The Coral-Derived Natural Products Eleutherobin and Sarcodictyins A and B: Effects on the Assembly of Purified Tubulin with and without Microtubule-Associated Proteins and Binding at the Polymer Taxoid Site

Ernest Hamel,*,‡ Dan L. Sackett,‡ Dionisios Vourloumis,§ and K. C. Nicolaou§

Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702, Department of Chemistry and Skaggs Institute for Chemical Biology, Scripps Research Institute, La Jolla, California 92037, and Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093

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ABSTRACT: We examined interactions with purified tubulin of synthetic sarcodictyins A and B and eleutherobin (coral-derived antimitotic agents) and of compound **1**, an analogue of sarcodictyin A methylated at the C-3 oxygen atom (i.e., the methyl ketal analogue of sarcodictyin A and thus structurally similar to eleutherobin but lacking the C-3 sugar moiety). Eleutherobin was much more active than sarcodictyins A and B, which were somewhat more active than compound **1**. Effects of eleutherobin did not differ greatly from those of paclitaxel and epothilone A. Eleutherobin and epothilone A were competitive inhibitors of the binding of radiolabeled paclitaxel to tubulin polymer (apparent K_i values of 2.1 and 2.6 μM, respectively). Tubulin assembly reactions induced by all compounds were similar to the paclitaxel-driven reactions in being enhanced by the addition of microtubule-associated proteins and/or GTP to the reaction mixture and by progressively higher reaction temperatures. Antiproliferative activity was studied in six human cancer cell lines, including two paclitaxel-resistant lines with point mutations in a β-tubulin gene. Except for compound **1**, effects on cell growth were generally in accord with effects on purified tubulin. Thus, sarcodictyins A and B had IC_{50} values in the 200−500 nM range; paclitaxel, <10 nM (except in the resistant lines); and eleutherobin and epothilone A, 10−40 nM. The antiproliferative activity of compound **1** was more comparable to that of eleutherobin than sarcodictyin A, despite its weak interaction with tubulin. The activities of the sarcodictyins, eleutherobin, and compound **1** in the mutant ovarian lines were similar to their activities in the parental line.

Antimitotic agents that interact with microtubule components are of interest not only for the insights they can provide into the roles of microtubules in cells and the subtleties of tubulin structure but also for their potential activity in the treatment of human neoplastic, inflammatory, and parasitic diseases. From the point of view of cytotoxic effects on tumor cells in culture, the most potent compounds are those derived from natural sources, with diverse marine organisms yielding a large number of interesting agents in recent years (for a review, see ref *1*). Examples of marine-derived compounds shown to have in vivo antitumor activity in animal models include the peptides dolastatin 10, dolastatin 15, and hemiasterlin, the macrocyclic polyether halichondrin B, and the lipid curacin A. While these agents all act as inhibitors of tubulin assembly, the marine environment has also yielded compounds that have taxoid-like activity, that is to say, compounds that hypernucleate tubulin assembly and prevent polymer disassembly. These marine-derived agents have been obtained from sponges [discodermolide from *Discodermia dissoluta* (*2*, *3*); laulimalide from *Hyattella* sp., *Fasciospongia rimosa*, and *Cacospongia mycofijiensis* (*4*)] and corals [sarcodictyins A−D from *Sarcodictyon roseum* (*5*, *6*) and *Eleutherobia aurea* (*7*); eleutherobin from *Eleutherobia* sp. (*8*, *9*)]. The structures of these compounds are shown in Figure 1, along with those of paclitaxel, docetaxel, and epothilones A and B, which are taxoid mimetics of bacterial origin (*10*).

The clinical success of paclitaxel (*11*) and docetaxel (*12*) has heightened interest in compounds that share their basic mechanism of action. Typically, however, compounds derived from marine organisms are available in very restricted supplies as natural products, and even animal studies require synthetic material. This is especially true of eleutherobin and the sarcodictyins. More extensive studies are now possible, however, since eleutherobin, sarcodictyins A and B, and a series of analogues have been successfully synthesized (*13*−*19*).

* Address correspondence to Dr. E. Hamel; Building 37, Room 5D02; National Institutes of Health; Bethesda, MD 20892-4255. Tel: (301) 496-4855. Fax: (301) 402-0752 or 496-5839.

‡ Frederick Cancer Research and Development Center.

§ Scripps Research Institute and University of California at San Diego.

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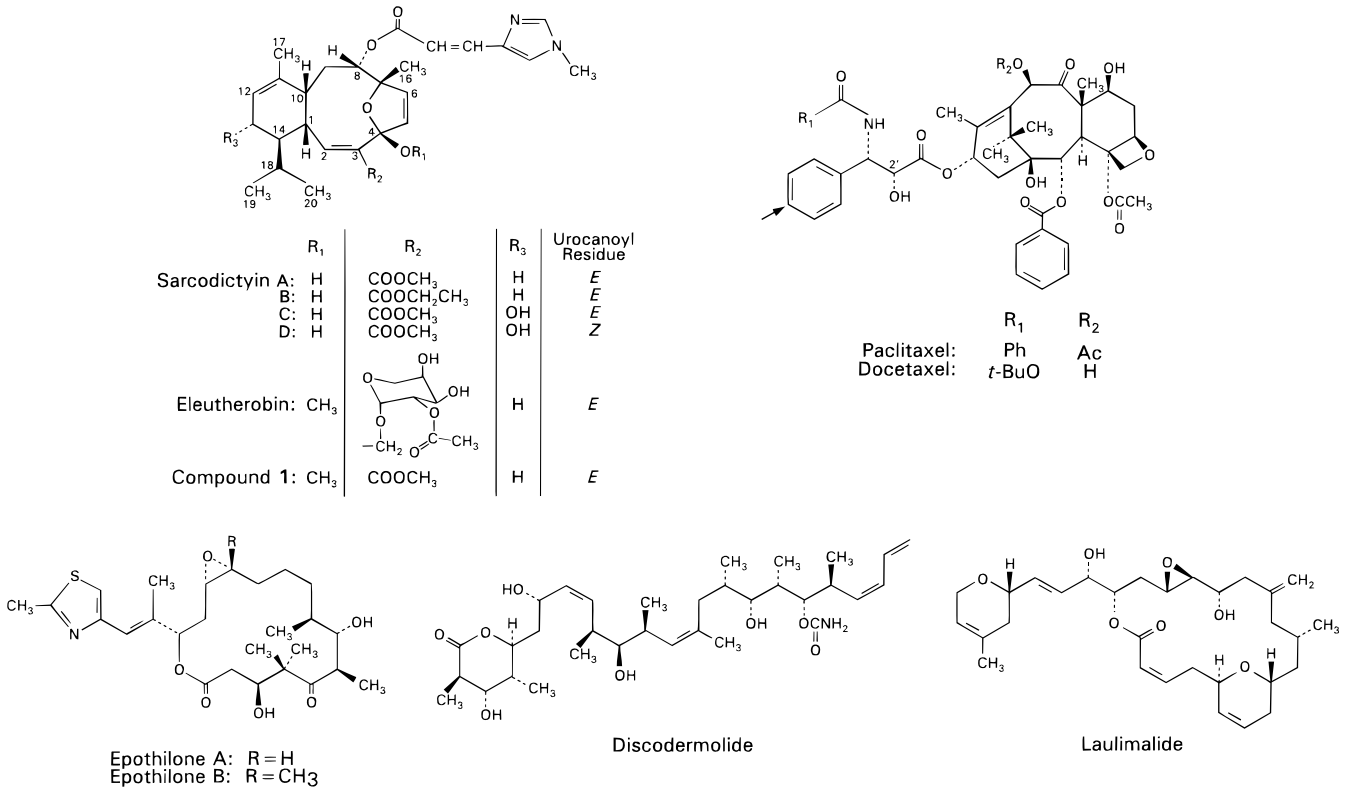

FIGURE 1: Structures of paclitaxel, docetaxel, eleutherobin, sarcodictyins A and B, compound **1**, epothilones A and B, discodermolide, and laulimalide. The urocanoyl residue is the substituent at C-8 in eleutherobin and the sarcodictyins. *E* and *Z* refer to the trans and cis isomers, respectively. The arrow in the taxoid structural diagram indicates the radiolabeled position in the [3"-^{3}H]paclitaxel used in some of the studies presented here.

The original report on taxoid-like activity in the sarcodictyins (*20*) described the compounds as equipotent with paclitaxel in a reaction condition using microtubule protein (unresolved tubulin + MAPs[1]) but of much lower relative cytotoxicity. In contrast, eleutherobin was described as having effects on cell growth not very different from those of paclitaxel, and it, too, was reported to be similar to paclitaxel in its effects on microtubule protein (*21*). In addition, the sarcodictyins (*20*) and eleutherobin (*21*) inhibited the binding of radiolabeled paclitaxel to polymerized microtubule protein, but the type of inhibition was not rigorously documented.

Preliminary studies with synthetic eleutherobin (*14*) and synthetic sarcodictyins A and B (*16*) were largely in agreement with data obtained with the natural products. A screening filtration assay with microtubule protein indicated that all compounds were similar in potency to paclitaxel in the ability to induce polymer formation. Synthetic sarcodictyin B, but not sarcodictyin A, appeared to have antiproliferative activity against an ovarian carcinoma cell line comparable to the activity of paclitaxel and epothilone A (*16*), in contrast to the report with the natural product (*20*).

Our goal in the studies reported here was to fully document that these compounds interacted with tubulin, as opposed to MAPs, by working with a highly purified tubulin preparation and to unambiguously demonstrate whether binding was at the paclitaxel site by kinetic analysis. We also wished to clarify the contradictory data obtained with the sarcodictyins by looking at suboptimal reaction conditions and performing more quantitative analyses, as well as to evaluate the antiproliferative activity of these agents in additional cell lines.

MATERIALS AND METHODS

Materials. Eleutherobin (*14*, *18*), sarcodictyin A (*13*, *17*), sarcodictyin B and compound **1** (structure in Figure 1) (*16*, *17*), epothilones A and B (*22*), and tubulin and heat-treated MAPs (*23*) were prepared as described previously. Unbound nucleotide was removed from the tubulin by gel filtration chromatography (*24*). Paclitaxel and [3"-^{3}H]paclitaxel (19.3 Ci/mmol) were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. Docetaxel and discodermolide were generous gifts, respectively, from Dr. D. G. I. Kingston, Virginia Polytechnic and State University, Blacksburg, VA, and Dr. R. E. Longley, Harbor Branch Oceanographic Institution, Fort Pierce, FL. GTP, from Sigma, and ddGTP, from Pharmacia, were repurified by anion exchange chromatography on DEAE-cellulose, and the samples used in these studies were >99% pure at the time of isolation.

Methods. Tubulin assembly was followed in Gilford model 250 recording spectrophotometers equipped with electronic temperature controllers. Temperature equilibration of cuvette contents occurs at about 0.5 °C/s when the temperature increases and about 0.1 °C/s when the temperature decreases. Unless otherwise indicated, 0.25 mL reaction mixtures contained 1.0 mg/mL (10 μM) tubulin, 0.75 mg/mL heat-treated MAPs, 0.1 mM GTP, 0.1 M Mes (0.5 M stock solution adjusted to pH 6.9 with NaOH), 4% dimethyl

[1] Abbreviations: MAPs, microtubule-associated proteins; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; Mes, 4-morpholineethanesulfonate.

sulfoxide as drug solvent, and 10 μM drug. Reaction mixtures without drug were added to cuvettes held at 0 °C, and drug was rapidly mixed into the reaction mixture. The turbidity of the reaction mixtures was followed for 10 min at 0 °C, but no reaction occurred under any of the reaction conditions shown. The temperature was subsequently changed as indicated.

Tubulin polymerization assayed by centrifugation was performed as described previously (*25*). Two reaction conditions were used. In the first the 100 μL reaction mixtures (in 0.5 mL microfuge tubes) contained 0.4 M monosodium glutamate (2.5 M stock solution adjusted to pH 6.6 with HCl), 1.0 mg/mL tubulin, 5% dimethyl sulfoxide, and varying drug concentrations. In the second, the glutamate concentration was 0.65 M and GTP at 0.1 mM was added to the reaction mixtures (other reaction components the same). Incubation was for 20 min at room temperature, and the polymer was removed by centrifugation in an Eppendorf model 5417C centrifuge (with tube adapters) for 10 min at 14 000 rpm at room temperature. The protein content of 20 μL of the supernatants was determined by the Lowry procedure.

The binding of [3"-^{3}H]paclitaxel to tubulin polymers was determined essentially as described previously (*26*), except that only the supernatant radiolabel content was determined. The solvent was evaporated from the radiolabeled paclitaxel, and the residue was mixed with nonradiolabeled paclitaxel to yield a 50 μM stock solution with a specific activity of 1760 cpm/pmol in 50% (v/v) dimethyl sulfoxide. This radiolabeled paclitaxel and inhibitor, as indicated in terms of final reaction concentrations, were mixed in 50 μL (0.75 M glutamate, 20% dimethyl sulfoxide) and warmed to 37 °C. Meanwhile, a reaction mixture containing 0.75 M glutamate, 0.25 mg/mL (2.5 μM) tubulin, and 25 μM ddGTP was prepared and incubated for 30 min at 37 °C. To each drug mixture was added 0.2 mL of the polymerized tubulin mixture, and incubation was continued for 30 min at 37 °C. During the incubation 0.15 mL of each reaction mixture was transferred to a 1.5 mL microfuge tube. These tubes were centrifuged at 14 000 rpm without adapters at room temperature for 20 min, and 100 μL of each supernatant was counted. We found that the pellets were very soft and easily aspirated, so that satisfactorily reproducible data could only be obtained from the supernatants. In some experiments the total reaction mixture as well as the supernatants was counted, but the counts were generally within 2% of the expected values. Thus, bound paclitaxel was calculated by subtracting the supernatant paclitaxel (assumed to be free drug) from the total added to each reaction mixture.

Effects of drugs on cell growth were examined after a 4 day incubation in the presence of drug. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. The medium used for all cell lines was RPMI-1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cell protein was the parameter measured, following staining with sulforhodamine B (*27*). The assays were performed in 96-well microtiter plates. The human ovarian carcinoma line 1A9 and the paclitaxel-resistant mutants derived from it were described previously (*28*). The other cell lines used were provided by the NCI drug-screening program. Prior to being plated, cells were grown from low density to less than half confluency to obtain an asynchronous population of cells growing exponentially. Cells were plated at a density of 1000 per well in 0.1 mL medium and allowed to grow for 24 h prior to drug addition.

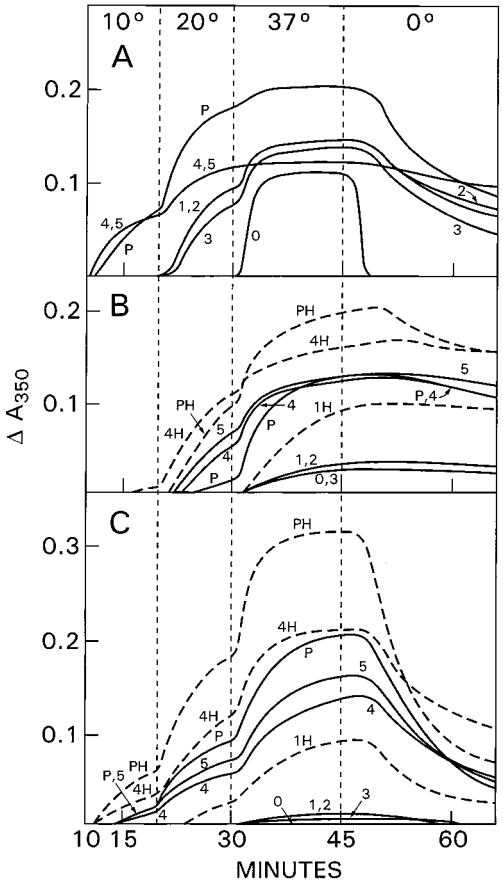

FIGURE 2: Comparison of the effects of eleutherobin, sarcodictyins A and B, and compound **1** with those of paclitaxel and epothilone A on tubulin assembly with both GTP and heat-treated MAPs (A), with heat-treated MAPs only (B), and with GTP only (C). In each panel drugs are indicated as follows: curve 0, no drug; curve P, paclitaxel; curve 1, sarcodictyin A; curve 2, sarcodictyin B; curve 3, compound **1**; curve 4, eleutherobin; curve 5, epothilone A. The letter H indicates that the drug concentration was 40 μM rather than 10 μM.

RESULTS

*Effects of Eleutherobin, Sarcodictyins A and B, and Compound **1** on Tubulin Assembly ± MAPs or GTP: Comparison with Paclitaxel and Epothilone A.* We found that reaction conditions had a considerable impact on the comparative activity observed with the "sarcodictyins" (we will use this term to encompass compound **1** and both sarcodictyins A and B) versus paclitaxel, and in these experiments it also became clear that the activity of eleutherobin was similar to that of paclitaxel and, particularly, to that of epothilone A. In the experiments shown in Figure 2, drug was always the final component added to a reaction mixture held at 0 °C, and tubulin and drug were both at 10 μM, unless otherwise indicated. In all cases, the reactions were followed for 10 min at 0 °C, and no reaction was observed in these experiments. To emphasize the differences among the compounds, the reaction temperature was increased stepwise to 10, 20, and 37 °C, with a final 20 min at 0 °C to evaluate the cold stability of the polymers formed.

When both MAPs and GTP were included in the reaction mixture (Figure 2A), reactions with paclitaxel, epothilone A, and eleutherobin all occurred at 10 °C, although the lag period was about 45 s longer with paclitaxel. At 20 °C reactions occurred with the three sarcodictyins, with compound **1** appearing slightly less potent than sarcodictyins A and B. At 20 °C the turbidity increased much more with paclitaxel than with eleutherobin or epothilone A (see below). At 37 °C assembly occurred without drug, and the sarcodictyin reaction mixtures became more turbid. There was little turbidity change with paclitaxel, eleutherobin, or epothilone A. When the temperature was returned to 0 °C, the normal polymer rapidly disappeared, as expected, while those formed with eleutherobin and epothilone A were almost completely stable. A slow drop in turbidity occurred in the reaction mixtures containing paclitaxel and the sarcodictyins.

With no GTP in the reaction mixtures (Figure 2B, MAPs only) with 10 μM drug there were no assembly reactions at 10 °C. At 20 °C there was a feeble reaction with paclitaxel, and brisker reactions with eleutherobin and epothilone A, which seemed slightly more active than eleutherobin. At 37 °C these reaction mixtures all became more turbid and reached similar plateaus. Relative to the reaction mixture without drug, there were negligible reactions with the sarcodictyins. The sarcodictyins, however, are not totally inert with tubulin + MAPs − GTP, since a reaction at 37 °C could be produced by increasing their concentrations (shown for sarcodictyin A at 40 μM in Figure 2B). Qualitatively similar enhanced reactions occurred with the more active drugs, too, as shown for both paclitaxel and eleutherobin at 40 μM (Figure 2B).[2] All drug-induced polymers were highly stable when the temperature was returned to 0 °C.

With no MAPs in the reaction mixtures (Figure 2C, GTP only) with 10 μM drug there were feeble reactions at 10 °C with paclitaxel, eleutherobin, and epothilone A, with progressive further increases in turbidity at 20 and 37 °C. Without MAPs the relative compound activity, based on turbidity readings, was paclitaxel > epothilone A > eleutherobin in contrast to without GTP, where it was epothilone A > eleutherobin > paclitaxel, and to the complete system, where eleutherobin and epothilone A behaved identically while paclitaxel showed a slightly longer lag phase but a higher plateau than the other drugs. In the tubulin + GTP − MAPs system the sarcodictyins were essentially inert at 10 μM, but reactions did occur with 40 μM drug, as shown for 40 μM sarcodictyin A (Figure 2C). A qualitatively similar enhancement also occurred with higher concentrations of the more active compounds (40 μM paclitaxel- and eleutherobin-induced reactions shown in Figure 2C). When the temperature was returned to 0 °C, there was extensive, but slow, disassembly observed with all drug-induced polymers.

[2] It should be noted that formulation studies at the National Cancer Institute (data of the Pharmaceutical Resources Branch, provided by Dr. E. Tabibi) demonstrated limited solubility of paclitaxel in a variety of aqueous media (5−9 μg/mL, equivalent to 6−11 μM). While paclitaxel does bind avidly and stoichiometrically to tubulin polymers (for example, see below in binding inhibition section), 40 μM probably exceeds the solubility of the drug in the reaction condition used in the studies of Figure 2, and this amount of paclitaxel probably represents saturating drug. The amounts of eleutherobin and the sarcodictyins prepared thus far have been inadequate to determine their solubility in aqueous buffer conditions. Epothilone A has been reported to be 30-fold more soluble than paclitaxel (*38*).

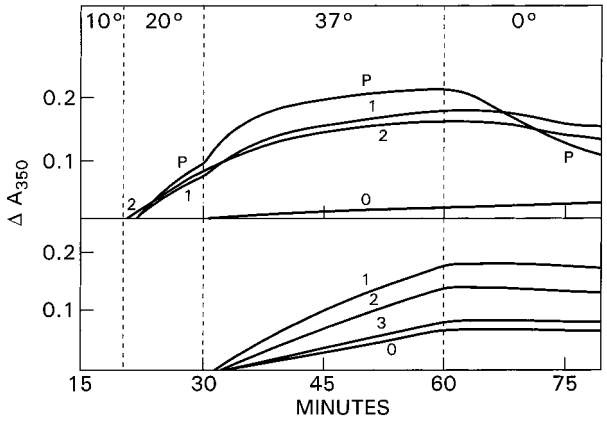

FIGURE 3: Drug-induced assembly of purified tubulin in the absence of both MAPs and GTP. (upper panel) Reaction mixtures contained 20 μM tubulin and 20 μM drug, as follows: curve 0, none; curve P, paclitaxel; curve 1, eleutherobin; curve 2, epothilone A. (lower panel) Reaction mixtures contained 60 μM tubulin and 40 μM drug, as follows: curve 0, none; curve 1, sarcodictyin A; curve 2, sarcodictyin B; curve 3, compound **1**.

With 10 μM tubulin alone (that is, without MAPs or GTP) none of these drugs at 10 μM induced an increase in turbidity relative to that observed without drug (data not presented). Reactions did occur with 20 μM tubulin + 20 μM paclitaxel, eleutherobin, and epothilone A (Figure 3, upper panel), and with 60 μM tubulin + 40 μM sarcodictyins A and B (Figure 3, lower panel; a minimal reaction relative to the control without drug also occurred with compound **1**). Except for the paclitaxel-induced polymer, there was relatively little loss of turbidity when the reaction temperature was reduced to 0 °C from 37 °C.

These last two reaction conditions unambiguously establish tubulin as the primary target for eleutherobin and the sarcodictyins. Both MAPs and GTP modulate the interaction, however, with effects on both the characteristics of enhancement of assembly and on polymer stability.

Morphological Evaluation of Drug-Induced Polymer Formed with MAPs and GTP. We investigated further the difference in turbidity levels observed at 20 °C in the MAPs/GTP system with paclitaxel versus eleutherobin or epothilone A (Figure 2A). One possibility was that assembly was much more extensive with paclitaxel as compared with the other drugs, but the measurement of protein remaining in the supernatant following centrifugation of reaction mixtures (methodology as described in ref *25*) demonstrated that this was not the case. In this experiment 28% of the total protein remained in the supernatant following polymerization with eleutherobin, 34% with epothilone A, and 35% with paclitaxel.

This result indicated that the turbidity differences probably resulted from differences in polymer morphology, so electron microscopic evaluation (negatively stained specimens) was performed on samples comparable to those shown in Figure 2A after the reaction mixtures had been at 20 °C for 15 min. Bundling of microtubules in these samples was not prominent with any drug. There was some evidence that the drugs had different potential for the induction of sheet polymers, in that these were rarely seen in the eleutherobin specimen and seen occasionally in the epothilone A specimen. In contrast, in the paclitaxel specimen a sheet polymer was observed in nearly every low power field. This difference between the

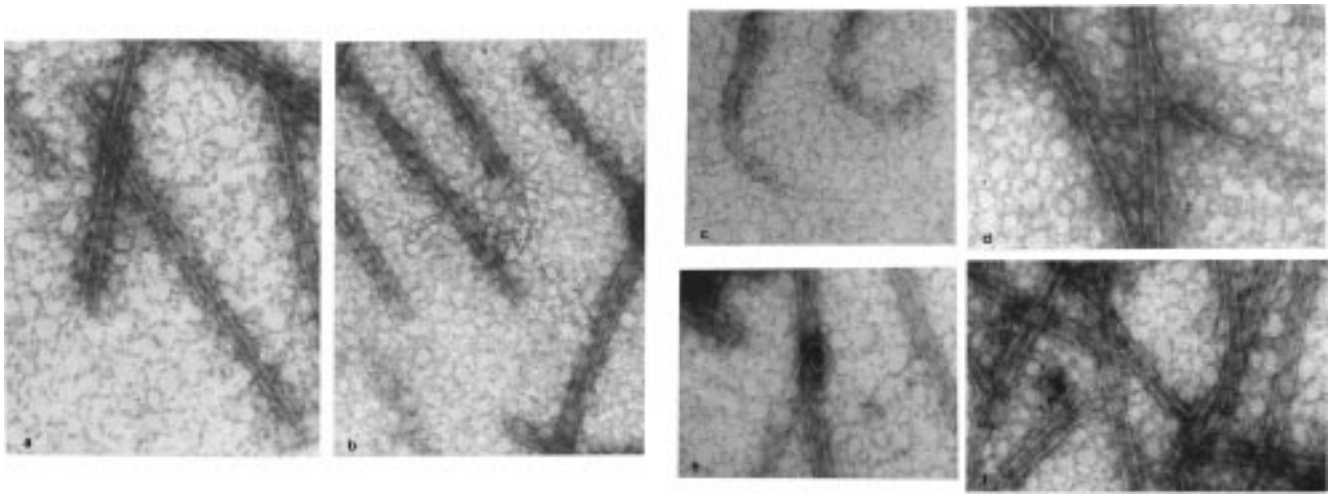

FIGURE 4: Morphology at polymer ends following assembly to 20 °C with MAPs and GTP as shown in Figure 2A. Incubation was at 20 °C for 15 min prior to preparation of grids. Drugs were 10 μM eleutherobin (A), 10 μM epothilone A (B), and 10 μM paclitaxel (C−F). Magnification: ×76000.

samples, however, did not appear to account fully for the differences in turbidity development.

The most striking morphological differences occurred at microtubule ends (Figure 4), and differences were also observed at the microtubule walls. With both eleutherobin (Figure 4A) and epothilone A (Figure 4B) virtually all microtubule ends were relatively blunt, and wall defects were rare. In contrast, with paclitaxel a large proportion of microtubule ends displayed attached sheet polymers or what appear to be splaying microtubule protofilaments (Figure 4C−F). Not infrequently protofilaments appeared to emerge from microtubule walls (Figure 4D), and in many cases these seemed to give rise to sheet polymers. We thus conclude that the higher turbidity levels that occur in the MAPs/GTP system with paclitaxel as compared with eleutherobin and epothilone A result from differences in polymer morphology rather than from more extensive assembly.

Quantitative Analysis of Eleutherobin and Sarcodictyin Effects on Tubulin Polymerization. It is probably unrealistic to expect any particular assay for paclitaxel-mimetic compounds to fully characterize the interaction of these drugs with tubulin. It is nonetheless useful to have conditions that allow a quantitative comparison of the interactions of drugs of this type with their target. We have analyzed glutamate as a tool for this purpose, since it allows extensive modulation of the tubulin−drug interaction and the design of assays in which near total assembly at room temperature is entirely drug-dependent (*25*).

By using the reaction condition we have studied most extensively (10 μM tubulin, 0.4 M glutamate, no GTP) that was designed in particular to identify compounds more active than paclitaxel (*25*, *29*), we obtained data (Figure 5A) entirely in accord with the experiments described above. Almost no assembly occurred with the three sarcodictyins at concentrations as high as 50 μM, while the polymerization reactions induced by paclitaxel, eleutherobin, and epothilone A were quantitatively almost identical (the experiment shown in Figure 5A yields EC_{50} values of 9.4, 11, and 9.6 μM for the three drugs, respectively).

However, we wished to demonstrate that a reaction condition could be designed with pure tubulin at room temperature in which relatively low EC_{50} values could be

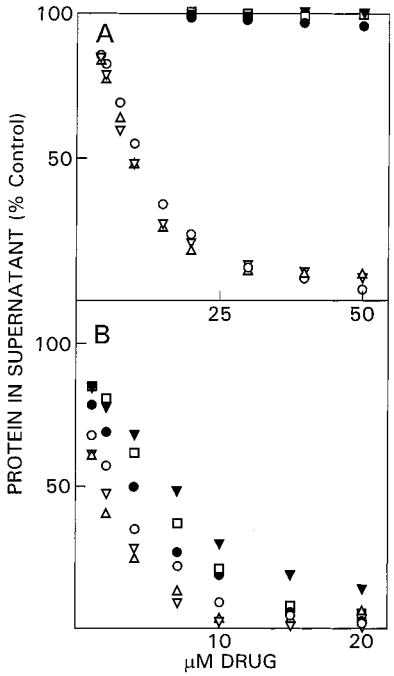

FIGURE 5: Induction of tubulin polymerization in glutamate at room temperature by eleutherobin, sarcodictyins A and B, compound **1**, paclitaxel, and epothilone A: (A) assembly with 0.4 M glutamate (no GTP); (B) assembly with 0.65 M glutamate + 0.1 mM GTP. In each experiment supernatant protein with drug was compared to supernatant protein in the absence of drug. In these experiments if drug was not added, over 95% of the added tubulin remained in the supernatant following centrifugation as described in the text: ○, eleutherobin; ▽, paclitaxel; △, epothilone A; ●, sarcodictyin A; □, sarcodictyin B; ▼, compound **1**.

obtained for the sarcodictyins, too. In previous studies, the similarity of the sarcodictyins to paclitaxel had been demonstrated at 37 °C in reactions in which MAPs were present (*16*, *20*). We found that this could not be achieved without GTP in the reaction mixture, but at high glutamate concentrations (0.8−1.0 M) with GTP there was some assembly at room temperature without drug. In short, we found that the most active system with 10 μM tubulin without assembly in the absence of drug consisted of 0.65 M glutamate + 0.1 mM GTP, and an experiment under this reaction condition

Table 1: Relative Activities of Selected Inducers of Microtubule Assembly as Inhibitors of the Binding of [3"-^{3}H]Paclitaxel to Polymers Formed from Purified Tubulin[a]

	% inhibition ± SD	
inhibitor added	4 μM inhibitor	20 μM inhibitor
eleutherobin	51 ± 6	
epothilone A	46 ± 3	
epothilone B	63 ± 5	
docetaxel	61 ± 6	
discodermolide	70 ± 4	
sarcodictyin A	21 ± 3	38 ± 0.5
sarcodictyin B	17 ± 3	25 ± 4
compound **1**	8.3 ± 5	4.5 ± 5

[a] The reaction conditions in the final reaction mixtures were 0.75 M glutamate, 2.0 μM tubulin, 2.0 μM [3"-^{3}H]paclitaxel, 4% dimethyl sulfoxide, inhibitor at 4.0 or 20 μM as indicated, and 20 μM ddGTP (prior to the assembly reaction). Reaction conditions are described in detail in the text. In these experiments 20-30% of the added radiolabeled paclitaxel was recovered in the supernatants in the absence of inhibitors. The 4.0 μM inhibitor data represent 4-8 repetitions with each drug; the 20 μM experiment was performed twice with sarcodictyins A and B and 8 times with compound **1**.

is shown in Figure 5B. Note the much reduced drug concentrations required for 50% assembly as compared with the experiment shown in Figure 5A. These data yield EC_{50} values of 1.7, 1.5, 2.5, 4.0, 5.2, and 6.5 μM, respectively, for paclitaxel, epothilone A, eleutherobin, sarcodictyin A, sarcodictyin B, and compound **1**. Although the more active agents still yield lower EC_{50} values under this reaction condition, there is now only a 2−3-fold difference between them and the sarcodictyins, and it is likely that further manipulations could yield comparable EC_{50} values.

Eleutherobin Is a Classic Competitive Inhibitor of the Binding of Radiolabeled Paclitaxel to Tubulin. In previous studies we found that we obtained the best data for analysis by driving most (about 90%) of the tubulin into polymer prior to adding radiolabeled paclitaxel ± inhibitor to the reaction mixtures. This was accomplished by inducing polymerization with ddGTP in 0.75 M glutamate (*26*). We wished this to be a microfuge-based assay for convenience, and higher glutamate concentrations, because of their viscosity, interfered with rapid polymer recovery in such an instrument since ddGTP-induced polymers are very small (*30*). In addition, since formulation studies at the National Cancer Institute had demonstrated a maximum aqueous solubility of paclitaxel of about 10 μM (see above), we felt that a meaningful assay required the use of relatively low concentrations of tubulin, paclitaxel, and inhibitors.

With 2 μM tubulin, initial studies with [3"-^{3}H]paclitaxel concentrations in the range 0.5−4 μM showed that 96% of the added paclitaxel bound to the polymer with 0.5 μM drug (stoichiometry, 0.24), 94% bound with 1.0 μM drug (stoichiometry, 0.47), 71% bound with 2.0 μM drug (stoichiometry, 0.71), 58% bound with 3.0 μM drug (stoichiometry, 0.87), and 52% bound with 4.0 μM drug (stoichiometry, 1.03). We selected 2.0 μM tubulin (in ddGTP-induced polymer) + 2.0 μM paclitaxel as the reaction condition for a preliminary study of inhibition of paclitaxel binding to polymer (Table 1). When potential inhibitors were compared at 4.0 μM, relative activities were consistent with their effects on tubulin assembly reactions. Thus, epothilone B was more effective than epothilone A, and discodermolide was the most inhibitory compound examined. The activities of eleutherobin and epothilone A were very similar. The sarcodictyins were the least active compounds examined in this assay. Even a 5-fold increase in their concentration did not result in inhibition comparable to that obtained with 4.0 μM eleutherobin, and compound **1** showed no significant inhibition of binding of [3"-^{3}H]paclitaxel to the tubulin polymer at either 4 or 20 μM.

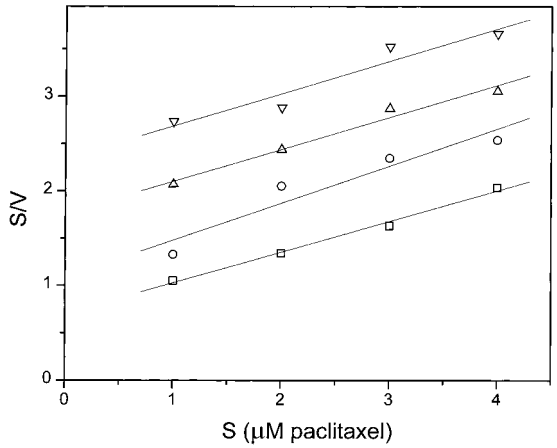

FIGURE 6: Competitive inhibition of the binding of [3"-^{3}H]paclitaxel to tubulin polymer by eleutherobin. Reaction mixtures contained the components described in the text, the indicated concentrations of paclitaxel, and the following concentrations of eleutherobin: □, none; ○, 2 μM; △, 4 μM; ▽, 6 μM. The data are presented in the Hanes format, with the lines drawn by linear regression using Origin Microcal Version 4.1. Ordinate units: micromolar total paclitaxel divided by micromolar bound paclitaxel.

This result led us to select eleutherobin for more detailed study, and the results are shown in Figure 6. The data are presented in the Hanes format (*31*), in which "substrate" (paclitaxel) concentration is plotted against substrate concentration divided by "reaction rate" (paclitaxel bound). Competitive-type inhibition is indicated by a pattern of parallel curves generated at different inhibitor (eleutherobin) concentrations, as opposed to other patterns (for example, with noncompetitive inhibition the curves intercept at the negative abscissa). Dixon analysis (*31*) of the data from three experiments yielded an apparent K_i value of 2.1 ± 0.3 μM for eleutherobin. A single experiment was performed with epothilone A for comparison. This confirmed our earlier report (*26*) that epothilone A was a competitive inhibitor of paclitaxel binding to tubulin polymer. Dixon analysis of the epothilone A experiment yielded an apparent K_i value of 2.6 μM (cf. data of Table 1).

*Eleutherobin and Compound **1**, but Not Sarcodictyins A and B, Significantly Inhibit the Growth of a Variety of Human Tumor Cells.* The published cytotoxicity data with eleutherobin (*21*) and the sarcodictyins (*16*) are consistent with the above observations with tubulin for eleutherobin and sarcodictyin A, but not for sarcodictyin B and compound **1**. Initial studies with the latter two compounds showed them to have significant effects on the growth of an ovarian carcinoma cell line. We therefore decided to evaluate these agents more extensively in a series of cancer lines, including two ovarian lines (1A9PTX10 and 1A9PTX22) that are resistant to paclitaxel on the basis of point mutations in a gene for β-tubulin (*28*).

We compared the effects of eleutherobin and the sarcodictyins with those of paclitaxel, docetaxel, and epothilones

Table 2: Comparison of Antiproliferative Properties of Eleutherobin and the Sarcodictyins with Those of Paclitaxel and Epothilones and to Quantitative Measures of Interactions with Tubulin

	effects on human cancer cell growth[a] IC_{50} (nM)						quantitative measures of interactions with tubulin	
	prostate carcinoma	melanoma	breast carcinoma	ovarian carcinoma			inhibition of taxol binding	promotion of assembly[b]
compound	PC3	LOX-IMV1	MCF-7	1A9	1A9PTX10	1A9PTX22	K_i (μM)	EC_{50} values (μM)
paclitaxel	4	6	2	4	60	60		9.4/1.7
docetaxel	0.9	0.9	0.5	2	10	20	1.3[c]	5.8/1.7
eleutherobin	20	30	10	40	60	30	2.1	11/2.5
sarcodictyin A	200	400	300	300	200	300		> 50/4.0
sarcodictyin B	200	500	400	300	300	300		> 50/5.2
compound **1**	50	80	300	20	20	10		> 50/6.5
epothilone A	10	10	5	10	40	10	2.6	9.6/1.5
epothilone B	0.9	0.9	0.4	1	3	1	1.7[c]	2.7/

[a] Cells were grown for 4 days as monolayer cultures in microtiter plates. The IC_{50} is the drug concentration that reduced cell protein by 50%. [b] Data from Figure 5, except for the values for docetaxel and epothilone B, which were normalized to the paclitaxel data from other experiments performed in the same manner. [c] These values are normalized to the apparent K_i value of epothilone A obtained in the current experiments. The actual values obtained for docetaxel, epothilone B, and epothilone A in previous experiments (*26*, *35*) were 0.3, 0.4, and 0.6 μM, respectively.

A and B. These cell growth data are summarized in Table 2, together with the quantitative measures of the interactions of these drugs with tubulin described above. Generally, epothilone B and docetaxel had similar effects on the growth of the cell lines examined and were more active than paclitaxel, in accord with their greater activity with tubulin. At the other extreme, we found sarcodictyins A and B to have limited ability to inhibit the growth of these cell lines, and there was little difference between these two compounds. This was in agreement with their reduced activities with tubulin. Eleutherobin and epothilone A had similar activities against the cell lines examined, but both compounds were less active than paclitaxel in all of the cell lines examined, with the exception of the paclitaxel-resistant lines. This was in contrast to the similar activity with tubulin of all three drugs.

However, it was with compound **1** that the discrepancy between effects on cell growth and effects on tubulin was greatest. This compound was probably the least active with tubulin of those examined here (Figure 5B; Table 1), but with the exception of the MCF-7 breast carcinoma line, its effects on cell growth did not differ greatly from those of eleutherobin. While it is not uncommon to identify compounds that have potent effects on tubulin assembly reactions with limited effects on cell growth (for example, see ref *32*, in which an extensive series of dolastatin 10 analogues was examined), it is unusual to observe analogues of active agents with good cytotoxicity despite a limited effect on tubulin-based reactions. Typically such observations can be readily explained in terms of the regeneration of an active compound in tissue culture medium. For example, the cytotoxicity of 2′-acetylpaclitaxel was rationalized in terms of a deacetylase in serum (*33*), and the cytotoxic activity of cryptophycin 8 has been attributed to rapid hydrolysis of its chlorhydrin components to regenerate a key epoxide moiety (*34*). A similar straightforward explanation for the activity of compound **1** is elusive, although intracellular metabolism at the C-3 substituent cannot be excluded.

Finally, our results with the paclitaxel-resistant mutants should be noted. In the experiments summarized in Table 2, both the 1A9PTX10 and 1A9PTX22 lines were 15-fold resistant to paclitaxel relative to the parental 1A9 line. There was some restoration of sensitivity with docetaxel (5- and 10-fold resistance in the two lines). As noted previously (*22*), with the epothilones 1A9PTX10 retained some resistance, while 1A9PTX22 was as sensitive to these agents as was the parental cell line. As was previously noted with discodermolide (*35*), full sensitivity of both mutant lines was observed with eleutherobin and compound **1**, as well as with sarcodictyins A and B. Our result showing full sensitivity of the 1A9PTX22 line to eleutherobin differs from the result of Long et al. (*21*), who found this cell line to be relatively resistant to eleutherobin.

DISCUSSION

In studies with tubulin plus MAPs and GTP with a temperature jump from 0 to 37 °C the sarcodictyins had activity in inducing assembly not that different from the activity that occurred with paclitaxel, consistent with the published studies almost entirely performed with microtubule protein (*16*, *20*). It was only as we made the assembly reaction conditions more stringent by lowering reaction temperature and/or omitting MAPs or GTP that it became clear that the sarcodictyins were much less potent than paclitaxel, eleutherobin, and epothilone A, while the activity of eleutherobin under virtually all conditions examined differed little from that of paclitaxel or epothilone A. These results emphasize that quantitative differences between paclitaxel-mimetic agents are most readily apparent when assembly reactions normally do not occur and, in our opinion, should be a routine aspect of the evaluation of this class of compounds.

Our use of MAP-free tubulin clearly establishes that the target of the sarcodictyins and eleutherobin is tubulin itself, with the MAPs having a modulating effect on the reaction. Qualitatively, these effects with eleutherobin and the sarcodictyins are similar to those observed with paclitaxel. Moreover, we were able to demonstrate with a polymer formed with pure tubulin that eleutherobin was a competitive inhibitor of the binding of radiolabeled paclitaxel to the polymer. We had previously shown similar competitive inhibition patterns with epothilones A and B and discodermolide, as well as docetaxel, and in the current studies we found only a minor difference in the apparent K_i values for eleutherobin and epothilone A (consistent with the assembly data). A competitive inhibition pattern is generally interpreted

as demonstrating that inhibitor and "substrate" bind in the same site, although allosteric effects cannot be completely excluded as a possibility. The recent molecular structure determination of paclitaxel-stabilized zinc sheet tubulin polymers (*36*) at least raises the possibility that binding of eleutherobin, epothilones, and/or discodermolide in the paclitaxel site or in an allosteric inhibitory site can be distinguished by a physical method.

Although this study was not designed as a structure−activity analysis of the interactions of the eleutherobin/sacrcodictyin class of drugs with tubulin and tubulin polymers, the weak activity of compound **1** relative to eleutherobin, together with the similarity of its activity to that of sarcodictyin A, indicates that either a sugar moiety or a bulky substituent at C-3 will be required for maximal interaction of a sarcodictyin analogue with tubulin.

We also performed a more extensive analysis of the antiproliferative effects of the sarcodictyins and eleutherobin than has been published thus far. Except for compound **1**, our cell growth data are in reasonable accord with the biochemical effects of these agents, although our results are not in complete accord with those obtained previously.[3] The most important observations from our studies are (1) that the two paclitaxel-resistant ovarian carcinoma cell lines retained complete sensitivity to eleutherobin and the three sarcodictyins relative to the parental 1A9 cells *and* (2) confirmation (cf. ref *16*) that compound **1** is an active agent despite its weak interaction with tubulin (most apparent from its inability to prevent binding of radiolabeled paclitaxel to tubulin polymers, Table 1).

Compound **1** structurally is a blend of sarcodictyin A and eleutherobin, in that it differs from the former only in having a methoxy instead of a hydroxy substituent at C-4 and from the latter in having a methylcarboxylate substituent at C-3 instead of the sugar moiety. Like sarcodictyin A compound **1** interacts poorly with tubulin, but like eleutherobin compound **1** has substantial antiproliferative activity. The cellular data reported here and previously (*16*) for compound **1** suggest that a sugar moiety may not be required for maximal interaction of this class of agent with tubulin and that compound **1** undergoes some intracellular transformation that enhances its interaction with tubulin. Alternative possibilites are that compound **1** might have a particularly potent effect on microtubule dynamics (cf. ref *37*) that would account for its disproportionate antiproliferative effects, that compound **1** might be more efficiently transported into cells as compared with sarcodictyins A and B, or that compound **1** could actually have an alternate intracellular target. The activity of this agent in cells suggests that further synthetic efforts with the sarcodictyin class would yield compounds with activities greater than that of paclitaxel in cells and perhaps with tubulin as well.

[3] The major differences were as follows: (1) eleutherobin had an IC_{50} value less than twice that of paclitaxel in 1A9 cells (called A2780 cells), as compared to our 10-fold lower value, while the 1A9PTX22 cells showed a relative resistance of 4.3, compared with our finding of no difference between mutant and parental lines (see ref *21*); and (2) sarcodictyin B yielded an IC_{50} value equivalent to that of paclitaxel in 1A9 cells, with the two mutant lines resistant to the agent (*16*), while in the current studies we find sarcodictyin B to have low activity, with similar low activity in mutant lines.

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