

# Synergistic Effects of Peloruside A and Laulimalide with Taxoid Site Drugs, but Not with Each Other, on Tubulin Assembly

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## ABSTRACT

Previous studies on the drug content of pelleted tubulin polymers suggest that peloruside A binds in the laulimalide site, which is distinct from the taxoid site. In a tubulin assembly system containing microtubule-associated proteins and GTP, however, peloruside A was significantly less active than laulimalide, inducing assembly in a manner that was most similar to sarcodictyins A and B. Because peloruside A thus far seems to be the only compound that mimics the action of laulimalide, we examined combinations of microtubule-stabilizing agents for synergistic effects on tubulin assembly. We found that peloruside A and laulimalide showed no synergism but that both compounds could act synergistically with a number of taxoid site agents [paclitaxel, epothilones A/B, discodermolide, dictyostatin, eleutherobin, the steroid derivative 17 $\beta$ -acetoxy-2-

ethoxy-6-oxo-B-homo-estra-1,3,5(10)-trien-3-ol, and cyclostreptin]. None of the taxoid site compounds showed any synergism with each other. From an initial study with peloruside A and cyclostreptin, we conclude that the synergism phenomenon derives, at least in part, from an apparent lowering of the tubulin critical concentration with drug combinations compared with single drugs. The apparent binding of peloruside A in the laulimalide site led us to attempt construction of a pharmacophore model based on superposition of an energy-minimized structure of peloruside A on the crystal structure of laulimalide. Although the different sizes of the macrocycles limited our ability to superimpose the two molecules, atom correspondences that were observed were consistent with the difficulty so far experienced in creation of fully active analogs of laulimalide.

The mechanism of action of paclitaxel was initially described in 1979 (Schiff et al., 1979), and for many years, taxoids were the only compounds known to interfere with the cellular microtubule system and cell division by causing tubulin hyperassembly. It was not until 1995 that epothilones A and B were reported as a second chemotype with a paclitaxel-like mechanism of action, including inhibition of the

binding of paclitaxel to polymer (Bollag et al., 1995). The epothilones were followed by many additional active compounds, including the sponge-derived discodermolide (Ter Haar et al., 1996), laulimalide (Mooberry et al., 1999), dictyostatin (Isbrucker et al., 2003), and peloruside A (Hood et al., 2002); the coral-derived eleutherobin and sarcodictyins (Long et al., 1998); the bacterial product cyclostreptin (Edler et al., 2005); the synthetic steroid analog AEH (Verdier-Pinard et al., 2000); and the taccalonolides, plant-derived steroid-like molecules (Tinley et al., 2003).

Most of these compounds bind in the taxoid site of tubulin polymer, competitively inhibiting the binding of radiolabeled

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**ABBREVIATIONS:** AEH, 17 $\beta$ -acetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-trien-3-ol; Flutax-2, 7-O-[N-(2,7-difluoro-4'-fluoresceincarbonyl)-L-alanyl]paclitaxel; MAP, microtubule-associated protein; MES, 4-morpholineethanesulfonate.

paclitaxel or the fluorescent taxoid derivative Flutax-2 to this site (Bollag et al., 1995; Buey et al., 2004, 2005; Edler et al., 2005; Hamel et al., 1999; Kowalski et al., 1997a, b). However, there are two exceptions to this generalization. Neither laulimalide (Pryor et al., 2002) nor peloruside A (Gaitanos et al., 2004) inhibits the binding of taxoids to tubulin polymer. Moreover, microtubules formed in the presence of paclitaxel and either laulimalide (Pryor et al., 2002) or peloruside A (Gaitanos et al., 2004) contain both paclitaxel and the second drug. No evidence was found for incorporation of laulimalide and peloruside A (Gaitanos et al., 2004), however, suggesting that both compounds bind at the same site on tubulin. Finally, paclitaxel and laulimalide can act synergistically in promoting tubulin assembly, as might be expected for drugs binding at different sites on polymer (Gapud et al., 2004).

Taxoid site drugs show a wide range of activity as inducers of tubulin assembly, ranging from the highly potent discodermolide (Ter Haar et al., 1996; Kowalski et al., 1997a) and dictyostatin (Madiraju et al., 2005) to the weakly active sarcodictyins (Hamel et al., 1999) and cyclostreptin (Edler et al., 2005). Thus far, peloruside A has been directly compared only with paclitaxel, and the available evidence suggests that it is less potent than paclitaxel in promoting assembly. Peloruside A yielded a tubulin-critical concentration more than 2-fold that obtained with paclitaxel (Gaitanos et al., 2004). In contrast, laulimalide yielded a tubulin critical concentration somewhat lower than that obtained with paclitaxel. In contrast, laulimalide yielded a tubulin critical concentration somewhat lower than that obtained with paclitaxel for assembly in 0.1 M MES without GTP (Gapud et al., 2004) and about 50% higher for assembly in 3.4 M glycerol with GTP (Paterson et al., 2005).

In this study we first evaluated the activity of peloruside A in a MAP and GTP-dependent tubulin assembly system in comparison with a variety of taxoid site drugs and with laulimalide. Next, we demonstrated the potential of peloruside A and of laulimalide to induce assembly synergistically with multiple compound chemotypes that bind at the taxoid site and determined that laulimalide and peloruside A were unable to synergize with each other. Finally, we constructed a laulimalide-peloruside A pharmacophore to examine structure-activity relationships among existing laulimalide analogs. Figure 1 presents the structures of peloruside A, laulimalide, and the taxoid site compounds we will examine here, with the exception of sarcodictyin A.

## Materials and Methods

**Materials.** Paclitaxel and natural epothilone B were generously provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Rockville, MD), and Merck Research Laboratories (Rahway, NJ), respectively. Natural peloruside A was purified as described previously (West et al., 2000). The remaining drugs were synthetic, prepared as described previously: epothilone A (Nicolaou et al., 1997b), eleutherobin (Nicolaou et al., 1997a), sarcodictyin A (Nicolaou et al., 1998), AEH (Wang et al., 2000), laulimalide (Ghosh et al., 2001), discodermolide (Paterson et al., 2000), cyclostreptin (Vanderwal et al., 2003), and dictyostatin (Shin et al., 2004). Two analogs of laulimalide, both with the epoxide moiety replaced with a *trans*-olefin bond and one with the C-2/C-3 *cis*-olefin bond replaced with a *trans* bond (called "*trans*-desoxylaulimalide" and "*bis-trans*-desoxylaulimalide") were prepared by modifications in the procedure

used to synthesize laulimalide (Ghosh et al., 2001). The nuclear magnetic resonance spectroscopic, mass spectrometric, and elemental analysis data of the two analogs were in agreement with the assigned structures. Tubulin and heat-treated MAPs were prepared as described previously (Hamel and Lin, 1984), and residual unbound GDP was removed from the tubulin by gel filtration chromatography on Sephadex G-50 (superfine). When MES was used, its concentration was 0.1 M (taken from a 1.0 M stock solution adjusted to pH 6.9 with NaOH).

**Methods.** Inhibition of MCF-7 human breast carcinoma cell growth was measured as described previously (Pryor et al., 2002). Tubulin assembly reactions were followed by either turbidimetric or centrifugal assays. In the former, Gilford (Oberlin, OH) model 250 recording spectrophotometers equipped with electronic temperature controllers were used, with apparent absorbance measured at 350 nm. Baselines were always established at 0°C in reaction mixtures without drug. The drug was rapidly mixed into the reaction mixture (final volume, 0.25 ml), and turbidity was continuously followed for the indicated times at the indicated temperatures. Temperature in the cuvettes rises at approximately 0.5°C/s and falls at approximately 0.1°C/s once a change is made in the setting. Reaction components are described for the individual experiments.

In the centrifugal assays with reaction mixtures containing 0.1 M MES, 0.1-ml reaction mixtures were prepared in polyallomer 7 × 20-mm tubes (Beckman Coulter, Fullerton, CA). Drug(s), dissolved in dimethyl sulfoxide, or offsetting volumes of the solvent to a final solvent concentration of 4% (v/v), were added to the tubes before addition of a mixture containing all other reaction components, as indicated in the individual experiments, except in experiments where critical concentrations of tubulin were determined. In these latter experiments, tubulin was the last component added to otherwise complete reaction mixtures. The complete reaction mixtures were briefly vortexed and incubated as indicated in the individual experiments. Centrifugation was in a TLA 100 rotor (Beckman Coulter) at 30,000 rpm for 10 min at the indicated temperature in an Optima TLX miniultracentrifuge (Beckman Coulter). An aliquot of each reaction mixture was taken from the top of the supernatant, and protein content was determined by the Lowry assay (Lowry et al., 1951). Total protein content of uncentrifuged samples and of centrifuged samples without drug were determined in each experiment. Pellet protein concentration was determined by subtracting supernatant protein concentration from total protein concentration.

In the centrifugal assays with reaction mixtures containing 1.0 M glutamate, 0.1-ml reaction mixtures were prepared in 0.5-ml Eppendorf (Westbury, NY) tubes (Lin et al., 1996). Drug(s), dissolved in dimethyl sulfoxide or offsetting volumes of the solvent to a final solvent concentration of 4%, were added to the tubes before addition of a mixture containing all other reaction components as indicated. Reaction mixtures were briefly vortexed and incubated for 15 min at 22°C. They were then centrifuged for 10 min at room temperature in an Eppendorf 5417C benchtop centrifuge. An aliquot of each reaction mixture was taken from the top of the supernatant, and protein content was determined by the Lowry assay. Total protein content of uncentrifuged samples and of centrifuged samples without drug were determined in each experiment.

**Molecular Modeling.** Models were built with the Cerius<sup>2</sup> program (v. 4.5; Accelrys, Inc., San Diego, CA), using the Merck Molecular Force Field (MMFF94) for molecular mechanics minimizations (adopted basis Newton-Raphson method), running on a Fuel workstation (SGI, Mountain View, CA). The model for laulimalide was taken from the X-ray diffraction structure (Jefford et al., 1996) and minimized by molecular mechanics. The model for peloruside A was built from the laulimalide structure by atomic replacement and subsequently minimized with MMFF94. Conformational searches of the peloruside A structure were performed using the grid scan and Boltzmann jump (5000 K) methods, all followed by minimization with MMFF94. Superimpositions were done by manual atom matching (up to 20 matched atoms per model) using both the flexible fit and

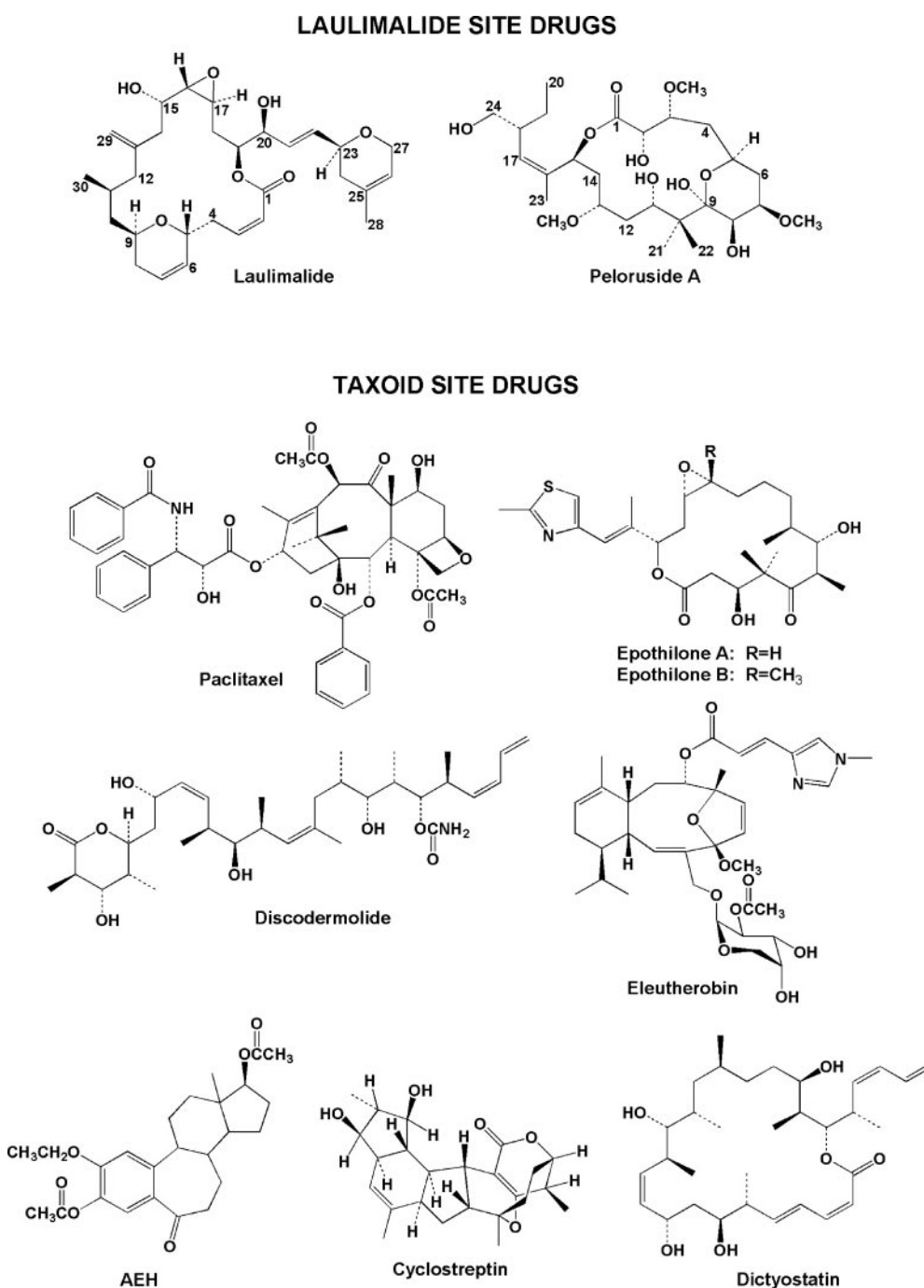
consensus alignment to the target strategies (for the model of laulimalide), with either rigid or flexible fit (for the model of peloruside A).

## Results

**Comparison of Peloruside A with Other Compounds As Inducers of Tubulin Assembly Dependent on MAPs and GTP.** Figure 2A summarizes assembly studies in which 10  $\mu$ M drug was combined with 10  $\mu$ M tubulin in the presence of MAPs and GTP in 0.1 M MES. Because extensive assembly occurs with some drugs at 0°C, as shown previously (Ter Haar et al., 1996; Kowalski et al., 1997a; Madiraju et al., 2005), the assembly studies presented in Fig. 2 were performed with a step-wise increase in temperature and with a

return of the samples to 0°C at the end of the experiments to evaluate the 0°C stability of the polymers formed.

As an example of an extensive 0°C assembly reaction, we show the effect of 10  $\mu$ M discodermolide (curve D) with assembly already underway at the earliest point we could measure. Assembly reactions induced by paclitaxel (curve T) and laulimalide (curve L) were similar, with extensive assembly occurring primarily at 10 and 20°C with both compounds (Pryor et al., 2002; Gapud et al., 2004). A significant reaction with 10  $\mu$ M peloruside A (curve P) did not occur until the temperature reached 20°C. This was comparable with what occurs with the coral-derived natural products sarcodictyins A and B (Hamel et al., 1999), and we therefore included

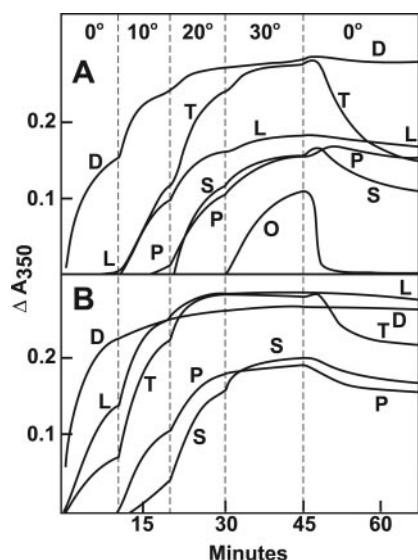




sarcodictyin A (curve S) here for comparison. Significant assembly in the absence of drug only occurred at 30°C (curve O). All polymers, except that formed without drug, persisted after return of the reaction temperature to 0°C. Thus, the drugs all induce formation of polymers that persist at 0°C.

Figure 2B summarizes analogous experiments performed with the drugs at 40  $\mu\text{M}$ , with all other reaction conditions unchanged. Assembly was already well underway with discodermolide at the earliest measurable time point and was almost complete after 10 min at 0°C. After brief lag periods, assembly began at 0°C with both laulimalide and paclitaxel, with the reaction with laulimalide more rapid and extensive than that with paclitaxel. With both drugs, assembly was almost complete during the 10°C incubation period. With 40  $\mu\text{M}$  peloruside A and sarcodictyin A, assembly began at 10°C, and the peloruside A-induced reaction was more extensive than that which occurred with sarcodictyin A. With both compounds, assembly was essentially complete during the 20°C incubation. Thus, the effect of increasing drug concentration 4-fold was to shift the temperature effects on assembly to a 10°C lower temperature. In addition, the assembly pattern with 40  $\mu\text{M}$  peloruside A was similar to that obtained with 10  $\mu\text{M}$  laulimalide, suggesting that the affinity of laulimalide is approximately 4-fold greater than that of peloruside A for the binding site.

When we omitted MAPs or GTP from the reaction mixture, conditions under which no assembly occurs with 10  $\mu\text{M}$  tubulin, we also found little or no assembly when the reaction mixture was supplemented with 10  $\mu\text{M}$  peloruside A (data not presented). This differs from the significant assembly that occurs with, for example, 10  $\mu\text{M}$  laulimalide or pacli-

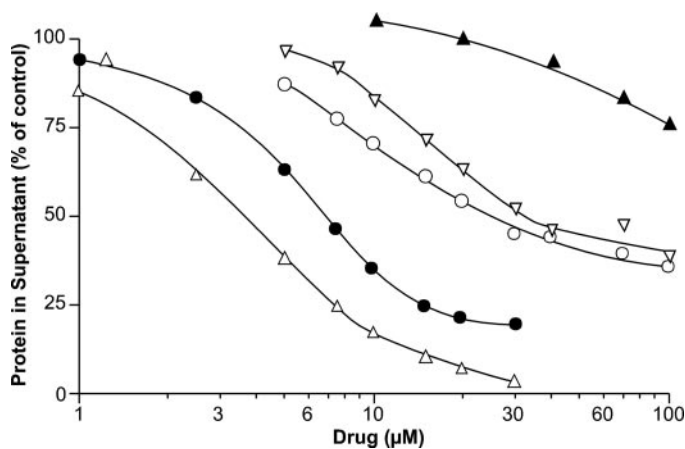


**Fig. 2.** Drug and temperature effects on tubulin assembly dependent on both MAPs and GTP. Each 0.25-ml (final volume) reaction mixture contained 10  $\mu\text{M}$  (1.0 mg/ml) tubulin, 0.75 mg/ml heat-treated MAPs, 100  $\mu\text{M}$  GTP, 0.1 M MES, 4% dimethyl sulfoxide as drug solvent, and drugs at either 10  $\mu\text{M}$  (A) or 40  $\mu\text{M}$  (B). Baselines were established without drug in the reaction mixture, and drugs were rapidly mixed into the mixtures (10–15 s). Turbidity was followed at 0°C as soon as possible after drug addition. Significant increase in turbidity had already occurred with discodermolide at the earliest time points that could be measured. Temperature changes were made at the times indicated by the dashed lines to the left of the temperatures. Drugs indicated as follows in both panels: curve D, discodermolide; curve L, laulimalide; curve T, paclitaxel (for “Taxol”); curve P, peloruside A; curve S, sarcodictyin A. In A only, curve O indicates no drug.

taxel in the MAP-only or GTP-only systems (Gapud et al., 2004). This is consistent with the higher critical concentration reported for assembly with peloruside A compared with paclitaxel (Gaitanos et al., 2004).

**Quantitative Comparison of Effects of Peloruside A with Those of Laulimalide and Paclitaxel on Assembly in 1.0 M Glutamate.** To obtain an idea of the relative affinities of peloruside A, laulimalide, and paclitaxel for tubulin polymer, we examined the three compounds in a quantitative pelleting assay (Lin et al., 1996). In this assay, each examined compound yields an  $\text{EC}_{50}$ , defined as the compound concentration that results in removal by centrifugation of 50% of the tubulin in the reaction mixture. The specific  $\text{EC}_{50}$  for a compound depends on the concentration of monosodium glutamate used in the experiment, with the  $\text{EC}_{50}$  varying inversely with the glutamate concentration. Because reaction mixtures contain no GTP, there is negligible pellet formation in the absence of assembly-inducing drug. Because peloruside A was relatively inactive in the previous studies, we compared the compounds with a relatively high concentration of glutamate (1.0 M) and obtained the results shown in Fig. 3. Combining these data with those obtained from a second experiment, we obtained an  $\text{EC}_{50}$  of  $4.3 \pm 1 \mu\text{M}$  (SD) for paclitaxel,  $6.7 \pm 0.5 \mu\text{M}$  for laulimalide, and  $24 \pm 0.1 \mu\text{M}$  for peloruside A. Thus, in this assay peloruside A was 3.6-fold less active than laulimalide and 5.6-fold less active than paclitaxel.

In a previous study (Pryor et al., 2002), *trans*-desoxylaulimalide (epoxide replaced by a *trans*-olefin bond) had activity in the MAPs/GTP assembly system similar to that described above for peloruside A. We therefore examined in the glutamate system (Fig. 3) this compound together with bis-*trans*-desoxylaulimalide (both the epoxide and the C-2/C-3 *cis*-olefin bond replaced by *trans*-olefin bonds). Although the latter had little activity in the glutamate system, the *trans*-desoxylaulimalide yielded an  $\text{EC}_{50}$  of  $31 \pm 2 \mu\text{M}$ . This analog



**Fig. 3.** Effects of drug concentrations on tubulin assembly at 22°C in 1.0 M glutamate. Each 0.1-ml reaction mixture contained 10  $\mu\text{M}$  tubulin, 1.0 M monosodium glutamate (pH adjusted to 6.6 with HCl in 2.0 M stock solution), 4% dimethyl sulfoxide, and drugs as indicated. Reaction mixtures were incubated for 15 min at 22°C and centrifuged for 10 min at room temperature at 14,000 rpm in an Eppendorf 5417C desktop centrifuge, with adaptors for 0.5-ml Eppendorf tubes. Protein concentration in supernatant aliquots was determined by the Lowry procedure, and drug-containing mixtures were compared with reaction mixtures without drug.  $\circ$ , peloruside A;  $\bullet$ , laulimalide;  $\Delta$ , paclitaxel;  $\nabla$ , *trans*-desoxylaulimalide;  $\blacktriangle$ , bis-*trans*-desoxylaulimalide

was thus 4.6-fold less active than laulimalide and approximately 30% less active than peloruside A in this assay.

**Comparison of Effects of Peloruside A and Laulimalide on MCF-7 Cell Growth.** Laulimalide has been examined in multiple human cancer cell lines by many workers, and it has yielded  $IC_{50}$  values in the 2 to 11 nM range (Mooberry et al., 1999; Pryor et al., 2002; Ahmed et al., 2003; Gallagher et al., 2004; Paterson et al., 2005; Wender et al., 2006). The  $IC_{50}$  values of peloruside A in human cancer cell lines range from 6 to 66 nM (Hood et al., 2001, 2002; Gaitanos et al., 2004), and thus it may be less active than laulimalide. As far as we know, the two compounds were never directly compared. We therefore examined them together, along with paclitaxel, *trans*-desoxylaulimalide, and *bis-trans*-desoxylaulimalide, in MCF-7 breast cancer cells. The  $IC_{50}$  values we obtained were  $10 \pm 0.7$  nM (SD) for laulimalide,  $20 \pm 5$  nM for peloruside A,  $4 \pm 0.7$  nM for paclitaxel,  $500 \pm 200$  nM for *trans*-desoxylaulimalide, and  $>5$   $\mu$ M for *bis-trans*-desoxylaulimalide. Thus, in MCF-7 cells, peloruside A was half as active as laulimalide and 5-fold less active than paclitaxel. However, despite the quantitative similarity between peloruside A and *trans*-desoxylaulimalide in both tubulin assembly assays (see above), the former was 25-fold more active than the latter in the cell-based assay. Moreover, in HL-60 myeloid leukemia cells peloruside A is 3-fold more active than paclitaxel (Hood et al., 2002).

**Drug Synergy Studies at 12°C.** An implication of peloruside A and laulimalide binding to tubulin polymer at a site distinct from the taxoid site is that assembly synergism might occur in the presence of two drugs that bind in different sites but not of two drugs that bind in the same site. In previous work, we examined laulimalide, paclitaxel, and epothilone A in detail, with preliminary examinations of other drugs. We selected a reaction condition in which single drugs caused negligible assembly, and impressive synergy occurred with the laulimalide-paclitaxel combination. The synergy could be measured either by turbidimetry or by separation of polymer and unpolymerized tubulin by centrifugation (Gapud et al., 2004).

When the reaction condition used (10  $\mu$ M tubulin in 0.1 M MES at 10°C) was examined in further detail, we found that it could not be applied without modification with all taxoid site compounds. For example, assembly with either discodermolide or dictyostatin alone was too extensive, whereas the peloruside A-paclitaxel combination showed little activity. However, we found that we could adequately demonstrate synergy between peloruside A or laulimalide, and all taxoid site chemotypes by varying reaction temperature and tubulin concentration. In no case was synergy observed between peloruside A and laulimalide or between any pair of taxoid site drugs.

The first condition we examined in detail varied only slightly from the reaction condition used previously (Gapud et al., 2004). By raising the reaction temperature to 12°C and the tubulin concentration to 15  $\mu$ M, synergy between peloruside A and epothilone A, paclitaxel, or eleutherobin was readily observed, as shown in Table 1. When any of the four drugs was present at 20  $\mu$ M, no more than 2% assembly occurred. When 20  $\mu$ M peloruside A was combined with any of the other three drugs at 20  $\mu$ M, 40 to 50% assembly occurred. With paclitaxel, epothilone A, or eleutherobin at 40  $\mu$ M, only 10 to 12% assembly occurred, and the 20  $\mu$ M combinations of these drugs (e.g., 20  $\mu$ M paclitaxel + 20  $\mu$ M epothilone A) all gave assembly reactions in the same range (6–14%). Laulimalide, too, had a negligible effect at 20  $\mu$ M and induced 7% assembly at 40  $\mu$ M. Potent synergy was observed when 20  $\mu$ M laulimalide was combined with 20  $\mu$ M paclitaxel, epothilone A, or eleutherobin (72–75% assembly), but the combination of laulimalide and peloruside A (each at 20  $\mu$ M) failed to induce significant assembly.

We also examined a few of the combinations of Table 1 across a wide concentration range, as shown in Fig. 4. Single drugs were examined at concentrations up to 100  $\mu$ M, whereas combinations were examined at up to 50  $\mu$ M each, for a total maximum drug concentration of 100  $\mu$ M. Because we had previously examined a concentration range for laulimalide combined with paclitaxel (Gapud et al., 2004), for the current study epothilone A was combined with laulimalide

TABLE 1  
Effects of taxoid site and laulimalide site drugs on tubulin assembly at 12°C

The 0.1-ml reaction mixtures contained one or two drugs, as indicated, 15  $\mu$ M tubulin, 0.1 M MES, and 4% dimethyl sulfoxide. Incubation was for 1 h at 12°C, followed by centrifugation for 10 min at 30,000 rpm at 12°C. Incubation was in the prechilled rotor in the centrifuge. Protein content of 12  $\mu$ l of each supernatant and of an uncentrifuged reaction mixture was determined. All data were corrected for the amount of protein removed by centrifugation of reaction mixtures containing 4% dimethyl sulfoxide but no drug. The amount of protein removed by centrifugation without drug averaged  $4.6 \pm 2\%$  in the experiments used to generate this table. Each experimental data point represents the average  $\pm$  S.D. from three to five independent assays. The values shown without brackets when a single drug was present in the assay mixture were obtained with the drug at 40  $\mu$ M. When drug mixtures were used, each drug was present at 20  $\mu$ M. The values shown with brackets when a single drug was present in the assay mixture were obtained with the drug at 20  $\mu$ M.

	PLA	LMD	PTX	EPA	ELR	CS	AEH
				% protein in pellet			
PLA	$3 \pm 2$ [2 $\pm$ 1]	$5 \pm 2$	$41 \pm 7$	$49 \pm 5$	$47 \pm 7$	$2 \pm 1$	$0.7 \pm 0.6$
LMD		$7 \pm 0.4$ [2 $\pm$ 2]	$73 \pm 0.7$	$75 \pm 4$	$72 \pm 3$	$17 \pm 2$	$29 \pm 3$
PTX			$12 \pm 5$ [2 $\pm$ 2]	$14 \pm 4$	$12 \pm 5$	$9 \pm 8$	$0.2 \pm 0.4$
EPA				$12 \pm 4$ [2 $\pm$ 4]	$6 \pm 4$	$4 \pm 2$	$0.6 \pm 1$
ELR					$10 \pm 5$ [0.3 $\pm$ 0.5]	$2 \pm 2$	$4 \pm 5$
CS						$1 \pm 2$ [0.9 $\pm$ 2]	$0.5 \pm 0.9$
AEH							$0.2 \pm 0.2$ [0.9 $\pm$ 0.9]

PLA, peloruside A; LMD, laulimalide; PTX, paclitaxel; EPA, epothilone A; ELR, eleutherobin; CS, cyclostreptin.

and with peloruside A, as well as with paclitaxel. The results presented in Fig. 4 are in accord with those of Table 1, with only the epothilone A-laulimalide and epothilone A-peloruside A combinations showing extensive assembly, with the former combination more active than the latter. With both peloruside A + epothilone A and laulimalide + epothilone A, near maximal assembly occurred when both drugs in a combination were present at 20  $\mu\text{M}$  (40  $\mu\text{M}$  total drug).

A few of the conditions summarized in Table 1 were evaluated by turbidimetry (Fig. 5), and essentially identical results were obtained. In this experiment, drugs were mixed into the reaction mixtures at 0°C, with no change in absorbance noted, and the temperature was then quickly raised to 12°C. Of the conditions examined, the most extensive reaction was observed with the combination of 20  $\mu\text{M}$  laulimalide with 20  $\mu\text{M}$  epothilone A (curve 8), followed by 20  $\mu\text{M}$  peloruside A and 20  $\mu\text{M}$  epothilone A (curve 7). In both these reaction mixtures, the increase in turbidity began as soon as the temperature was increased. A much slower reaction was observed with the combination of 20  $\mu\text{M}$  paclitaxel and 20  $\mu\text{M}$  epothilone A (curve 6); this is analogous to the results shown in Table 1, where a 6-fold increase in the reaction occurred when the concentration of either paclitaxel or epothilone A was increased from 20 to 40  $\mu\text{M}$ , and a similar increase occurred with both drugs at 20  $\mu\text{M}$ . There was little turbidity change when laulimalide and peloruside were both added to the tubulin (curve 5), in agreement with the centrifugation data (Table 1).

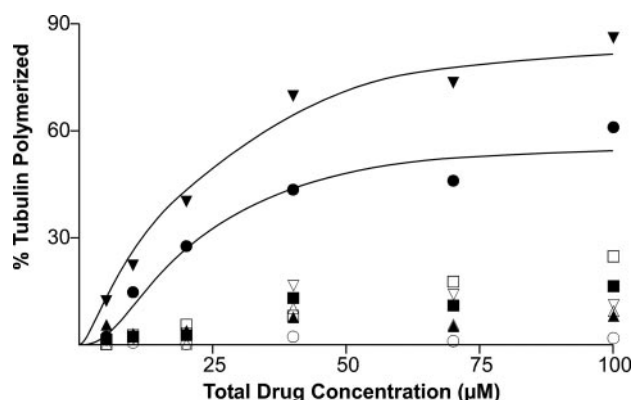
**Drug Synergy Studies at 0°C.** We next examined by centrifugation the assembly effects of peloruside A and laulimalide at 0°C with discodermolide, dictyostatin, and epothilone B (Table 2). The chief problem with demonstrating synergy by centrifugation at a single time point under this reaction condition was that a significant reaction (20% assembly) occurred with 20  $\mu\text{M}$  discodermolide and, to a lesser extent, with dictyostatin (8%). There was only minimal en-

hancement when the concentration of either compound was increased to 40  $\mu\text{M}$ . The other compounds, including epothilone B, examined in the series of experiments summarized in Table 2, had negligible effects on assembly at 0°C, as measured in the centrifugation assay.

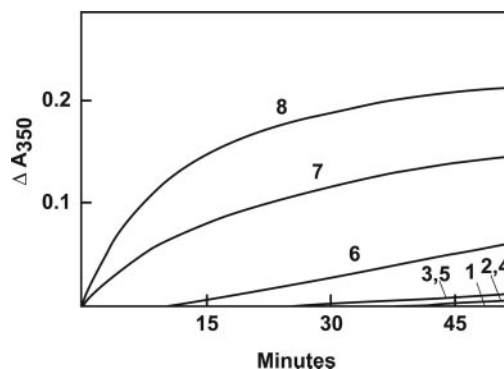
Combining discodermolide, dictyostatin, or epothilone B with each other or with the other taxoid site drugs examined produced no evidence of synergy. Assembly with all three drugs was enhanced by addition of peloruside A or, especially, laulimalide. Thus, the 20 to 25% assembly with discodermolide was increased to 43% with peloruside A and 62% with laulimalide addition to the reaction mixture. Likewise, the 8 to 13% assembly with dictyostatin and the 0 to 3% assembly with epothilone B was increased to 24% with addition of peloruside A to the reaction mixture and to 44 to 47% with addition of laulimalide. As at 12°C, there was no synergistic effect when peloruside A and laulimalide were added together to the reaction mixture.

More convincing evidence of synergy between laulimalide or peloruside A and discodermolide at 0°C was obtained in a turbidimetry study (Fig. 6). The reaction observed with 20  $\mu\text{M}$  discodermolide (curve 4) was not enhanced by 20  $\mu\text{M}$  paclitaxel (curve 6). In contrast, there was dramatic stimulation of the discodermolide reaction, particularly for the first 30 min, by either 20  $\mu\text{M}$  peloruside A (curve 7) or 20  $\mu\text{M}$  laulimalide (curve 8). There was little or no change in turbidity with 20  $\mu\text{M}$  peloruside A (curve 1), 20  $\mu\text{M}$  laulimalide (curve 2), 20  $\mu\text{M}$  paclitaxel (curve 3), or with the combination of 20  $\mu\text{M}$  peloruside A and 20  $\mu\text{M}$  laulimalide (curve 5).

**Drug Synergy Studies at Higher Temperatures.** With two of the taxoid site chemotypes, AEH and cyclostreptin, we could only demonstrate synergistic effects on assembly with peloruside A or laulimalide when the temperature and tubulin concentration were increased still further. In the 12°C study presented in Table 1, there was borderline synergy between each of these compounds and laulimalide, but not peloruside A. Synergism of laulimalide, but not of peloruside A, with AEH and cyclostreptin was enhanced at 14°C with 20  $\mu\text{M}$  tubulin (90 min incubation). Single drugs at 20  $\mu\text{M}$  led to 1 to 7% assembly, whereas 28% and 42% assembly occurred



**Fig. 4.** Effects of drug combinations on tubulin assembly at 12°C as a function of drug concentration. Each 0.1-ml reaction mixture contained one or two drugs, as described below, at varying concentrations, 15  $\mu\text{M}$  tubulin, 0.1 M MES, and 4% dimethyl sulfoxide. When reaction mixtures contained two drugs, they were present at equimolar concentrations. Incubation was for 1 h at 12°C (in the centrifuge in the rotor, which had been prechilled to 12°C), followed by centrifugation for 10 min at 30,000 rpm at 12°C. Protein content of 12  $\mu\text{l}$  of each supernatant and of an uncentrifuged reaction mixture was determined. All data are corrected for the amount of protein removed by centrifugation of reaction mixtures containing 4% dimethyl sulfoxide but no drug (average 4.6%). ●, peloruside A + epothilone A; ▼, laulimalide + epothilone A; ▲, laulimalide + peloruside A; ■, epothilone A + paclitaxel; ○, peloruside A; □, laulimalide; △, epothilone A; ▽, paclitaxel.



**Fig. 5.** Drug effects on tubulin assembly at 12°C. Each 0.25-ml (final volume) reaction mixture contained 15  $\mu\text{M}$  tubulin, 0.1 M MES, 4% dimethyl sulfoxide, and drugs as follows: curve 1, 20  $\mu\text{M}$  peloruside A; curve 2, 20  $\mu\text{M}$  laulimalide; curve 3, 20  $\mu\text{M}$  paclitaxel; curve 4, 20  $\mu\text{M}$  epothilone A; curve 5, 20  $\mu\text{M}$  peloruside A + 20  $\mu\text{M}$  laulimalide; curve 6, 20  $\mu\text{M}$  paclitaxel + 20  $\mu\text{M}$  epothilone A; curve 7, 20  $\mu\text{M}$  peloruside A + 20  $\mu\text{M}$  epothilone A; curve 8, 20  $\mu\text{M}$  laulimalide + 20  $\mu\text{M}$  epothilone A. Baselines were established in the spectrophotometers at 0°C before drug addition, drugs were added to the reaction mixtures, and the temperature was jumped to 12°C at zero time.



when 20  $\mu$ M laulimalide was combined with 20  $\mu$ M cyclostreptin or AEH, respectively. A further increase in temperature to 25°C (2-h incubation) provided evidence for synergism between peloruside A and either cyclostreptin or AEH. These drugs singly at 20  $\mu$ M led to only 4 to 5% assembly, whereas 43 and 38% assembly occurred when 20  $\mu$ M peloruside A was combined with 20  $\mu$ M cyclostreptin or AEH, respectively. As before, no evidence of synergism was found with any combination of taxoid site compounds examined or with laulimalide and peloruside A in combination.

**Effect of a Drug Combination on Tubulin Critical Concentration.** The observation that altering the tubulin concentration could have a significant effect on the extent of synergy observed with different combinations of agents suggested that one result of combining a taxoid and laulimalide site drug might be to lower the critical tubulin concentration required for assembly. To examine this possibility, we selected two of the less active compounds, peloruside A and cyclostreptin, for evaluation.

Figure 7 summarizes experiments performed with 20 and 40  $\mu$ M concentrations of each compound and the combination at 20  $\mu$ M each. Without drug, a small amount of protein sedimented, and pellet size was a linear function of the protein concentration in the reaction mixture. Because no polymer is in these reaction mixtures without drug, as determined by electron microscopy, we conclude that this background pellet is caused by inactive protein aggregates. We take the critical concentration in the presence of an assembly-inducing compound to be the point at which the drug-containing samples deviate from the background curve. The data obtained with either 20  $\mu$ M peloruside A or 20  $\mu$ M cyclostreptin yielded critical concentrations of 0.74 mg/ml, whereas the combination of the two compounds at 20  $\mu$ M each had a critical concentration of 0.24 mg/ml. With 40  $\mu$ M

peloruside A or 40  $\mu$ M cyclostreptin, we obtained intermediate critical concentrations of 0.63 and 0.47 mg/ml, respectively. Thus, combining the two drugs in equimolar amounts resulted in a lower tubulin critical concentration than was obtained by doubling the concentration of either compound.

**Attempt at Pharmacophore Construction.** Molecular models were built from the crystal structure of laulimalide (Jefford et al., 1996) and a priori for peloruside A with extensive searches for low energy conformers, which were matched to available conformational information provided by nuclear magnetic resonance data (West et al., 2000). The two compounds are both macrocyclic lactones containing 2,6-substituted di- or tetrahydropyran rings, and both compounds possess moderately flexible side chains of similar length. However, the peloruside A macrocycle contains four fewer atoms than that of laulimalide. Moreover, the macrocycle of peloruside A is more heavily substituted than that of laulimalide, particularly with polar groups.

Many superimpositions were performed, including overlaying the tetrahydropyran ring of peloruside A on both the side chain and macrocycle dihydropyran rings of laulimalide. No superimposition attempted resulted in substantial overlap of the macrocycles. However, as shown in Fig. 8, the side chains could be readily superimposed, with matching atoms all within 1.15 Å, and this model shows the greatest superimposition observed. Matching atoms in the side chains included the branch points in the macrocycles (C-15 in peloruside A and C-19 in laulimalide). The overlapping of the side chains included the apparent correspondence between the C-20 hydroxyl oxygen atom of laulimalide and C-23 of peloruside A (1.01 Å separation in the model), and, in addition, C-20 of peloruside A occupied space close to two atoms, C-24 and C-25, in the laulimalide side chain. The only side chain atoms without matches were C-26, C-27, and C-28 of laulimalide.

TABLE 2

Effects of taxoid site and laulimalide site drugs on tubulin assembly at 0°C

The 0.1-ml reaction mixtures contained one or two drugs, as indicated, 10  $\mu$ M tubulin, 0.1 M MES, and 4% dimethyl sulfoxide. Incubation was for 90 min at 0°C, followed by centrifugation for 10 min at 30,000 rpm at 2°C (lowest temperature setting on the centrifuge). Incubation was on ice, and all mixing operations were performed in a 4°C cold room. Protein content of 20  $\mu$ l of each supernatant and of an uncentrifuged reaction mixture was determined. All data were corrected for the amount of protein removed by centrifugation of reaction mixtures containing 4% dimethyl sulfoxide but no drug. The amount of protein removed by centrifugation without drug averaged  $5.7 \pm 2\%$  in the experiments used to generate this table. Each experimental data point represents the average  $\pm$  S.D. from three independent assays, except that only two data points were obtained for each assay condition containing laulimalide. The values shown without brackets when a single drug was present in the assay mixture were obtained with the drug at 20  $\mu$ M. When drug mixtures were used, each drug was present at 10  $\mu$ M. The values shown with brackets when a single drug was present in the assay mixture were obtained with the drug at 10  $\mu$ M.

	PLA	LMD	DCD	DCT	EPB	PTX	ELR	CS	AEH
	% protein in pellet								
PLA	$1 \pm 0.7$ [0.7 $\pm$ 1]	$2 \pm 2$	$43 \pm 3$	$24 \pm 1$	$24 \pm 1$	0	N.D.	N.D.	N.D.
LMD		$2 \pm 2$ [2 $\pm$ 0.8]	$62 \pm 3$	$47 \pm 6$	$44 \pm 5$	N.D.	N.D.	N.D.	N.D.
DCD			$25 \pm 3$ [20 $\pm$ 1]	$23 \pm 2$	$23 \pm 1$	$19 \pm 2$	$21 \pm 4$	$23 \pm 2$	$22 \pm 2$
DCT				$13 \pm 3$ [8 $\pm$ 2]	$12 \pm 1$	$8 \pm 4$	$9 \pm 0.7$	$9 \pm 2$	$9 \pm 0.4$
EPB					$3 \pm 3$ [0] <sup>a</sup>	$0.2 \pm 0.3$	$0.9 \pm 2$	$1 \pm 1$	$0.1 \pm 0.2$
PTX						$0.9 \pm 0.9$ [0.4 $\pm$ 0.7]	N.D.	N.D.	N.D.
ELR							$1 \pm 2$ [0.6 $\pm$ 1]	N.D.	N.D.
CS								$0.5 \pm 0.6$ [0.7 $\pm$ 1]	N.D.
AEH									$0.3 \pm 0.6$ [0.5 $\pm$ 0.9]

N.D., not determined; PLA, peloruside A; LMD, laulimalide; PTX, paclitaxel; ELR, eleutherobin; CS, cyclostreptin; DCD, discodermolide; DCT, dictyostatin; EPB, epothilone B.

<sup>a</sup> Same value obtained in all experiments.

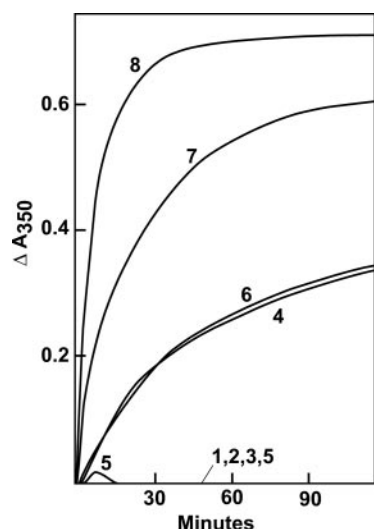
In the Fig. 8 model, the carboxylic ester regions of the macrocyclic portions of the molecules adopt different orientations and could be superimposed only partially. Likewise, because of the different-sized macrocycles, the di/tetrahydropyran units in the macrocycles occupy different spatial regions in the model. Nevertheless, the C-5/C-9 dihydropyran oxygen atom of laulimalide occupied a similar spatial area (2.34 Å separation) as the peloruside A methoxyl oxygen at

C-3. The C-5/C-9 tetrahydropyran oxygen atom of peloruside A was close to two atoms of laulimalide: 1.40-Å separation from C-14 and 2.05-Å separation from the oxygen atom of the C-15 hydroxyl group.

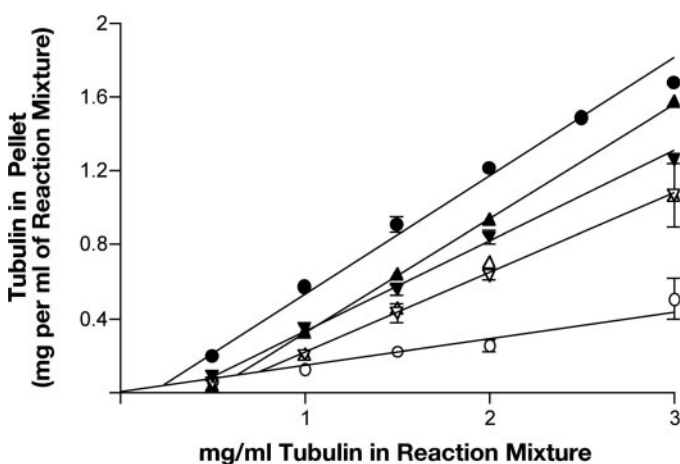
There are several other corresponding atoms in the maximal overlap model: 1) the methoxyl carbon atom at C-7 in the tetrahydropyran ring of peloruside A with the carbon atom of the C-30 methyl group of laulimalide (2.45 Å separation); 2) the C-22 methyl group of peloruside A with C-29, the exocyclic alkenyl methylene moiety of laulimalide (2.16 Å separation); 3) C-12 of peloruside A with the epoxide oxygen atom of laulimalide (<0.25 Å separation); 4) C-11 of peloruside A with C-16 of laulimalide (1.45 Å separation; the latter atom is bonded to the epoxide oxygen); and 5) C-13 of peloruside A with C-17 of laulimalide (<0.25 Å separation; the latter atom is also bonded to the epoxide oxygen).

## Discussion

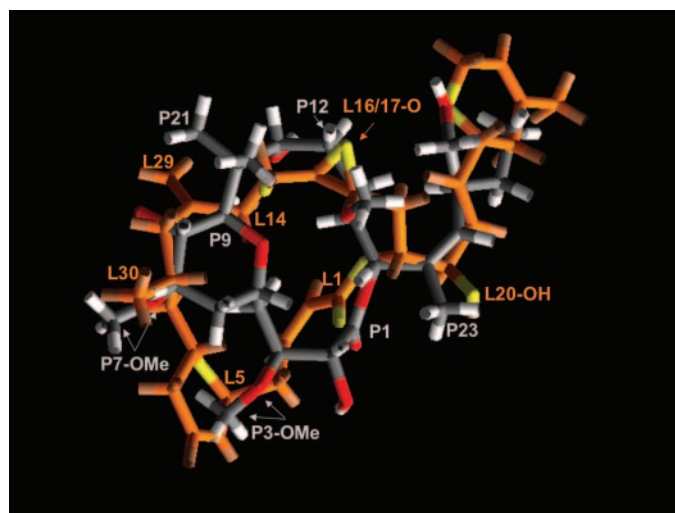
Our findings here confirm the proposal that peloruside A and laulimalide bind in the same site on tubulin polymer (Gaitanos et al., 2004), a site distinct from the taxoid site (Pryor et al., 2002). We found no evidence for any synergistic effects on tubulin assembly when either peloruside A and laulimalide were combined or with multiple combinations of taxoid site drugs (compounds examined: discodermolide, dictyostatin, paclitaxel, epothilones A and B, eleutherobin, the steroid derivative AEH, and cyclostreptin). In contrast, we were able to show by centrifugal analysis and turbidimetry that both laulimalide and peloruside A synergistically enhanced assembly induced by all taxoid site chemotypes examined. This synergy probably derives, at least in part, from the active drug combinations having a lower critical concentration for tubulin than the individual drugs. This was specifically shown for the peloruside A/cyclostreptin combination, where the individual drugs yielded critical concentrations of 0.74 mg/ml (7.4 μM) tubulin and the combination, 0.24 mg/ml (2.4 μM). Definitive demonstration that laulimalide and peloruside A do bind at



**Fig. 6.** Drug effects on tubulin assembly at 0°C. Each 0.25-ml (final volume) reaction mixture contained 20 μM tubulin, 0.1 M MES, 4% dimethyl sulfoxide, and drugs as follows: curve 1, 20 μM peloruside A; curve 2, 20 μM laulimalide; curve 3, 20 μM paclitaxel; curve 4, 20 μM discodermolide; curve 5, 20 μM peloruside A + 20 μM laulimalide; curve 6, 20 μM paclitaxel + 20 μM discodermolide; curve 7, 20 μM peloruside A + 20 μM discodermolide; curve 8, 20 μM laulimalide + 20 μM discodermolide. Baselines were established in the spectrophotometers at 0°C before drug addition, drugs were added to the reaction mixtures, and turbidity development at 0°C was followed.



**Fig. 7.** Determination of critical tubulin concentrations in assembly induced by peloruside A or cyclostreptin, alone or in combination. Each 0.1-ml reaction mixture contained 0.1 M MES, pH 6.9, 4% dimethyl sulfoxide, tubulin as indicated, and drugs as indicated by the symbols below. Tubulin was the last component added to the reaction mixtures in Beckman polyallomer 7 × 20 mm tubes. Incubation was for 2 h at 25°C, followed by centrifugation at 30,000 rpm for 10 min at 25°C in a TLA 100 rotor in an Optima TLX minilultracentrifuge. An aliquot of each reaction mixture was taken from the top of the supernatant, and protein content was determined by the Lowry procedure. ○, none (average protein in pellet, 14 ± 2% of total in reaction mixture); ●, 20 μM peloruside A + 20 μM cyclostreptin; △, 20 μM peloruside A; ▲, 40 μM peloruside A; ▼, 20 μM cyclostreptin; ▽, 40 μM cyclostreptin.



**Fig. 8.** Superimposition model of an energy-minimized conformer of peloruside A and the crystal structure of laulimalide. Laulimalide carbon atoms are shown in orange, oxygen in yellow, and hydrogen in beige. Peloruside A carbon atoms are shown in gray, oxygen in red, hydrogen in white. Laulimalide position numbers are in orange, preceded by "L". Peloruside position numbers are in white, preceded by "P". See Fig. 1 for details of position numbers.



the same site on tubulin polymer, however, requires that at least one of these compounds be available in a radiolabeled form or that an active fluorescent analog be prepared.

We also wanted to determine the relative activity of peloruside A in direct comparison with laulimalide. Not surprisingly, in view of the drug-dependent tubulin critical concentrations in the literature (Gaitanos et al., 2004; Gapud et al., 2004; Paterson et al., 2005), we found that peloruside A was significantly less potent than laulimalide. In terms of relative activities, laulimalide A, as was noted previously (Pryor et al., 2002), has activity similar to paclitaxel, epothilone A, and eleutherobin, whereas the activity of peloruside A, as shown here, is most similar to that of sarcodictyins A and B and not very different from the less active laulimalide analog *trans*-desoxylaulimalide. Reaction components required for peloruside A-induced assembly were thus significantly more stringent than those required for laulimalide-induced assembly in terms of temperature and the presence of MAPs and GTP as reaction components.

We attempted to construct a laulimalide-peloruside A pharmacophore to gain insight into important features of their molecular structures and to understand better why laulimalide has thus far proved resistant to structural modifications that do not cause loss of biological activity. In general, in analog studies published to date, introduction of a single modification into laulimalide has resulted in moderate to severe reduction in cytotoxic activity, whereas introduction of more than one modification generally has yielded almost inert compounds (Pryor et al., 2002; Ahmed et al., 2003; Wender et al., 2003, 2006; Gallagher et al., 2004, 2005; Mooberry et al., 2004; Paterson et al., 2005). Important findings thus far: 1) if the epoxide moiety of laulimalide is replaced with a *trans*-olefin bond, activity is reduced 23- to 51-fold against MCF-7 (Pryor et al., 2002; Ahmed et al., 2003; see above), 11-fold against MaTu (Ahmed et al., 2003), and 19- to 21-fold against MDA-MB-435 breast cancer cells (Wender et al., 2003; Mooberry et al., 2004) [a larger loss of activity against MDA-MB-435 cells was reported by Gallagher et al. (2004)]; 2) if the C-2/C-3 olefin bond is changed from *cis* to *trans*, activity is reduced 10- to 14-fold against MaTu and MCF-7 cells, respectively (Ahmed et al., 2003) [again, a larger loss of activity against MDA-MB-435 cells was reported by Gallagher et al. (2004)]; 3) if the C-30 methyl substituent at C-11 is removed, activity is reduced 9-fold against MDA-MB-435 cells (Wender et al., 2006) and 15-fold against A2780 ovarian cancer cells (Paterson et al., 2005); 4) if the hydroxyl at C-20 is methylated or acetylated, activity is reduced 40- to 42-fold against MDA-MB-435 cells (Wender et al., 2003; Gallagher et al., 2004; Mooberry et al., 2004); 5) if the hydroxyl at C-15 is acetylated, activity is reduced 10-fold against MDA-MB-435 cells, with larger reductions with other ester groups (Gallagher et al., 2004); 6) if the C-15 hydroxyl group is methylated, activity is reduced over 400-fold against MDA-MB-435 cells (Gallagher et al., 2004); 7) if an alkyne bond is introduced between C-2 and C-3, activity is reduced over 400-fold against MDA-MB-435 cells (Paterson et al., 2005); 8) possibly, if the side chain is eliminated or modified, activity is substantially reduced (Paterson et al., 2005); and 9) possibly, if configuration is reversed at C-15, activity is substantially reduced (Gallagher et al., 2004). Analogs that lack the C-30 methyl group and possess modified side chains have activities reduced by more than 2500-fold compared

with laulimalide against A2780 cells (Paterson et al., 2005). Likewise, analogs with reversal of configuration at C-15 and changes in the epoxide moiety have activities reduced over 75-fold compared with laulimalide against MDA-MB-435 cells (Gallagher et al., 2004).

There are few published structure-activity data for peloruside A. Near quantitative disruption of the tetrahydropyran ring is readily achieved (Hood et al., 2001), leaving the macrocycle intact. This change causes a 26- to 32-fold reduction in cytotoxic activity in two cell lines relative to peloruside A (Hood et al., 2001, 2002).

Assuming that atoms of laulimalide that superimpose on atoms of peloruside A in our model are important for binding to tubulin polymer, our model is consistent with the structure-activity findings that demonstrate the importance of the laulimalide side chain, the epoxide moiety, the C-30 methyl group, and the C-15 hydroxyl group for full activity. Our model also predicts that modification of the C-29 substituent of laulimalide would be deleterious. Furthermore, the model is consistent with the observed activity loss after disruption of the tetrahydropyran ring of peloruside A, but the model does suggest that at least some of the multiple hydroxyl groups of peloruside A might be alkylated without reducing its activity. If so, this would be highly useful in the synthesis of radiolabeled and/or reactive analogs that would permit exploration of the mechanism of binding to tubulin.

Unlike some other chemotypes that interfere with tubulin assembly, including a series of C-2-substituted paclitaxel analogs (Kingston et al., 1998), there is poor correlation thus far between assembly effects and cellular effects of laulimalide site compounds. This seems to be the case whether the net effect on assembly is measured, as shown here (Fig. 3 data versus MCF-7 cytotoxicity data), or whether drug effects on the tubulin critical concentration are quantitated (Paterson et al., 2005). These studies show that very small reductions in apparent affinity for the presumptive binding site on tubulin and/or microtubules are associated with major loss of cytotoxicity with a number of laulimalide analogs. Interestingly, however, the relative activities of the two natural products in the tubulin assays we used are reflected more closely in relative effects on MCF-7 cell proliferation. Thus, both with MAPs + GTP in 0.1 M MES (Fig. 2) and in 1.0 M glutamate without GTP (Fig. 3), peloruside A was 3- to 4-fold less active than laulimalide, and it is half as effective as an inhibitor of growth of the breast cancer cell line.

Despite the reduced biochemical activity of peloruside A, the compound is not that different from paclitaxel and laulimalide in either its quantitative or qualitative effects on cells, in that all three compounds lead to bundled intracellular microtubules and mitotic arrest. Although initial experiments failed to demonstrate synergistic effects on cell growth by combining laulimalide and paclitaxel (Pryor et al., 2002), the synergy observed in inducing tubulin assembly by combining taxoid and laulimalide site compounds suggests other combinations are worth investigating.

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