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Microtubule Interactions with Chemically Diverse Stabilizing Agents: Thermodynamics of Binding to the Paclitaxel Site Predicts Cytotoxicity

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Summary

The interactions of microtubules with most compounds described as stabilizing agents have been studied. Several of them (lonafarnib, dicumarol, lutein, and jatrophane polyesters) did not show any stabilizing effect on microtubules. Taccalonolides A and E show paclitaxel-like effects in cells, but they were not able to modulate in vitro tubulin assembly or to bind microtubules, which suggests that other factors are involved in their cellular effects. The binding constants of epothilones, eleutherobin, discodermolide, sarcodictyins, 3,17β-diacetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-triene, and dictyostatin to the paclitaxel site; the critical concentrations of ligand-induced assembly; and their cytotoxicity in carcinoma cells have been measured, and correlations between these parameters have been determined. The inhibition of cell proliferation correlates better with the binding enthalpy change than with the binding constants, suggesting that large, favorable enthalpic contribution to the binding is desired to design paclitaxel site drugs with higher cytotoxicity.

Introduction

Tubulin assembly modulators represent an important class of antitumor drugs, as they have been proven to be an effective tool for cancer chemotherapy [1]. There are two major classes of tubulin assembly modulators: (1) compounds like vinblastine or colchicine, which depolymerize microtubules and block the mitotic spindle [2, 3], thus arresting dividing cells in mitosis with condensed chromosomes; and (2) compounds like paclitaxel (Taxol [Bristol-Myers Squibb], 4,10-diacetoxy-2a-(benzoyloxy)-5b,20-epoxy-1,7b-dihydroxy-9-oxotax-11-en-13a-yl(2*R*, 3*S*)-3[(phenylcarbonyl) amino]-2-hydroxy-3-phenylpropionate), which promote tubulin polymerization into microtubules (so called microtubule-stabilizing agents, MSAs) and block microtubule dynamics, leading to abnormal mitosis and subsequent apoptosis [4, 5]. Both microtubule depolymerizers and microtubule stabilizers at low concentrations can arrest dividing cells in mitosis, without altering microtubule mass, by blocking microtubule dynamics [6]. Paclitaxel was the first MSA discovered [7]; it promotes the in vitro assembly of tubulin in the absence of GTP, which is normally required for microtubule assembly; and the microtubules formed are stable against conditions favoring depolymerization, such as cold temperature, Ca^{2+}, and dilution [7]. In cells, paclitaxel induces microtubule stabilization and characteristic microtubule bundles [8]. However, despite the clinical success of paclitaxel and docetaxel (a closely related analog; Taxotere [Aventis RP56976], 4-acetoxy-2a-(benzoyloxy)-5b,20-epoxy-1,7b,10b-trihydroxy-9-oxotax-11-en-13a-yl(2*R*,3*S*)-3-[(tert-butoxycarbonyl) amino]-2-hydroxy-3-phenylpropionate) against ovarian, metastatic breast, head and neck, and lung cancer [9], both have two factors that limit their applicability: they have relatively low aqueous solubility, and they develop pleiotropic drug resistance mediated by the overexpression of the P-glycoprotein [10, 11]. Moreover, it has been shown that cultured ovarian tumor cells exposed to paclitaxel develop resistance through mutations in β-tubulin [12].

The clinical successes of paclitaxel (Taxol) and docetaxel (Taxotere) have triggered the search for new agents with similar mechanisms of action but without their disadvantages, i.e., higher solubility in aqueous solutions, poorer substrates of P-glycoprotein, and easier total chemical synthesis. Screenings of natural products from various sources (including corals, marine sponges, bacteria, and plants) have found an increasing number of structurally unrelated compounds that stabilize microtubules and mimic the activity of paclitaxel/docetaxel [13].

These MSA compounds can be classified into three groups with respect to their mechanism of action. The first group of compounds includes those that bind to the paclitaxel site in microtubules. Epothilones, eleutherobin, discodermolide, sarcodictyins, an estradiol analog (3,17β-diacetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-triene), cyclostreptin (also called FR182877 and WS9885B), dictyostatin, paclitaxel, and docetaxel bind to the same, or overlapping, taxoid binding site, as has been demonstrated by competition with ^{3}H-paclitaxel or Flutax-2 (a fluorescent derivative of paclitaxel; (7-*O*-[*N*-(2,7-difluoro-4'-fluoresceincarbonyl)-L-alanyl]paclitaxel) [14–20] (see the chemical formulas in Figure 1A).

The second group of compounds is the group formed by those that bind the laulimalide binding site; this group comprises two ligands, laulimalide itself [20] and peloruside A, which has recently been described to bind to this site [21].

The third group would include those compounds that have been described as having, actually or potentially,

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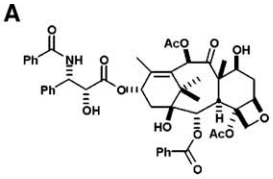
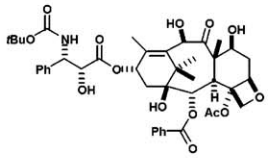
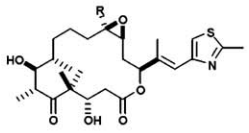
A



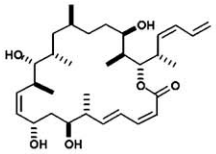
Paclitaxel (1)



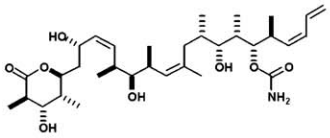
Docetaxel (2)



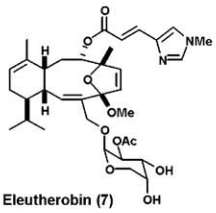
Epothilone A (3): R = H
Epothilone B (4): R = Me



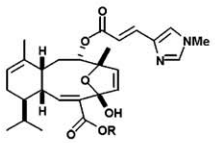
Dictyostatin (5)



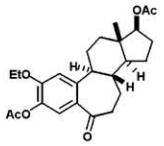
Discodermolide (6)



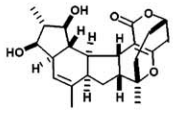
Eleutherobin (7)



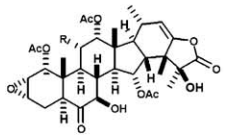
Sarcodictyin A (8): R = Me
Sarcodictyin B (9): R = Et



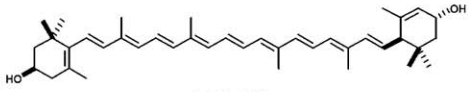
Estradiol analog (10)



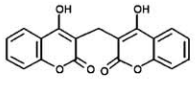
Cyclostreptin (11)



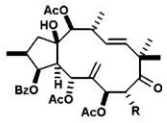
Taccalonolide A (12): R = OAc
Taccalonolide E (13): R = H



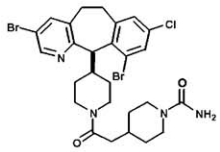
Lutein (14)



Dicumarol (15)

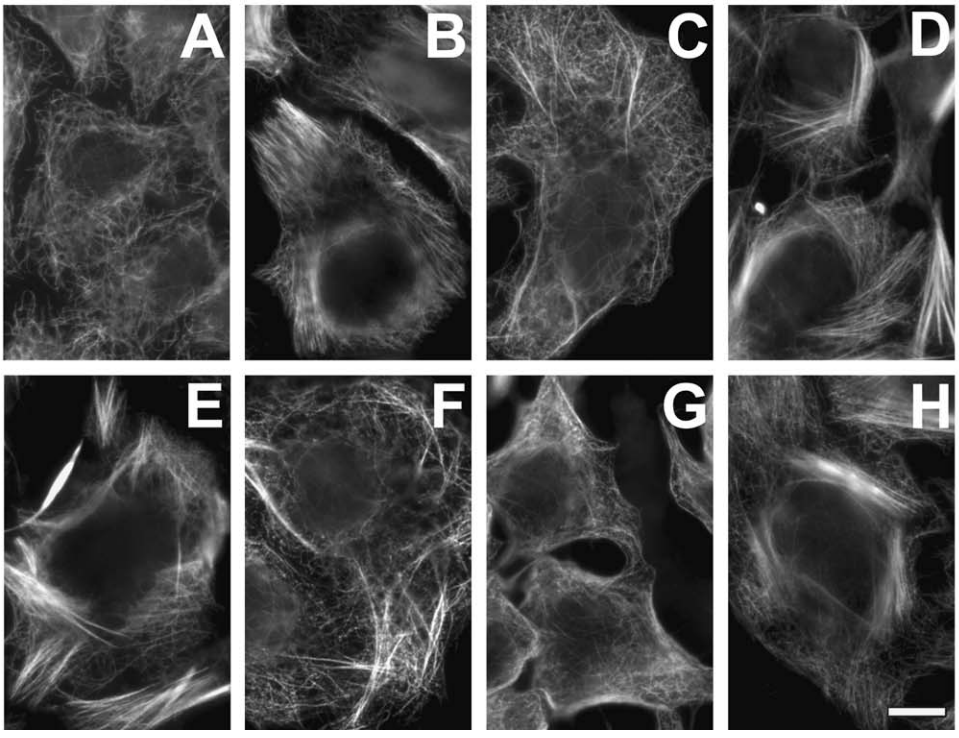


Jatrophane A (16): R = H
Jatrophane B (17): R = O*i*Bu
Jatrophane C (18): R = OAc



Lonafarnib (19)

B



microtubule stabilizing activity but whose binding to microtubules has not been described; this group would include the plant steroids taccalonolides A and E [22]; some xanthophylls that have been described as possible endogenous ligands for the paclitaxel binding site in the macula [23]; dicumarol and its analog coumadin [24], which have anticoagulant activity [25]; jatrophane polyesters [26]; and lonafarnib (Sch66336), a farnesyl transferase inhibitor that has been recently described to bundle microtubules in cells and seems to act by inhibiting tubulin deacetylase [27] (see the chemical formulas in Figure 1A).

Our first goal was to characterize the molecular mechanisms underlying the microtubule stabilization induced by each of these compounds in order to determine whether they directly bind to the microtubules and thermodynamically stabilize them. To do this, we have tested all of these ligands for their ability to induce or modulate tubulin assembly in vitro, and we have checked to see if they are able to bind to microtubules and to the paclitaxel site.

From the 19 compounds studied in this work, we have found that only 11 of them were able to modify tubulin assembly in vitro and bind to the paclitaxel site at the microtubule. We have measured the binding of these 11 ligands of the paclitaxel binding site, the critical concentrations of the ligand-induced in vitro tubulin-GTP and tubulin-GDP assembly, and the cytotoxicities in 1A9 and A549 human carcinoma cells, as well as their effect on A549 interphase microtubules, nuclear morphology, mitotic aberrations, and cell cycle progression of U937 leukemia cells. We also explored the correlations between binding to the paclitaxel site and these microtubule-mediated effects, in search of useful hints for rational drug design. In this way, we have found a predictive value of the enthalpic contribution to the free energy of binding for the rational design of new paclitaxel site-directed drugs with higher cytotoxicity.

Results and Discussion

Epothilones A and B (7,11-Dihydroxy-8,8,10,12-tetramethyl-3-[1-methyl-2-(2-methyl-thiazol-4-yl)-vinyl]-4,17-dioxa-bicyclo [14.1.0] heptadecane-5,9-dione), sarcodictyins A and B, eleutherobin, a estradiol analog, discodermolide, dictyostatin, and docetaxel, have been described to bind to the paclitaxel site at the microtubules, either by competing with ^{3}H-paclitaxel [15, 16, 28] or by displacing a fluorescent labeled paclitaxel (Flutax-2) [20, 29], but little is known about the mechanisms of action of these and the rest of the compounds that stabilize microtubules. In this work, we have classified most of the compounds described as microtubule-stabilizing agents on the basis of their mechanisms of action. Furthermore, we have studied their binding and explored the relationships between binding thermodynamics, tubulin assembly, and cytotoxicity of all of the paclitaxel site ligands. Laulimalide and peloruside A, which bind to a distinct site, have been studied separately [20, 21].

Effect of the Ligands on Interphase Microtubules, Mitotic Spindles, Nuclear Morphology, and Cell Cycle

First of all, the effect of the ligands on the interphase microtubules, mitotic spindles, nuclear morphology, and cell cycle of tumor cells was examined comparatively.

Human lung carcinoma A549 cells, incubated for 24 hr with three serial dilutions of each ligand, were used to characterize the effects of the ligands on the microtubule cytoskeleton and nuclear morphology. Indirect inmunofluorescences (Figure 1B) showed that 100 nM paclitaxel (Figure 1B[B]), 100 nM docetaxel (not shown), 100 nM epothilone B (Figure 1B[C]), 500 nM epothilone A (not shown), 100 nM dictyostatin (Figure 1B[D]), 100 nM discodermolide (Figure 1B[E]), 500 nM eleutherobin (Figure 1B[F]), and 500 nM cyclostreptin (Figure 1B[H]) induced many bundles and also multipolar spindles and micronucleated cells (not shown). Both 100 nM dictyostatin and 100 nM discodermolide were found to be more active in inducing bundles than the other ligands. Sarcodictyin A (1, 5, and 10 μM, [Figure 1B[G]]) and sarcodictyin B (1, 5, and 10 μM, not shown) induced few bundles made up of long microtubules; the cells treated with those ligands had no multipolar spindles and few multiple micronuclei. With 20 μM of the estradiol analog, neither effects on interphase microtubules nor multipolar spindles or micronucleated cells were observed. Taccalonolides also showed paclitaxel-like effects on A549 cells: 5 μM taccalonolide A and 10 μM taccalonolide E induced bundles, multipolar spindles, and multiple micronuclei (results not shown).

Lonafarnib (5, 10, 20, and 40 μM), luteine, dicumarol, and jatrophane polyesters (Figures 1B [B–D], up to 20 μM) did not induce bundles, and neither micronucleated cells nor multipolar spindles were observed in our preparations, which were similar to those of control, untreated cells (Figure 1B[A]).

Cell cycle analysis was performed on monocytic leukemia U937 cells incubated for 20 hr in the presence of five serial dilutions of each ligand. The smallest concentration that gave maximal accumulation of cells in the G2+M phase (Table S1; see the Supplemental Data available with this article online) was 5 nM paclitaxel, 2.5 nM docetaxel, 25 nM epothilone A, 5 nM epothilone B, 7.5 nM dictyostatin, 25 nM discodermolide, 50 nM eleutherobin, 0.5 μM sarcodictyin A, 0.5 μM sarcodictyin B, 100 nM cyclostreptin, 2.5 μM taccalonolide A, and 10 μM taccalonolide E. In all cases, a sub-G1 peak, presumably of cells undergoing apoptosis, appeared (Table S1). With the estradiol analog, no accumulation was observed in the G2+M phase at concentrations up to 40 μM.

Lonafarnib (up to 20 μM), lutein (up to 40 μM), dicumarol (up to 40 μM), and jatrophane polyesters B, C, and D

Figure 1. Compounds Used in the Study and Their Effect on Interphase Microtubules

(A) Chemical formulas of the ligands studied.

(B) Comparative effect of the ligands on interphase microtubules. A549 cells were incubated for 24 hr in the presence of drug vehicle (A) or 100 nM paclitaxel (B), 100 nM epothilone B (C), 100 nM dictyostatin (D), 100 nM discodermolide (E), 500 nM eleutherobin (F), 1 μM sarcodictyin A (G), and 500 nM cyclostreptin (H). The bar indicates 10 μm.

Table 1. Critical Concentrations of the Assembly Reactions and Cytotoxicity of the Paclitaxel Site Ligands

Ligand	Cr-GTP (μM)	Cr-GDP (μM)	IC_{50} 1A9 (nM)	IC_{50} A549 (nM)
Paclitaxel (1)	4.23 ± 0.2[a]	10.29 ± 0.7	1.0 ± 0.4	1.4 ± 0.2
Docetaxel (2)	1.52 ± 0.1[a]	5.92 ± 1.0	0.4 ± 0.2	3.0 ± 0.3
Epothilone A (3)	5.11 ± 0.5[a]	8.98 ± 0.4	4.1 ± 1.0	7.5 ± 1.4
Epothilone B (4)	1.17 ± 0.6[a]	5.60 ± 0.1	1.2 ± 0.3	0.8 ± 0.1
Dictyostatin (5)	0.66 ± 0.1	2.40 ± 0.2	3.6 ± 1.0	5.6 ± 1.0
Discodermolide (6)	0.92 ± 0.04	3.81 ± 0.9	17.6 ± 2.7	6.6 ± 0.8
Eleutherobin (7)	6.50 ± 0.1	12.8 ± 1.0	13.2 ± 7.0	3.3 ± 0.2
Sarcodictyin A (8)	>60[b]	Inactive	32 ± 21	36 ± 12
Sarcodictyin B (9)	>60[b]	Inactive	103 ± 18	50 ± 15
Estradiol analog (10)	>60[b]	Inactive	43,000 ± 3,000	>20,000
Cyclostreptin (11)	~30[b]	Inactive	40 ± 5	37 ± 8

Critical concentrations of the ligand-induced GTP- or GDP-tubulin assembly in buffer PEDTA4 with 1 mM GTP or 1 mM GDP in the presence of the corresponding ligand (data ± standard error). IC_{50} of the ligands against 1A9 human ovarian carcinoma cells and A549 human lung carcinoma cells (data ± standard error; average of at least four independent experiments).

[a] Data from [33].

[b] Although microtubules were observed in the pellets, much higher concentrations of tubulin and drugs would be required for a precise measurement of critical concentrations.

(up to 40 μM) did not significantly arrest cells in the G2+M phase in our experimental conditions, but a sub-G1 peak of hypodiploid cells appeared (Table S1).

We concluded from these cellular results that the only compounds that behaved like paclitaxel were docetaxel, epothilone A, epothilone B, dictyostatin, discodermolide, eleutherobin, sarcodictyin A, sarcodictyin B, cyclostreptin, and taccalonolides A and E.

Ligand-Induced Assembly of Tubulin

The characteristic effect of these ligands is microtubule thermodynamic stabilization, so we checked if the ligands were able to induce tubulin assembly in a pure GTP-tubulin or GDP-tubulin system and then measured the critical concentrations (Cr) of the assembly reaction (Table 1), in a buffer in which tubulin is unable to self-assemble in the absence of ligand (Cr > 200 μM for GTP-tubulin; [30]).

Paclitaxel (compound 1), docetaxel (compound 2), epothilones A (compound 3) and B (compound 4), dictyostatin (compound 5), discodermolide (compound 6), eleutherobin (compound 7), sarcodictyin A (compound 8) and sarcodictyin B (compound 9), an estradiol analog (compound 10), cyclostreptin (compound 11), taccalonolides A (compound 12) and E (compound 13), lutein (compound 14), dicoumarol (compound 15), jatrophane polyesters 13B (compound 16), jatrophane polyesters 13C (compound 17), jatrophane polyesters 13D (compound 18), lonafarnib (Sch66336; compound 19), and, as control, the solvent in which the drugs are dissolved (DMSO, called compound 20) were tested for their ability to induce purified tubulin assembly.

The strongest assembly inductor is dictyostatin, with a Cr of 0.66 ± 0.06 μM for GTP-tubulin and one of 2.40 ± 0.2 μM (Table 1) for GDP-tubulin, more than five times below than that for paclitaxel (Cr of 4.23 ± 0.22 μM for GTP-tubulin and 10.29 ± 0.69 μM for GDP-tubulin). Both sarcodictyins A and B, cyclostreptin, and the estradiol analog weakly induced assembly at high concentrations of GTP-tubulin (we checked by electron microscopy that the pelleted polymers were microtubules), but their critical concentrations were not precisely determined since no constant concentration of tubulin was reached in the supernatants. Precise measurements of these Cr would require higher concentrations of tubulin and drugs, which were not possible to reach because of the limited amounts of available drug. Furthermore, no assembly was detected in GDP-tubulin experiments for these ligands.

No ligand-induced polymerization was observed in the absence of Mg^{+2} for ligands 1–11, indicating that the assembly induced by all of these ligands is dependent on Mg^{+2}, as is the case of paclitaxel and docetaxel, requiring free mM concentrations of Mg^{+2} [30]. Nevertheless, a weak assembly has been observed for discodermolide with much lower concentrations of Mg^{+2} in the nanomolar range by using high concentrations of tubulin (data not shown).

Electron microscopy was performed on all of the induced polymers, and these were normal microtubules. Figure 2A shows morphologically normal microtubules induced by the weakest ligands (both sarcodictyins A and B and the estradiol analog) compared to paclitaxel-induced microtubules.

Taccalonolides A and E; jatrophane polyesters 13B, 13C, and 13D; lutein; lonafarnib; and dicoumarol were not able to induce assembly at concentrations as high as 60 μM GTP-tubulin (and 66 μM ligand), as checked by centrifugation and electron microscopy.

Effects of the Ligands on Microtubule Assembly

In order to check if the ligands that cannot induce purified tubulin assembly could at least modulate tubulin assembly, i.e., modify the critical concentration in conditions in which purified tubulin is able to assemble (GAB [glycerol assembly buffer; 3.4 M glycerol, 10 mM sodium phosphate, 1 mM EGTA, 6 mM $MgCl_2$ (pH 6.5)]-1 mM GTP buffer), 15 and 20 μM pure GTP-tubulin were incubated at 37ºC in this buffer in the presence of 16.5 and 22 μM ligand, respectively. Under these conditions, tubulin assembles in the absence of exogenous ligands with a critical concentration of 3.3 ± 0.02 μM. Figure 2B shows how the compounds modify the critical concentration of the assembly. Compounds 1–11 decrease the critical concentration necessary for polymerization, as expected from their capacity to induce GTP-tubulin assembly (Table 1). Compounds 12 and 13 and

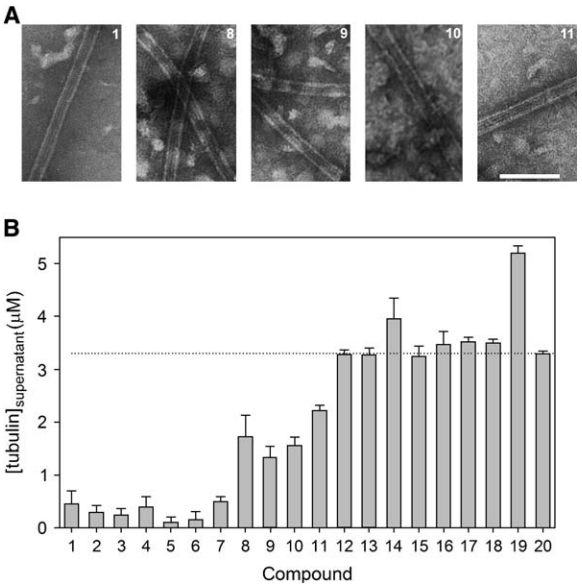


Figure 2. Ligand-Induced Microtubule Assembly

(A) Electron micrographs of ligand-induced microtubules. 30 μM tubulin and 33 μM ligand were incubated at 37°C for 30 min, and 20 μl of the samples was adsorbed onto formvar/carbon-coated grids and stained with 1% uranyl acetate. The scale bar indicates 100 nm.
(B) Critical concentration of the GTP-tubulin assembly at 37°C in buffer GAB-1 mM GTP in the presence of 10% excess of the ligands as compared with the control (20, DMSO, dotted line). Ligands: paclitaxel (1) and docetaxel (2), epothilone A (3), epothilone B (4), dictyostatin (5), discodermolide (6), eleutherobin (7), sarcodictyin A (8), sarcodictyin B (9), an estradiol analog (10), cyclostreptin (11), taccalonolide A (12), taccalonolide E (13), lutein (14), dicoumarol (15), polyester jatrophane 13B (16), polyester jatrophane 13C (17), polyester jatrophane 13D (18), lonafarnib (19), and, as control, the solvent in which the drugs are dissolved (DMSO, [20]).
The error bars indicate standard error.

15–18 are inactive, and lutein 14 and lonafarnib 19 behave as very weak depolymerizers.

Finally, we checked if those ligands that do not affect microtubule assembly in pure tubulin systems could be affecting other proteins in the in vitro microtubule assembly system. Thus, we performed similar experiments with microtubular protein, i.e., in the presence of microtubule-associated proteins (MAPs). Ligands that do not affect microtubule assembly in purified tubulin assembly systems were inactive, and lonafarnib behaved as a weak depolymerizer (data not shown).

On the basis of the microtubule assembly results, we can conclude that ligands 12–19 (including taccalonolide A and E; jatrophane polyesters 13B, 13C, and 13D; lonafarnib; lutein; and dicoumarol) do not induce or promote microtubule assembly in our in vitro systems, at least under the conditions that we have assayed in this work, suggesting that any microtubule stabilizing activity of some of these ligands would come from the interaction with other factors in any of the signaling pathways that may be regulating microtubule stability.

Binding of the Ligands to the Paclitaxel Site

We next checked the ligands for their ability to displace Flutax-2 (a bona fide probe of the paclitaxel binding site) [20, 29, 31]. To do so, increasing concentrations of ligand up to 100 μM against a mixture of 50 nM crosslinked microtubules and 50 nM Flutax-2 were used (Figure 3A). Compounds 1–11 were able to displace Flutax-2 from its site, indicating that all of them bind to the paclitaxel or an overlapping site at the microtubule. As expected, compounds 14–19 were not able to displace Flutax-2, indicating that they do not interact with the paclitaxel binding site.

Taccalonolides A and E (compounds 12 and 13, respectively) weakly and slowly displaced Flutax-2 from its site in our standard displacement test. Full displacement of 50 nM Flutax-2 by 50 μM taccalonolide A takes

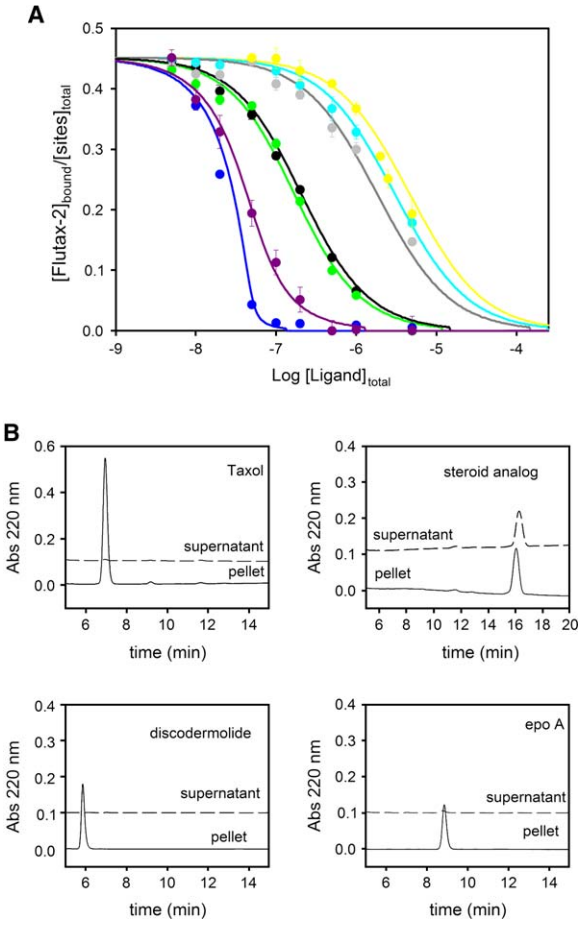


Figure 3. Binding of Ligands to the Paclitaxel Site

(A) Competition between Flutax-2 and the ligands for the paclitaxel binding site. Displacement of the fluorescent taxoid Flutax-2 (50 nM) from microtubule binding sites (50 nM) by the ligands at 35°C. The points are data (and standard errors), and the lines were generated with the best-fit value of the binding equilibrium constant of each ligand, assuming a one to one binding to the same site. Dark blue, discodermolide; purple, dictyostatin; green, eleutherobin; black, paclitaxel; gray, sarcodictyin B; light blue, sarcodictyin A; and yellow, estradiol analog.
(B) Binding of ligands to crosslinked microtubules. 30 μM of paclitaxel binding sites in crosslinked microtubules were incubated with equimolecular amounts of the desired ligands and harvested by centrifugation, the bound ligand was extracted from the protein pellet, and the supernatant and analyzed by HPLC as described in the text. Each injection contains equivalent amounts of extract. Supernatant traces have been displaced 0.1 absorbance units for presentation purposes.

around 50 min at 35ºC, which is far from the range of time (less than 4 min for 99% equilibrium) that can be calculated from the kinetic dissociation constant of Flutax-2 (1.92×10^{-2} s^{-1} [32]). (The observed kinetic rate of dissociation of Flutax-2 from its binding site corresponds to the slowest reaction, which is either the dissociation of Flutax-2 from its site or the association of the taccalonolide to the sites left empty. In the second case, the displacement kinetics should be dependent upon the concentration of taccalonolide, which is not the case.) So, to confirm that we were observing a true displacement, we performed similar experiments by centrifugation. No Flutax-2 displacement by taccalonolides or inhibition of Flutax-2 binding after preincubation of the microtubules with taccalonolides was seen, indicating that the observed decrease of the Flutax-2 anisotropy was artifactual. Moreover, since neither effects on the polymerization nor direct binding to microtubule pellets, or decreasing the free ligand-free concentration in the supernatants after incubation with microtubules, was observed by HPLC analysis (see below), we should consider that both taccalonolides A and E are not in vitro microtubule-stabilizing agents and that the effect observed on cells is not due to their binding to microtubules.

Binding to Microtubules

We next checked whether direct evidence of the binding to microtubules of the ligands that were able to induce or modulate tubulin assembly could be obtained. Microtubule pellets both from crosslinked (Figure 3B) and non-crosslinked microtubules (not shown) were extracted with dichloromethane and measured by HPLC; the ligands were found bound to the microtubules in the pellet (except in the case of the low-affinity ligands, in which part of the ligand was found in the supernatant). Appropriate controls were performed to confirm the solubility of the ligands under the conditions of the experiment (30 μM ligand and crosslinked microtubules was used for these experiments). Figure 3B shows representative results with paclitaxel, the estradiol analog, discodermolide, and epothilone A. Similar results were obtained with docetaxel, epothilone B, dictyostatin, eleutherobin, and sarcodictyins A and B.

Interestingly, cyclostreptin was not observed in the extracts of the microtubule pellets in these experiments, even at higher concentrations (since this compound has very low absorbance in the UV range, HPLC-mass spectrometry was used to detect it). However, a decrease in the amount of ligand in the supernatant was observed after incubation with microtubules. Since Flutax-2 displacement had been observed for this compound (not shown), we have investigated the nonreversible binding of this compound to the microtubules, which has led to the discovery of the covalent binding of cyclostreptin to its binding site in the microtubules, which will be reported separately (unpublished data).

Similar experiments were performed with taccalonolides A and E, and no evidence of binding to crosslinked or native microtubules was found.

Thermodynamic Parameters of Ligand Binding to the Paclitaxel Site

The binding constants and thermodynamic parameters of compounds reversibly displacing Flutax-2 were calculated from the displacement isotherms (Figure 3A; Experimental Procedures) at different temperatures (Table 2).

The ligand with the highest binding affinity is discodermolide (K_{app} discodermolide = 526×10^7 M^{-1}; Table 2), with almost 500-fold higher affinity than paclitaxel (K_{app} paclitaxel = 1.07×10^7 M^{-1}) and around 150-fold affinity than docetaxel (K_{app} docetaxel = 3.09×10^7 M^{-1}) at 37ºC. Discodermolide has around 30 times more affinity for the site than its cycled analog dictyostatin (K_{app} dictyostatin = 18.0×10^7 M^{-1}) and 8 times more affinity for the site than epothilone B (K_{app} epoB = 60.8×10^7 M^{-1}). The weakest ligands are the estradiol analog and the sarcodictyins (K_{app} estradiol = 0.047×10^7 M^{-1}, K_{app} sarcA = 0.16×10^7 M^{-1}, K_{app} sarcB = 0.20×10^7 M^{-1}). Eleutherobin has a binding affinity in between those of paclitaxel and docetaxel (K_{app} eleutherobin = 1.36×10^7 M^{-1}). Compatible values for epothilones A and B (5×10^7 and 50×10^7 M^{-1}, respectively) [33] and discodermolide (K_{app} discodermolide = 500×10^7 M^{-1}) were obtained by using direct sedimentation measurements, which further validates the Flutax-2 displacement method.

As for the case of paclitaxel and docetaxel, all of the ligands show a dependence of the apparent binding constant on temperature. The binding reaction is exothermic for all of the ligands; the higher the temperature, the lower the binding constant. Binding is enthalpy driven for paclitaxel, docetaxel, and epothilones A and B, and it has a positive contribution (disfavorable) of the entropic term ($-T\Delta S$) to the free energy of binding, while all the rest have a negative (favorable) entropic contribution to the free energy. This is especially remarkable for discodermolide, a ligand that should have a strong conformational freedom in solution, which shows a large unexpected favorable entropic contribution to the free energy of binding.

Comparison between Ligand Binding Affinities and Tubulin Assembly Activity

In order to quantitatively compare microtubule assembly induced by these ligands, we explored the correlation between this activity and the binding affinity. The comparison between the apparent affinity constants (or free energy of binding, ΔG^0_{app}) of the ligands to their site and the equilibrium constants for the microtubule growth reaction, which is approximately the inverse of the critical concentration [34], shows that they are correlated ($r^2 = 0.58$ for GTP-tubulin [Figure 4A]; $r^2 = 0.50$ for GDP-tubulin [Figure 4B]). Changes in the apparent free energy of elongation are much smaller than those in the free energy of binding (slope = 0.30 for GTP-tubulin and 0.16 for GDP-tubulin). Similar results were obtained for a set of chemically related epothilone analogs [33]. Although the elongation constant and binding affinity would be expected not to be correlated, since they are different reactions with different intrinsic equilibrium binding constants (see the Supplemental Data in [33]), the experimental results indicate that they are somehow related, even for these chemically different compounds. Docetaxel and dictyostatin, which are the most powerful ligands inducing assembly, are over the best regression line in both GTP- and GDP-tubulin assembly experiments. The rest of the ligands lie inside the 95%

Table 2. Apparent Thermodynamic Parameters of Binding of Ligands to the Paclitaxel Binding Site of Microtubules, Determined from the Displacement of the Fluorescent Taxoid Flutax-2

Ligand	K_b^{app} (10^7 M^{-1})								ΔG^0_{app} (kJ mol^{-1}),	ΔH^0_{app} (kJ mol^{-1})	ΔS^0_{app} (J mol^{-1} K^{-1})
	26°C	27°C	30°C	32°C	35°C	37°C	40°C	42°C	37°C		
Paclitaxel (1)[a]	2.64 ± 0.17	2.19 ± 0.05	1.83 ± 0.09	1.81 ± 0.21	1.43 ± 0.17	1.07 ± 0.11	0.96 ± 0.14	0.94 ± 0.23	−41.7 ± 0.2	−51.4 ± 4.2	−29.3 ± 13.1
Docetaxel (2)[a]	6.95 ± 0.42	6.57 ± 0.52	5.42 ± 0.42	4.89 ± 0.38	3.93 ± 0.27	3.09 ± 0.22	2.89 ± 0.17	2.38 ± 0.11	−44.4 ± 0.2	−52.5 ± 2.3	−25.5 ± 7.5
Epothilone A (3)[a]	7.48 ± 1.00	6.94 ± 1.08	5.81 ± 1.08	5.00 ± 0.49	3.63 ± 0.51	2.93 ± 0.44	2.32 ± 0.25	2.08 ± 0.21	−44.3 ± 0.4	−65.6 ± 2.4	−68.3 ± 7.9
Epothilone B (4)[a]	150 ± 15	144 ± 32	120 ± 13	129 ± 30	75.0 ± 7.4	60.8 ± 10.1	49.4 ± 8.8	35.6 ± 2.6	−52.1 ± 0.4	−70.7 ± 7.0	−59.7 ± 22.8
Dictyostatin (5)	35.5 ± 7.4	32.0 ± 5.5	24.8 ± 2.9	23.1 ± 2.8	19.2 ± 2.5	16.8 ± 2.0	14.1 ± 1.8	14.2 ± 1.7	−48.8 ± 0.3	−45.9 ± 2.6	9.5 ± 8.5
Discodermolide (6)	872 ± 82	837 ± 77	958 ± 134	704 ± 95	555 ± 111	526 ± 72	547 ± 60	321 ± 73	−57.7 ± 0.3	−33.2 ± 7.6	79.5 ± 2.5
Eleutherobin (7)	3.43 ± 0.21	3.26 ± 0.25	2.57 ± 0.08	2.47 ± 0.02	2.01 ± 0.11	1.36 ± 0.62	1.92 ± 0.02	1.7 ± 0.17	−43.6 ± 0.03	−33.2 ± 3.2	32.9 ± 10.3
Sarcodictyin A (8)	0.23 ± 0.09	0.23 ± 0.09	0.22 ± 0.09	0.21 ± 0.09	0.18 ± 0.07	0.16 ± 0.06	0.44 ± 0.25	0.15 ± 0.05	−36.8 ± 0.8	−23.6 ± 2.6	43.12 ± 8.6
Sarcodictyin B (9)	0.34 ± 0.14	0.33 ± 0.15	0.28 ± 0.12	0.29 ± 0.11	0.24 ± 0.08	0.20 ± 0.07	0.21 ± 0.06	0.19 ± 0.05	−37.9 ± 0.7	−29.3 ± 3.1	27.1 ± 9.9
Estradiol analog (10)	0.077 ± 0.021	0.077 ± 0.023	0.078 ± 0.026	0.07 ± 0.021	0.057 ± 0.016	0.047 ± 0.013	0.056 ± 0.020	0.063 ± 0.022	−33.4 ± 0.8	−18.2 ± 6.8	51.6 ± 22.1

Data ± standard error; average of at least four independent measurements.
[a] Data from [33].

Figure 4. Comparison between Binding Affinity and Microtubule Stabilization

(A and B) Dependence of the elongation constant of ligand-induced (A) GTP- or (B) GDP-tubulin assembly, K_{el} (K_{el} = 1/Cr) on the free energy of binding of the ligands to the paclitaxel site. Solid lines are the best linear regression to the experimental data; dashed lines are the 95% confidence intervals of the regression. The error bars indicate standard error.

confidence interval of the regression, showing a good linear correlation.

Comparison between Binding Thermodynamics and Cytotoxicity

The IC_{50} of the ligands were determined (Table 1) in 1A9 and A549 cells. The most cytotoxic ligand in 1A9 cells is docetaxel (IC_{50} = 0.4 ± 0.2 nM), which is very similar to paclitaxel (IC_{50} = 1.0 ± 0.4 nM) and epothilone B (IC_{50} = 1.2 ± 0.3 nM). The ligand with the largest affinity, discodermolide, has a relatively low cytotoxicity (IC_{50} = 17.6 ± 2.7 nM in 1A9 cells and 6.35 ± 0.8 in A549 cells), while eleutherobin, a ligand with much lower affinity for the site than paclitaxel, has a similar or even lower IC_{50} (13.2 ± 7 nM in 1A9 cells and 3.3 ± 0.2 nM) than discodermolide.

Figure 5A shows a weak correlation of cytotoxicity (IC_{50}) with the binding affinity (free energy of binding) (r^2 = 0.31 for 1A9 cells; r^2 = 0.27 for A549 cells; data not shown). This type of correlation has been previously observed for a group of chemically related compounds (epothilones) in a previous work of our group [33]. These findings were apparently unexpected since we do not know the total equilibrium concentration of the drugs

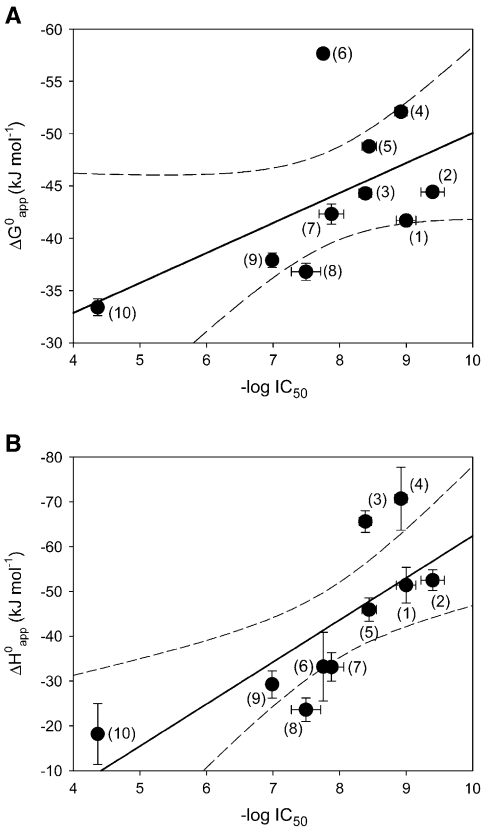


Figure 5. Comparison between Binding Affinity and Cytotoxicity in 1A9 Cells

(A) Dependence of the IC_{50} of ligands against 1A9 human ovarian carcinoma cell lines on the free energy of binding to the paclitaxel binding site of microtubules.

(B) Dependence of the IC_{50} of ligands on the enthalpic term of the free energy of binding to the paclitaxel site. Solid lines are the best linear regression to the experimental data; dashed lines are the 95% confidence intervals of the regression.

The error bars indicate standard error.

in the cells, their metabolism, or whether there is any other target or binding site for the drugs in the cells.

It is especially noticeable that the IC_{50} values of many of the compounds are much lower than their dissociation constants (1 nM IC_{50} in 1A9 cells for paclitaxel for 100 nM Kd at 37ºC), which would imply that, unless the local concentration inside the cell is higher, only a very small fraction of the tubulin molecules would be bound by paclitaxel. A possible reason would be drug accumulation due to the high, local tubulin concentration. For compounds with similar physicochemical properties, the binding affinity would be the driving force to pass the membrane.

However, the observed correlation is much weaker for this set of chemically different compounds than for those observed for epothilones (r^2 = 0.76) [33]. It can be noticed that discodermolide (6) has much lower cytoxicity than expected from its binding affinity. As will be discussed below, this compound has a strong entropic contribution to binding (Table 2). Previous results for a set of epothilone analogs suggested that compounds with a high, favorable entropic contribution to binding are less effective at killing tumoral cells than those with a high, favorable enthalpic contribution [33]. This would be the case for discodermolide.

Since compounds with a high, favorable entropic contribution to binding are less effective at killing tumoral cells than those with a favorable enthalpic contribution, it could be suggested that the enthalpic term is more important in order to get higher cytotoxicity. In fact, although we have to take with caution the dissection of binding free energies into their entropic and enthalpic contributions because of enthalpy-entropy compensations [35], it can be shown that IC_{50} is better correlated with ΔH_{app} than with ΔG_{app} for all of the MSA (Figure 5B; r^2 = 0.58 for 1A9 cells; r^2 = 0.50 for A549 cells; data not shown).

As observed in Figure 5B, there are two groups of compounds: the first group includes those compounds with high cytotoxicity (including compounds 1–5), and the other group contains those with medium cytotoxicity (including compounds 6–9). The estradiol analog (10) has very low cytotoxicity. Interestingly, the first group with high cytotoxicity includes compounds with negative (or only slightly positive) entropic contributions to the binding; the binding reaction is enthalpy driven. All of the ligands in the second group with low cytotoxicities have strong positive entropic contributions to binding, which leads to a more entropy-driven reaction. Nevertheless, ΔG^0_{app} is still high for some of the compounds in the second group, including eleutherobin or even discodermolide, the compound with the highest affinity for the site. One factor that could be involved in the low cytotoxicity of discodermolide is its predicted hydrophilicity. As the ligand becomes more hydrophilic, it will be more difficulty for it to cross the cellular membrane by passive diffusion, and its cytotoxicity will decrease.

The binding affinity, expressed as the Gibbs free energy change (ΔG) ($K_a = \exp[-\Delta G/RT]$), arises from the sum of two terms ($\Delta G = \Delta H - T\Delta S$): the enthalpic ($\Delta H$) and the entropic one ($-T\Delta S$). Obviously, an increase of the binding affinity can be accomplished by making either ΔH more negative or ΔS more positive. Different compounds may have similar binding constants, which may arise from different contributions of the enthalpic and the entropic terms to the free energy, which, in turn, may be reflected in differential effects into the microtubule assembly properties and cytotoxicity of the compounds.

From the results obtained, it can be deduced that ΔH is a better predictor of the IC_{50} than ΔG for these chemically unrelated ligands. (Interestingly, the cytotoxic effect is not related to the microtubule thermodynamic stabilizing effect of the ligands; there is no correlation between Kel and IC_{50} [r^2 = 0.008], which indicates that in vitro microtubule stabilization is not a predictor of cytotoxicity). Since the binding enthalpy is the term that predominantly reflects the strength of the interactions of the ligand with its target relative to the solvent [36], it could be suggested that ligands with a high enthalpic contribution to binding will produce stronger effects on tubulin and that this will be reflected in the subsequent microtubule-mediated effects, leading to cytotoxicity. Since no relationship between microtubule stabilization/bundling in cells and IC_{50} was qualitatively observed, the results suggest that the cytotoxic effect might be related

to effects on microtubule dynamics. In conclusion, for rational drug design, complete thermodynamic binding profiles of ligand binding must be examined, and the enthalpic contribution to binding must be maximized in order to get compounds with higher cytotoxic properties.

Significance

The biochemical mechanisms and the thermodynamics of the interaction of microtubules with paclitaxel-site ligands have been studied. Laulimalide and peloruside A are known to thermodynamically stabilize microtubules by binding to a site distinct from the paclitaxel site. Taccalonolides A and E, dicumarol, lutein, and jatrophane polyesters have not been found to be able to induce or modulate in vitro microtubule assembly or to displace a fluorescent taxoid (Flutax-2) from its binding site, suggesting that the microtubule-stabilizing activity of these compounds, if any, comes through interactions with other factors regulating cellular microtubule polymer mass rather than by direct binding to microtubules. The ligands that bind to the paclitaxel site, including epothilones, eleutherobin, discodermolide, sarcodictyins, a estradiol analog, dictyostatin, and cyclostreptin (which binds covalently), require Mg^{+2} to induce assembly and were able to induce GTP-tubulin assembly under conditions in which tubulin itself is unable; moreover, the strongest compounds also induced GDP-tubulin assembly. The equilibrium binding constants of the compounds to the paclitaxel site, the critical concentrations of ligand-induced assembly, and their IC_{50} in 1A9 and A549 cells have been measured. Correlations between these parameters have also been explored. The binding is exothermic for all ligands. Taxoids and epothilones have unfavorable entropic contributions to binding, whereas the rest (including discodermolide, which is expected to have a large conformational freedom in solution) have favorable entropy changes. The binding constants weakly correlate with cytotoxicity (IC_{50}) values in two cell lines: 1A9 and A549 (r^2 = 0.31 for 1A9 cells and r^2 = 0.27 for A549 cells). Interestingly, IC_{50} correlates better with the apparent enthalpy of binding (r^2 = 0.58 for 1A9 cells and r^2 = 0.50 for A549 cells), suggesting a predictive value of enthalpic contributions to the free energy of binding in designing paclitaxel-site drugs with higher cytotoxicity.

Experimental Procedures

Proteins and Ligands

Purified calf brain tubulin, bovine brain microtubule proteins, and chemicals were prepared as described [37, 38]. Paclitaxel (Taxol) was provided by the late M. Suffness from the National Cancer Institute (Bethesda, MD). Docetaxel (Taxotere) was kindly provided by Rhône Poulenc Rorer, Aventis (Schiltigheim, France). Epothilones, sarcodictyins, and eleutherobin were provided by K.C. Nicolaou from the Department of Chemistry and Biochemistry, University of California, San Diego and the Scripps Research Institute (La Jolla, CA). Lonafarnib was provided by W.R. Bishop (Schering-Plough Research Institute). Cyclostreptin was provided by E.J. Sorensen (Princenton University, Princenton, NJ), and the estradiol analog was provided by E. Hamel from the National Institutes of Health (Frederick, MD). Jatrophane polyesters were provided by G. Appendino, Università del Piemonte Orientale, Italy. Lutein and dicumarol were purchased from Sigma. Flutax-2 was provided by F. Amat-Guerri from Instituto de Química Orgánica (Madrid, Spain). Taccalonolides were isolated as described [22]. Dictyostatin and discodermolide were synthesized as described [19, 39]. All of the compounds were diluted in DMSO to a final concentration of 10 mM and were stored at −20ºC.

Ligand-Induced Tubulin Assembly

The critical concentrations of ligand-induced tubulin assembly were measured in PEDTA4-GTP (10 mM sodium phosphate, 1 mM EDTA, 1 mM GDP, 1 mM GTP [pH 6.7] with 4 mM $MgCl_2$) or PEDTA4-GDP (10 mM sodium phosphate, 1 mM EDTA, 1 mM GDP [pH 7.0] with 4 mM $MgCl_2$). Control experiments have shown that, in these buffers, tubulin concentrations of up to 200 μM do not assemble in the absence of exogenous ligands [37]. GTP- or GDP-tubulin (prepared as described in [37]) at several concentrations (usually 5–8) ranging between 1 and 60 μM (depending on the ligand) were incubated at 37ºC for 30 min in the presence of the ligand at a concentration 10% in excess of the tubulin. The polymers were sedimented at 90,000 × g for 20 min in a TLA 100 rotor that was preequilibrated at 37ºC in a Beckman Optima TLX ultracentrifuge. The supernatants were carefully removed by pipetting, and the pellets were resuspended in 10 mM phosphate, 1% SDS (pH 7.0). The pellets and the supernatants were diluted 1:10 in the same buffer, and their concentrations were fluorimetrically measured by employing a Fluorolog 3 spectrofluorimeter (excitation wavelength 285 nm and emission wavelength 320 nm using slits of 2 and 5 nm, respectively). Tubulin concentration standard curves were constructed for each experiment by using spectrophotometrically measured concentrations of purified tubulin. Considering a noncovalent nucleation-condensation polymerization, the critical concentration is equal to the concentration of tubulin in the supernatant (that should be equal to the tubulin concentration given by the extrapolation to zero of the plot concentration of tubulin in the pellets versus total concentration) [34]. Apparent polymer growth equilibrium constants were estimated as the reciprocal critical concentrations for polymerization [34]. The assembled polymers were observed by electron microscopy as described below.

Effects of Ligands on Microtubule Assembly and Stability

The effects of the ligands on tubulin assembly were monitored by incubating concentrations from 10 to 20 μM tubulin in buffer GAB-1 mM GTP in the presence of 11 or 22 μM ligand. In this buffer, tubulin can assemble without ligand with a critical concentration of 3.3 μM. The samples were processed as described above, and the tubulin concentrations were also measured fluorometrically as described above.

The effect of the ligand on polymerization was also tested in a MAP-containing system; microtubular protein (1 mg/ml) was incubated in buffer AB-1 mM GTP (100 mM MES, 1 mM EGTA, 1 mM MgSO4, 2 mM 2-mercaptoethanol, 1 mM GTP [pH 6.5]) for 30 min at 37ºC in the presence of 11 μM ligand. The polymers were then pelleted at 90,000 × g for 20 min in a TLA 100 rotor that was preequilibrated at 37ºC in a Beckman Optima TLX ultracentrifuge. The supernatants were taken, the pellets were resuspended in 10 mM sodium phosphate (pH 7.0), and microtubular protein concentrations were measured by the method described by Bradford [40] by using BSA (bovine serum albumin) as standard.

Preparation of Stabilized Microtubules

Stabilized, crosslinked microtubules were prepared by mild crosslinking with 0.2% glutaraldehyde; the excess of glutaraldehyde was quenched with $NaBH_4$ (Fluka), and the solution was dialyzed overnight by using Slide-A-Lyzer 10K dialysis cassettes (Pierce) against the desired buffer and drop frozen in liquid nitrogen [29, 31]. After this treatment, 90% of the tubulin was found to have been incorporated into the microtubules that are stable against dilution and low temperatures. The concentration of paclitaxel binding sites in these crosslinked microtubules was determined as described [29]; 100% of the assembled tubulin dimers were found to bind taxoids immediately after dialysis.

Binding of Ligands to Microtubules

Samples containing ligand and either stabilized, crosslinked microtubules (prepared as described before) or native ones (polymerized

in GAB-1 mM GTP buffer) were incubated for 30 min at 25ºC in polycarbonate centrifuge tubes (Beckman) in GAB/0.1 mM GTP or GAB/1 mM GTP buffer (DMSO concentration was always kept under 2%). The samples were then centrifuged at 90,000 × g for 10 min at 25ºC in a TLA100 rotor employing a Beckman Optima TLX ultracentrifuge. The supernatants were collected by pipetting, and the pellets were resuspended in 10 mM phosphate (pH 7.0). Both the pellets and supernatants were extracted three times with an excess volume of dichloromethane, dried in vacuum, and dissolved in 25 μl of a methanol/water (v/v: 70/30) mixture. Ligands bound to pelleted polymers and free in the supernatant were determined by HPLC. HPLC analysis of the samples was performed in a C-18 column (Supercosil, LC18-DB, 250 × 4.6 mm, 5 μM bead size) developed isocratically with 70% methanol, or 50% acetonitrile, in water (v/v) at a flow rate of 1 ml/min.

Binding of Flutax-2 to stabilized, crosslinked microtubules in the presence of taccalonolides was also measured by centrifugation. Samples containing 1 μM taxoid binding sites and 500 nM Flutax-2 in GAB/0.1 mM GTP buffer were incubated at 37ºC for 2 hr with 100 μM taccalonolide A or E and an equal amount of DMSO. The samples were then centrifuged at 90,000 × g for 10 min at 37ºC in a TLA100 rotor employing a Beckman Optima TLX ultracentrifuge. The supernatants were collected by pipetting, and the pellets were resuspended in 10 mM phosphate (pH 7.0). The concentrations of Flutax-2 were fluorimetrically measured by employing a Fluorolog 3 spectrofluorimeter (excitation wavelength 495 nm, emission wavelength 520 nm using slits of 2 and 5 nm respectively). Flutax-2 concentration standard curves were constructed for each experiment by using spectrophotometrically measured concentrations of Flutax-2.

Equilibrium Binding Constants of the Ligands to Microtubules

The equilibrium binding constants of the different ligands to the paclitaxel binding site of assembled microtubules were measured as described [29]. Binding constants for compounds reversibly displacing Flutax-2 were calculated by using a PC program written by us (*Equigra v5*, J.F.D., unpublished software). This program fits the experimental data by least-squares to the equilibrium binding constant of the ligand investigated, by employing the known values of the reference ligand Flutax-2. The thermodynamic parameters (apparent ΔG^0, ΔH^0, and ΔS^0) were calculated from the binding constants at different temperatures.

The binding affinity constant for discodermolide was also measured by HPLC as follows: 65 ml of 10 nM taxoid binding sites in crosslinked microtubules were incubated for 30 min at 25ºC with increasing concentrations of the ligand. The mixtures were then centrifuged for 55 min at 45,000 rpm in a Ti45 rotor in a Beckman XL-90 ultracentrifuge. The supernatants were removed, and the pellets were resuspended in 20 ml of 10 mM sodium phosphate and left for 1 hr at 4ºC. The supernatants were extracted with 2 × 50 ml dichloromethane, and the pellets were extracted with 2 × 20 ml of the same solvent. After vacuum evaporation of the dichloromethane, the residues were dissolved in 30 μl 70% methanol in water (v/v). HPLC analysis was performed as described above.

Cell Culture

Human ovarian carcinoma 1A9, non-small cell lung carcinoma A549 cells, and monocytic leukemia U937 cells were cultured at 37ºC in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 40 μg/ml gentamycin, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 air atmosphere.

Indirect Immunofluorescence

A549 cells were plated at a density of 100,000 cells/ml onto 12 mm round coverslips, cultured overnight, and then treated with the ligands at different concentrations or drug vehicle (DMSO) for 24 hr. Residual DMSO was less than 0.5%. Attached A549 cells were permeabilized with Triton X-100 and fixed with 3.7% formaldehyde, as previously described [31, 41]. Cytoskeletons were incubated with a DM1A monoclonal antibody reacting with α-tubulin, washed twice, and incubated with FITC goat anti-mouse immunoglobulins. The coverslips were washed, 1 μg/ml Hoechst 33342 was added to stain chromatin, washed, examined, and photographed with a Zeiss Axioplan epifluorescence microscope, and the images were recorded with a Hamamatsu 4742-95 cooled CCD camera.

Cell Cytotoxicity Assay

1A9 or A549 cells were seeded in 96-well plates at a density of 10,000 cells in 0.05 ml per well. On the next day, cells were exposed to 0.05 ml serial dilutions (0.005 nM–40 μM) of ligands for 72 hr, at which time an MTT assay [42] was performed to determine viable cells with some modifications. Briefly, 20 μl of 2.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, incubated for 4 hr at 37ºC, and then treated with 0.1 ml MTT solubilizer (10% SDS, 45% dimethylformamide [pH 5.5]). Plates were again incubated overnight at 37ºC to solubilize the blue formazan precipitate before measuring the absorbance at 595/690 nm in an automated Multiscan microplate reader. Control wells containing medium without cells were used as blanks. The MTT response is expressed as a percentage of the control (untreated) cells. The IC_{50} was calculated from the log-dose response curves and expressed as the mean of at least four independent experiments ± standard deviation.

Cell Cycle Analysis

Progression through the cell cycle was assessed by flow cytometry DNA determination with propidium iodide. U937 cells (300,000 per ml) were incubated with several concentrations of the drugs for 20 hr. The cells were fixed with 70% ethanol, treated with RNase, and stained with propidium iodide as previously described [31]; analysis was conducted with a Coulter Epics XL flow cytometer.

Electron Microscopy

Aliquots of GTP-tubulin solutions (10–20 μM) incubated with the ligand in PEDTA4-1 mM GTP buffer at 37ºC for 30 min were adsorbed onto formvar/carbon-coated 300 mesh copper grids, stained with 1% uranyl acetate, and observed with a Jeol 1230 transmission electron microscope (JEOL, Tokyo, Japan).

Supplemental Data

Supplemental Data including cell cycle distribution of U937 cells treated with ligands are available at http://www.chembiol.com/cgi/content/full/12/12/1269/DC1/.

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