines, MDA-MB-435 and SK-OV-3, and in a multidrug-resistant cell line, SKVLB-1. Laulimalide is a potent inhibitor of cell proliferation with IC50 values between 5–12 nM (Table 1). Isolaulimalide is less potent with IC50 values in the low μM range.

Both laulimalide and isolaulimalide inhibited the proliferation of the SKVLB-1 cell line that overexpresses the drug efflux pump P-glycoprotein. The resistance factors between the parental, drug-sensitive line (SK-OV-3) and the drug-resistant cell line (SKVLB-1) were 105 and 1.03 for laulimalide and isolaulimalide, respectively (Table 1).

We did not achieve >70% inhibition of the SKVLB-1 cell line with concentrations of paclitaxel up to 100 μM. Laulimalide and isolaulimalide are significantly more effective against the SKVLB-1 cell line than paclitaxel. These data confirm that these new agents are poor substrates for transport by P-glycoprotein.

### Table 1  Inhibition of proliferation in drug sensitive and resistant cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Laulimalide (nM)</th>
<th>Isolaulimalide (nM)</th>
<th>Paclitaxel (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-435</td>
<td>5.74 ± 0.58</td>
<td>1,970 ± 97</td>
<td>1.02 ± 0.25</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>11.53 ± 0.53</td>
<td>2,570 ± 290</td>
<td>1.71 ± 1.07</td>
</tr>
<tr>
<td>SKVLB</td>
<td>1,210 ± 490</td>
<td>2,650 ± 1,384</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>Resistance factor</td>
<td>105</td>
<td>1.03</td>
<td>&gt;58,480</td>
</tr>
</tbody>
</table>

**Fluorescence**

Fig. 4. Effects of laulimalide and paclitaxel on micronuclei formation. A-10 cells were treated for 18 h with paclitaxel or laulimalide. The cells were fixed, and nuclei were visualized by DAPI staining. The percentages of cells containing micronuclei were counted in 20 microscope fields/treatment in two experiments.

Fig. 3. Effects of laulimalide on nuclear structure. DNA was visualized by staining with DAPI in A-10 cells after 18-h treatments with a vehicle control (A) or 2 μM laulimalide (B).

Fig. 5. Effects of laulimalide on cell cycle distribution. Log phase growth cultures of MDA-MB-435 cells were treated with vehicle control (A) or 20 nM laulimalide for 9 h (B) or 18 h (C). After treatment, the cells were fixed, stained, and analyzed on a Coulter EPICS XL-MCL flow cytometer and plotted as events versus propidium iodide fluorescence intensity.
The IC_{50} for inhibition of proliferation was also determined in the A-10 cell line, a nontransformed line that was used to show the effects of microtubule-stabilizing agents on cellular structures (Figs. 2 and 6). The IC_{50} for laulimalide was 51 μM and for paclitaxel was 40 μM. The nontransformed A-10 cell line was much less sensitive to the inhibitory effects of both paclitaxel and laulimalide, illustrating the need to use micromolar concentrations in these cells to induce abnormal microtubules.

**Initiation of Apoptosis by Laulimalide.** The ultimate mechanism of action of many cytotoxic cancer chemotherapeutic agents is the initiation of pathways of gene and protein expression leading to apoptosis (reviewed in Refs. 21 and 22). Antimicrotubule drugs including paclitaxel, vinblastine, and cryptophycin 1 initiate apoptosis both *in vitro* and *in vivo* (18, 23–25). The flow cytometry data (Fig. 5) show a doubling of the subdiploid peak at 18 h (Fig. 5C), suggesting the initiation of apoptosis. The loss of cellular DNA is detected by the appearance of the subdiploid peak when apoptotic cells are analyzed by flow cytometry (26). Studies were undertaken to determine whether laulimalide initiates a gene-driven program of cellular suicide.

During apoptosis, specific cysteine proteases called the caspases are activated (34). Activation of the caspase cascade leads to the proteolytic degradation of specific cellular proteins. The activation of caspase 3 and the proteolysis of the DNA repair enzyme PARP, a downstream substrate of caspase 3, were examined in cell lysates from laulimalide-treated cells. Activation of caspase 3 leads to the loss of the 32 kDa proenzyme and the formation of the activation products p17 and p12 (28). Analysis of immunoblot data from the cell lysates (Fig. 7) shows the formation of the p17 activation product at 24, 42, and 48 h after laulimalide treatment. The loss of the p32 proenzyme is seen at 48 h. The specific proteolysis of PARP by caspase 3 leads to the formation of two products, an 89 kDa COOH-terminal fragment and a 24 kDa N-terminal fragment (28). The proteolysis of PARP and the appearance of the 89 kDa degradation product coincided with the activation of caspase 3 (Fig. 7). Caspase 3 was activated and PARP proteolytically cleaved in cell lysates from cells treated with laulimalide for 42 and 48 h. These data are consistent with laulimalide-induced apoptotic cell death.

**Effects of Laulimalide on Tubulin Polymerization in Vitro.** One characteristic of the microtubule-stabilizing agents paclitaxel, disodermolide, epothilones A and B, and eleutherobin is the ability of these agents to initiate the polymerization of tubulin in the absence of polymerization promoters, such as glycerol. Studies were undertaken to determine the effects of laulimalide on tubulin polymerization. Both isolaulimalide and laulimalide stimulated tubulin polymerization. Fig. 8A shows the effects of laulimalide on tubulin polymerization, and the accompanying Fig. 8B shows the effects of paclitaxel. Comparisons between the effects of laulimalide and paclitaxel on tubulin polymerization (Fig. 8, A and B) show that, at low micromolar concentrations, paclitaxel is more potent than laulimalide (Table 2). At low micromolar concentrations more tubulin polymer was formed in the presence of paclitaxel, and the rate of polymerization was faster than was seen with equivalent concentrations of laulimalide.

A very different relationship was seen when comparing the 20-μM concentrations of laulimalide and paclitaxel. The 20-μM concentration of laulimalide was more effective at stimulating the formation of tubulin polymer than was the 20-μM concentration of paclitaxel (Fig. 8, A and B). Laulimalide promoted the polymerization of approximately 30% more tubulin polymer than was formed in the presence of paclitaxel, and the kinetics of tubulin formation were twice as fast as the rate measured in the presence of 20 μM paclitaxel.

The tubulin polymers formed in the presence of laulimalide were insensitive to cold and CaCl_{2}-induced depolymerization (data not shown). Neither paclitaxel nor laulimalide promoted the polymeriza-
tion of tubulin at 0°C, and laulimalide stabilized tubulin polymer formed in the presence of GTP.

Samples of the tubulin polymer formed were examined by electron microscopy to determine whether the increase in turbidity measured during the polymerization experiments was due to the formation of microtubule-like polymers or the formation of other structures. Under high magnification (×63,000) the tubulin polymers formed by paclitaxel and laulimalide were indistinguishable (Fig. 9, A and B). Both agents formed structures resembling tubules with evidence of longitudinal symmetry. Examination of tubulin polymers formed in the presence of the microtubule-stabilizing agents at lower magnification (×4,000) showed that the polymers formed with laulimalide were very long structures with rounded curves (Fig. 9D). The polymers formed with paclitaxel were not only shorter and exhibited no rounded curves, but instead formed linear structures with angular branches (Fig. 9C).

DISCUSSION

Laulimalide and isolaulimalide were first isolated on the basis of their cytotoxicity, however, the mechanism of action was not elucidated. We now report that these agents are paclitaxel-like stabilizers of microtubules that cause alterations of both interphase and mitotic microtubules. Laulimalide is a potent inhibitor of cell proliferation and initiates mitotic arrest, micronuclei formation, and ultimately apoptosis. These compounds are superior to paclitaxel in their ability to circumvent P-glycoprotein-mediated drug resistance. The laulimalides represent a new class of paclitaxel-like microtubule-stabilizing agents with properties that may provide advantages over the taxanes.

Laulimalide and isolaulimalide are chemically related compounds, with isolaulimalide being a decomposition product of laulimalide. The difference between these two compounds is in the size and attachment points of the oxygen-containing ring within the top portion of the molecules. Laulimalide contains a three-membered epoxide ring involving carbons C-16 and C-17, whereas isolaulimalide contains a five-membered tetrahydrofuran ring linking carbon C-17 with side chain carbon C-20. This slight chemical difference between laulimalide and isolaulimalide results in a difference in potency of greater than two orders of magnitude in their ability to inhibit cell proliferation. Furthermore, this site also seems to be crucial for recognition by the multidrug efflux pump P-glycoprotein. Laulimalide had a resistance factor of 105 when comparing the IC50 values in SK-OV-3 cells and SKVLB-1 cells, whereas isolaulimalide was equally sensitive in both cell lines. These data suggest that the epoxide moiety of laulimalide is critical for its interaction with both tubulin and P-glycoprotein.

Among the five groups of known antimicrotubule agents having paclitaxel-like microtubule-stabilizing properties, laulimalide most closely resembles the epothilones. Although the ring size of laulimalide is two carbons bigger (18-membered versus 16-membered), both contain a similar structural motif that incorporates the epoxide ring, an unsaturated side chain bearing a methylated heterocyclic ring, and the ester of the macrocyclic lactone ring. This similarity seems to translate to similar activities. Both stabilize microtubules, and like laulimalide, the epothilones are poor substrates for P-glycoprotein-mediated transport (9).

The cellular effects of laulimalide are similar to, but distinct from, the cellular effects of paclitaxel. The increase in the density of cellular microtubules observed at low concentrations of laulimalide closely resembled the changes induced by paclitaxel at the same concentrations. However, at concentrations above 2 μM, the effects diverged and laulimalide initiated short thick bundles of microtubules that were more prevalent in the cell periphery and appeared to form from many nucleation centers. These effects are consistent with the greater efficacy of 20 μM laulimalide in promoting the polymerization of purified tubulin. In contrast, paclitaxel-induced microtubule bundles were long and thick and aligned in the central areas of the cells surrounding the nucleus, consistent with nucleation from one or two centers. Long microtubule bundles were not seen in cells treated with a wide range of laulimalide concentrations. These data suggest that in cells, laulimalide was not as effective as paclitaxel at elongation of microtubules, but was more effective at stimulating the formation of microtubules from multiple nucleation centers, resulting in shorter microtubule bundles in the periphery rather than the long microtubule bundles surrounding the nucleus.

The microtubules that form the mitotic spindle are highly dynamic structures and are more sensitive to disruption by antimicrotubule agents than are the less dynamic interphase microtubules. Agents that target microtubules disrupt mitotic spindle dynamics, thereby preventing normal mitosis, leading to mitotic arrest (1). Mitotic spindles formed in the presence of laulimalide were abnormal and formed unique starburst arrays in contrast to the short thickened tri- and tetra-polar spindles formed in the presence of paclitaxel. Laulimalide-treated mitotic cells exhibited
chromatin condensation, loss of the nuclear envelope and abnormal chromatin alignment. The aberrant mitotic spindles were associated with circular chromatin arrays, suggesting that the microtubules were coordinating a specific, but abnormal structuring of the chromatin. Disruption of the mitotic apparatus by laulimalide treatment lead to mitotic arrest, followed by the initiation of apoptosis, as determined by the increase in cells in G2-M and the activation of the caspase cascade.

Cells treated with laulimalide exhibited vesicle-like structures in the central region of the cell. DAPI staining revealed that these structures were composed of DNA and that laulimalide initiated the formation of multiple micronuclei. In A-10 cells, micronuclei were found in the majority of cells with treatments of 20 nM - 2 μM laulimalide. Micronuclei formations occur as a consequence of treatment with either paclitaxel or epothilones and are thought to be the result of abnormal mitosis leading to abnormal chromosome segregation (4, 20).

One characteristic of the paclitaxel-like microtubule-stabilizing agents is their ability to promote the polymerization of tubulin. Normally tubulin will not polymerize without tubulin promoters; however, laulimalide, like paclitaxel, stimulated the polymerization of tubulin in the absence of microtubule-associated proteins and glycerol. The rate and extent of polymerization in the presence of laulimalide was dependent on concentration and differed significantly from paclitaxel, suggesting that there are differences in their mechanisms of action. Further experimentation in the presence of different tubulin and laulimalide concentrations and in the presence and absence of microtubule-associated proteins will allow more comprehensive comparisons of the tubulin polymerizing effects of laulimalide as compared with paclitaxel, discodermolide, and the epothilones.

The tubulin polymers formed in the presence of laulimalide were indistinguishable from paclitaxel-induced polymers under high magnification, but were noticeably longer than the paclitaxel-induced polymers when visualized under lower magnification. Under these conditions, laulimalide seems to promote polymer-elongating activity more readily than paclitaxel. These in vitro...
effects with purified tubulin differ from the effects in cells where paclitaxel promotes longer microtubules, whereas laulimalide promotes short microtubules. The initial studies suggest that there are intriguing differences in the mechanisms of action of laulimalide and paclitaxel.

The clinical success of the taxanes in treating a wide range of tumors has lead to the search for new agents with a similar mechanism of action. The recent discovery of four additional classes of compounds, discodermolide, the epothilones, eleutheroerin, and now the laulimalides, provides hope that new drugs may be found to treat cancer.

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