Epothilones, a New Class of Microtubule-stabilizing Agents with a Taxol-like Mechanism of Action

Daniel M. Bollag, Patricia A. McQueney, Jian Zhu, Otto Hensens, Lawrence Koupal, Jerrold Liesch, Michael Goetz, Elias Lazarides,1 and Catherine M. Woods2


ABSTRACT

Tubulin polymerization into microtubules is a dynamic process, with the equilibrium between growth and shrinkage being essential for many cellular processes. The antineoplastic agent taxol hyperstabilizes polymerized microtubules, leading to mitotic arrest and cytotoxicity in proliferating cells. Using a sensitive filtration-calorimetric assay to detect microtubule nucleating activity, we have identified epothilones A and B as compounds that possess all the biological effects of taxol both in vitro and in cultured cells. The epothilones are equipotent and exhibit kinetics similar to taxol in inducing tubulin polymerization into microtubules in vitro (filtration, light scattering, sedimentation, and electron microscopy) and in producing enhanced microtubule stability and bundling in cultured cells. Furthermore, these 16-membered macrolides are competitive inhibitors of 3H)taxol binding, exhibiting a 50% inhibitory concentration almost identical to that of taxol in displacement competition assays. Epothilones also cause cell cycle arrest at the G2-M transition leading to cytotoxicity, similar to taxol. In contrast to taxol, epothilones retain a much greater toxicity against P-glycoprotein-expressing multiple drug resistant cells. Epothilones, therefore, represent a novel structural class of compounds, the first to be described since the original discovery of taxol, which not only mimic the biological effects of taxol but also appear to bind to the same microtubule-binding site as taxol.

INTRODUCTION

Taxol and taxotere are novel cancer chemotherapeutic agents that stabilize cellular MTs.3 Taxol, a complex diterpene with a taxane ring system, was discovered in 1971 in a screen for anticancer activity (1). Following the elucidation of the mechanism of action of taxol (2), clinical trials have established taxol as an anticancer agent with significant activity against various human solid tumors (3). With earlier drug supply problems resolved, vigorous synthetic efforts under way to modify the structure of taxol, and trials of taxol efficacy in combination therapy in progress, further improvements in taxane-based chemotherapy are likely. Nevertheless, the complexity of the taxol structure presents a major obstacle to facile chemical modification aimed at improving the solubility characteristics and side effect profile of taxol. A novel class of drugs which stabilizes MTs might stimulate the development of more effective cancer chemotherapeutics with this mechanism of action.

Microtubules are one of the fundamental structures comprising the cytoskeleton of eukaryotic cells and are involved in such diverse cellular processes as cell division, locomotion, and intracellular transport (4). MTs are highly dynamic polar structures, with growth favored at the plus end and shrinkage more prevalent at the minus end. However, both MT ends experience periods of growth and shrinkage, a phenomenon described as dynamic instability (5). In vertebrate cells, the centrosome acts as the major site of MT nucleation (microtubule-organizing center) by lowering the critical concentration of tubulin required for polymerization and anchoring the minus ends of the resultant MTs (6, 7).

Taxol preferentially binds the polymeric MT form of tubulin in a 1:1 stoichiometry with the αβ-tubulin heterodimer subunits, with a KD of ~1 μM (8). Taxol binding markedly reduces the rate of αβ-tubulin dissociation, hence augmenting and stabilizing the MT pool (9). In vitro taxol has also been shown to nucleate tubulin polymerization into MTs, eliminating the requirement for GTP in normal polymerization (2, 10). Within cells this effect is manifested by micromolar taxol levels overriding the centrosomal microtubule-organizing center function and inducing the appearance of many short non-centrosomally linked bundles throughout the cytoplasm (11). In rapidly cycling human cells, taxol induces a block at the transition between G2 and M phase (12). Recent studies have clearly indicated that it is this mitotic arrest rather than the disruption of interphase MT function which is the actual mechanism behind the antineoplastic activity of taxol (13, 14).

Although taxol has shown efficacy against refractive ovarian (15), metastatic breast (16), head and neck (17), melanoma (18), and lung (19) cancer, its clinical usefulness is limited by its side effect profile (neutropenia, peripheral neuropathy, and alopecia) (20) and the fact that its low solubility necessitates that taxol be delivered in Cremophor. Cremophor delivery in itself can affect cardiac function and cause severe hypersensitivity responses (20). Furthermore, taxol is a substrate for P-glycoprotein which pumps many cytotoxic compounds out of MDR cells (21). Multiple drug resistance represents a major limitation of many cancer interventions. The complex taxane ring structure of taxol is not readily amenable to chemical manipulation to improve the therapeutic index and solubility properties of this class of compounds. We therefore sought to screen for a different structural class of compounds that would mimic the MT-stabilizing properties of taxol.

Here we report that epothilones A and B, 16-membered macrolides, mimic all the biological effects of taxol, both in vitro and in cultured cells. Independently, Hoefle et al. (22) have described epothilones A and B as having antifungal and cytotoxic activity. Competition studies reveal that epothilones act as competitive inhibitors of taxol binding to MTs, consistent with the interpretation that they share the same MT-binding site and possess a similar MT affinity as taxol. Epothilones appear to possess one advantage over taxol; namely, they exhibit a much lower drop in potency compared to taxol against a multiple drug-resistant cell line. The epothilones represent the first class of compounds to be described in the two decades following the original discovery of taxol which mimic the MT-stabilizing effect of the taxane ring structure.
Materials and Methods

Taxol and GTP were purchased from Sigma. Uranyl acetate was obtained from Ernest F. Fullam, Inc. For immunofluorescence microscopy, anti-chick β-tubulin monoclonal (Amersham) and FITC-conjugated anti-mouse (Cappel) antibodies were used. HeLa cervical epithelioid carcinoma cells and HS57ST and HS578Bst breast carcinoma and Ralt cells were obtained from the American Type Culture Collection. The multiple drug-resistant human carcinoma cell line KBV-1 and its parental line, KB3-1, were provided by I. Pastan (23).

Tubulin Purification

Microtubule protein was purified from bovine brains essentially as described by Asnes and Wilson (24). Brains were rinsed in cold saline solution within 1 h of sacrifice. Whole brains were minced and homogenized with 500 ml lysis buffer per brain [100 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.75-1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid-1 mM MgCl2-1 mM DTT-100 μg/ml phenylmethylsulfonyl fluoride] for 60 s in an Osterizer blender and then incubated on ice for 30 min. The extract was centrifuged at 4°C for 45 min at 30,000 × g. GTP was added to the supernatant at a final concentration of 1 mM and the mixture was incubated at 37°C for 30 min. This extract was centrifuged at 37°C for 30 min at 10,000 × g and the pellet was resuspended in one-tenth the 4°C supernatant volume of lysis buffer. This solution was incubated on ice for 30 min and then centrifuged at 4°C for 40 min at 30,000 × g. The supernatant was adjusted to 2.5 mM GTP, incubated at 37°C for 15 min, and centrifuged at 100,000 × g for 20 min at 37°C. The supernatant was removed and the pellets were frozen in liquid nitrogen and stored at −80°C. On the day of the assay, the microtubule pellets were resuspended in MEM buffer at 4°C and spun at 100,000 × g for 20 min. The supernatant protein concentration was determined by the bicinchoninic acid assay (Pierce).

Turbidity and Sedimentation Assays

To monitor the change in turbidity in the microtubule protein solution, the test compound was added to a cuvet containing 1 mg/ml microtubule protein. The cuvet was incubated at 37°C for 30 min in a Beckman D640 spectrophotometer equipped with a Peltier heating unit, and the absorbance was measured at 340 nm. The change in absorbance over the 30-min incubation was measured by the method of Schaffner and Weissmann (25). Briefly, 200 μl of elution solution (25 mM NaOH-0.05 mM EDTA, pH 12.0; NaNO2, 0.25; MgSO4·7H2O, 0.25; HEPES, 2.4; CaCl2, 0.01; Difo agar, 15.0). The pH was adjusted to 7.0-7.2 with dilute HCI. After solidification of the medium in sterile 90-mm Petri dishes, sterile Whatman No. 1 filter paper was applied to the surface of the agar. Dried fruiting bodies characterized required an incubation period of about 10-12 days before germination and the emergence of cell swarms was seen. Growing cells were transferred from the edges of the colony onto fresh medium before 4 weeks of incubation.

Microtubule protein (0.25 ml of 1 mg/ml) was placed into an assay tube and 2.5 μl of the test compound were added. The sample was mixed and incubated at 37°C for 30 min. Sample (150 μl) was transferred to a well in a 96-well Millipore Multiscreen Durapore hydrophilic 0.22-μm pore size filtration plate which has previously been washed with 200 μl of MEM buffer under vacuum. The well was then washed with 200 μl of MEM buffer.

To stain the trapped protein on the plate, 50 μl amido black solution [0.1% naphthol blue black (Sigma)/45% methanol/10% acetic acid] were added to the filter for 2 min; then the vacuum was reapplied. Two additions of 200 μl methanol wash; these afforded 42 mg of residue upon evaporation. Further purification was achieved by gel filtration on a 70-car Pharmacia Sephadex LH-20 column in methanol; the target compounds eluted after 0.7-0.8 column volume (15 mg residue). Semi-preparative HPLC followed on a 9 × 250-mm Zorbax Rx-C8 column eluted at 4 ml/min with a gradient of acetonitrile in water (40-60% acetonitrile). This afforded pure preparations of A and B (2.7 and 0.9 mg, respectively).

The homogeneity of the isolated materials was ascertained in several TLC systems [e.g., E. Merck Silica Gel 60 column, eluting with methylene chloride containing increasing amounts of methanol. Epothilone A and B were found in fractions corresponding to the 3% methanol wash; these afforded 42 mg of residue upon evaporation. Further purification was achieved by gel filtration on a 70-cm3 Pharmacia Sephadex LH-20 column in methanol; the target compounds eluted after 0.7-0.8-column volume (15 mg residue). Semi-preparative HPLC followed on a 9 × 250-mm Zorbax Rx-C8 column eluted at 4 ml/min with a gradient of acetonitrile in water (40-60% acetonitrile). This afforded pure preparations of A and B (2.7 and 0.9 mg, respectively).

Structure Determination. 13C NMR analysis of compound A in CD2Cl2, indicated the presence of 20 carbons (6 in CH3, 5 in CH2, 8 in CH, 1 in COX),
samples were incubated for an additional 30 min. MTs were then pelleted by GTP and 7.5 nM taxol at 37°C. Preliminary experiments had defined these albumin and 0.05% Triton X-100 was preincubated for 20 min with 100 μM GTP and calculated relative to a 1 mM GTP control incubated at 37°C for 60 min. To The microtubule protein solution was adjusted to 3 mM CaCl₂ and the test transference buffer lacking ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. On the basis of extensive two dimensional NMR [1H]-[1H] (correlated spectroscopy and total correlated spectroscopy) and [1H]-[13C] (HMQC, HMBC) correlation studies the 2-methylthiazole 16-membered macroclide A was proposed (Fig. 1). The structure was confirmed by HR-EIMS analysis.

Similarly, component B was assigned as the methyl homologue on the basis of the HRMS-derived molecular formula C₃₂H₄₉NO₁₅S (m/z: found, 507.2654; calculated, 507.2658) and the extra methyl singlet resonance at δ 1.26 in the 1H NMR spectrum. The position of the methyl group on the epoxide ring followed clearly from the COSY 1H NMR data. But for these modified features the spectra were almost identical to those of A. HR-EIMS fragmentation data also clearly allowed the methyl group to be located at C-12. Comparison of the structures and spectroscopic data with those of epothilone A and B recently reported in the patent literature (22) suggests that they are the same compounds.

Cold and Calcium Stability Studies

For the cold stability studies, microtubule protein was treated with the test compound and incubated at 37°C for 30 min. One portion of the sample was transferred to a test tube on ice for 30 min while the remainder was maintained at 37°C. Both portions of the sample were then centrifuged as described above for the sedimentation assay. The percentage of maximal polymerization was calculated relative to a 1 mM GTP control incubated at 37°C for 60 min. To assay calcium stability, microtubule protein was prepared in MEM buffer lacking ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. The microtubule protein solution was adjusted to 3 mM CaCl₂ and the test compound was added. The turbidity of the solution during a 30-min 37°C incubation was monitored and compared to the turbidity produced by 1 mM GTP addition to microtubule protein in MEM buffer.

Competition Studies

Microtubule protein (0.4 mg/ml) in MEM containing 0.1% bovine serum albumin and 0.05% Triton X-100 was preincubated for 20 min with 100 μM GTP and 7.5 nM taxol at 37°C. Preliminary experiments had defined these conditions as inducing essentially maximal microtubule polymerization, thus providing a roughly constant number of taxol binding sites over the range of drug concentrations used in the displacement studies. Tritiated taxol (100 nM; Moravek Biochemicals) and the competing compound were then added and the samples were incubated for an additional 30 min. MTs were then pelleted by centrifugation for 10 min at 100,000 x g in a TLA 100 rotor in a Beckman TLX benchtop ultracentrifuge. The pellets were carefully washed twice with 100 μL MEM containing 0.1% Triton X-100, resuspended in 100 μL 0.1 n NaOH, and transferred to a scintillation vial. After neutralization with 100 μL of 0.1 n HCl, 5 ml of ReadyProtein™ scintillation fluid (Beckman) were added and the sample was counted in an LKB model 1209 Rackbeta scintillation counter. Competitor taxol concentrations above 10 μM resulted in nonspecific sample precipitation.

Electron Microscopy

Microtubule protein solution (0.3 mg/ml) was incubated at 37°C for 30 min after addition of test compound. The protein sample was stained with 0.5% uranyl acetate as described by McEwen and Edelstein (26) and the grids were viewed on a Philips CM12 electron microscope.

Immunofluorescence

Cells were incubated for 4 h in 25 or 1 μM taxol. Cells were processed for immunofluorescence as described previously (27) using a monoclonal antibody against chicken brain β-tubulin.

Mitotic Block and Cytotoxicity Studies

Mitotic block, aberrant mitosis, and cytotoxicity were evaluated as described elsewhere. Briefly, cells were plated either in 48-well plates (for trypsin blue and cell counting) or onto No. 1 coverslips. After 24 h, the cells were treated with drug and were scored at regular intervals. For the cytotoxicity analysis, both attached cells and cells floating in the media were counted and scored as trypsin blue positive or negative. Concurrently, coverslips and aliquots of cells in the culture supernatant were fixed and stained with Hoechst 33342 (Boehringer Mannheim) in PBS. These cells were scored for cells blocked at the G₂-M transition and aberrant mitosis.

DNA Labeling and Agarose Gel Electrophoresis

Isolation of genomic cellular DNA is described by Sambrook et al. (28). End labeling of DNA using terminal transferase was carried out as described by Tilly et al. (29) except that the transerase reaction was incubated for 30 min.

RESULTS

A Novel Filtration-Colorimetric Assay Can Detect Microtubule Polymerization Induced by Low Concentrations of Taxol. We developed a novel sensitive microtubule filtration assay to enable large scale screening for compounds that would mimic the ability of taxol to induce tubulin polymerization. Tubulin solutions comprised of αβ heterodimers incubated in the presence of either GTP or taxol yield microtubule polymer. Tubulin solution filtration through a 0.22-μm pore size filter was found to separate tubulin heterodimer subunits effectively from MT polymer, the latter being readily visualized in a quantifiable manner by amido black staining. With the conditions defined in “Materials and Methods,” this filtration-colorimetric assay could readily detect the MT-nucleating activity of taxol down to 100 nm (Fig. 2A), which is markedly more sensitive than previously described assays. Standard turbidity or sedimentation assays require at least 1 μM taxol in the absence of GTP for detection of polymerization (Fig. 2B; Ref. 9). To distinguish between nonspecific protein aggregation or precipitation versus bona fide MT induction, follow-up electron microscopy analysis of incubation mixes was carried out (see below). The filtration-colorimetric assay was readily adapted to large scale screening with the use of 96 well plate technology. One worker could easily test over 300 samples in 1 day. Thus, the filtration-colorimetric assay provides a more sensitive method for detecting tubulin polymerization as a high throughput screen.

[Diagram of molecular structure of epothilones]

Fig. 1. Molecular structure of epothilones.

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4 C. Woods et al., manuscript in preparation.
Epothilones, a New Structural Class of Compounds with Tubulin-Polymerizing Properties. Subsequent screening of various plant, marine, insect, and fermentation extracts for tubulin polymerization activity revealed an extract from S. cellulosum to be an effective promoter of GTP-independent tubulin polymerization into MTs. This extract was fractionated with an initial silica gel column followed by a gel filtration step. Two pure peaks of activity eluted from a final reverse phase chromatography step. Subsequent analysis by mass spectrometry and NMR resulted in the identification of epothilones A and B (Fig. 1; Mr 493.25 and 507.27, respectively). The epothilones induced tubulin polymerization in the filtration assay at concentrations paralleling those of taxol (Fig. 3). As with taxol, the dose-response curve for the epothilones is very steep in this assay, reflecting extensive MT polymerization occurring above the minimal nucleating drug concentration. Turbidity and sedimentation assays of the epothilones also showed induction of tubulin polymerization at concentrations similar to those of taxol (data not shown). To determine whether the epothilones induced bona fide MT formation rather than nonspecific protein precipitation, samples of tubulin protein incubated in the presence of GTP, taxol, or epothilone A or B were analyzed by electron microscopy. All four treated samples displayed filamentous arrays while untreated protein samples showed no protein organization (Fig. 4). At higher magnification (Fig. 5), the tubulin filaments induced by treatment with epothilones A and B were shown to have a diameter of approximately 25 nm and possessed 8–9 protofilament-like striations, a structure identical to that of MTs induced by taxol.

A search for compounds with homology to the epothilones revealed a family of 16-membered macrolide antibiotics. Erythromycin, chalcomycin, carbomycin, and rosamycin were tested with the filtration-colorimetric assay, but none of these compounds displayed activity (results not shown). Interestingly, a different 16-membered macrolide, the MT-depolymerizing drug rhizoxin, possesses tubulin binding properties (K_D, 1.7 × 10^{-7} M; Ref. 30).

Epothilones Confer Resistance against Cold- or Calcium-induced MT Destabilization. Taxol has been shown to stabilize MTs against cold-induced and calcium-induced depolymerization (2). To determine whether epothilones could also mimic these effects, we tested for the ability to confer stability to microtubules exposed to cold or calcium. Microtubules treated with 1 μM epothilone A, epothilone B, or taxol were resistant to depolymerization after incubation at 4°C for 30 min whereas samples treated with 1 mM GTP depolymerized (Table 1). Similarly, 10 μM taxol or epothilone A or B induced tubulin polymerization in the presence of 3 mM calcium, whereas 1 mM GTP did not. Thus, the epothilones share with taxol the ability to confer resistance to calcium or cold treatment.

Epothilones Are Competitive Inhibitors of Taxol Binding to MTs. Competition assays of [3H]taxol binding to preformed MTs revealed that both epothilone A and epothilone B displaced 100 nM [3H]taxol with a similar 50% inhibitory concentration and slope to unlabeled taxol (Fig. 6). Preliminary experiments had established that under the conditions of the assay (100 μM GTP and a minimum of 10^{-8} M up to 3 × 10^{-6} M taxol), the amount of MT polymer (therefore, the number of taxol-binding sites) remained essentially constant in a consistent and reproducible manner. At 10^{-5} M taxol, the amount of MT polymer increased dramatically, and consequently the maximum competing taxol concentration that could be used was 10^{-5} M. Saturation studies of [3H]taxol binding to preformed MTs confirmed the K_D for taxol binding to MTs to be 0.55 μM, similar to the original observations of Parness and Horwitz (Ref. 8; data not shown). Under conditions of 100 nM [3H]taxol, the 50% inhibitory concentration values for displacement by taxol, epothilone A, and epothilone B were 3.6, 2.3, and 3.3 μM, respectively. The fact that all three compounds gave very similar slopes is consistent with the interpretation that the epothilones compete for the same binding site as taxol.

Epothilones Induce All the Classical Effects of Taxol on MT Arrays in Cultured Cells. Taxol-treated cells develop characteristic, extensive MT bundles that arise randomly throughout the cytoplasm. At lower concentrations (10^{-8} to 10^{-7} M), the interphase MT arrays remain generally unaffected, and taxol selectively affects mitotic spindle MT formation and function, leading to mitotic arrest and cytotoxicity (13). As illustrated in Fig. 7, epothilones A and B induced identical changes in intracellular MT arrays as taxol. Rat1 cells, chosen here for their large flattened morphology, treated for 4 h with 25 μM taxol (Fig. 7B), epothilone A (Fig. 7C), and epothilone B (Fig. 7D), all developed extensive MT bundles. Note that these bundles arise independently from the centrosome. Thus, as reported previously for taxol (11), this new class of MT-stabilizing agent overrides the
MT-nucleating activity of the centrosome in interphase cells. Similar bundling activity was observed in HeLa, Hs578T, and Hs578Bst cells (data not shown). At 5 × 10^{-9} to 10^{-6} M taxol (Fig. 7E), epothilone A (Fig. 7F), and epothilone B (Fig. 7G), the primary effect was seen in cells entering mitosis. All three agents induce similar multipolar spindles in HeLa cells, eventually resulting in arrest at the G2-M transition. As noted previously for taxol (13), it is striking that even at concentrations that override centrosomal MT nucleation (10^{-6}–10^{-7} M), these taxol- or epothilone-stabilized MTs still undergo massive reorganization to form spindle arrays, albeit abnormal ones. In addition to the gross rearrangements of cellular MT arrays described above, epothilones also stabilized cellular MTs from cold-induced depolymerization similarly to the action of taxol as shown in Fig. 8.

The cytotoxic effects of taxol in human cells have been shown to correlate with arrest at the G2-M transition (Refs. 12, 13, 14, and 31; see also Footnote 4). Therefore, to determine whether epothilones could also mediate similar effects, we analyzed G2-M block by Hoechst staining and cell death by trypan blue exclusion following exposure of 100 nM taxol, epothilone A, or epothilone B to HeLa or Hs578T cells over a 72-h period. Care was taken to score unattached cells in the supernatant also, since cells detach from the substrate following a few h of mitotic arrest. As shown in Fig. 9, all three agents
induced identical kinetics of G2-M block and identical lag and kinetics of cytotoxicity. A small percentage of cells, more evident in the Hs578T cells, appeared to passage through aberrant mitosis, as manifested by multimininucleation in the subsequent G1 stage. Aberrant alignment of chromosomes at the metaphase plate preceded and seemed to underlie multimininucleation. Multimininucleated cells also died but with much slower kinetics. Careful analysis of the time course of these changes showed that multimininucleation became prevalent only at later time points and appeared to represent the fraction of cells entering mitosis after ~24–30 h following taxol addition. Epothilone B showed a slight difference in that a larger percentage of cells showed multimininucleation (up to 38% compared with 10–15% for epothilone A or taxol with Hs578T cells) around the EC50 in both cell lines. As described by Kung et al. (32) for taxol, the majority of rodent Rat1 cells were tolerant of the prolonged abnormal mitosis induced by all three drugs, becoming arrested in the subsequent G1 stage as multimininucleated cells.

The EC50 for these effects was determined by scoring these parameters for a range of drug concentrations. Table 2 outlines the EC50.
isolated DNA confirmed that epothilone induced the DNA ladders consistent with internucleosomal nicking typical of apoptosis as also seen with taxol (Fig. 10). In situ TUNEL labeling revealed that DNA nicking occurred specifically in the G2-M-blocked cells (data not shown).

Epothilones Are Cytotoxic to Multiple Drug-resistant Cells. Since one major form of acquired resistance to taxol is by increased expression of the P-glycoprotein pump which serves to lower the intracellular drug concentration in MDR cells (21), we compared the potency of taxol versus the epothilones in cell lines lacking or expressing P-glycoprotein (Table 3). In the parental KB3-1 cell line which does not express elevated P-glycoprotein, the sensitivity to all three drugs was very similar, with EC50 values of 1.2 nm (taxol), 13 nM (epothilone A), and 15 nM (epothilone B). However, in the KBV-1 P-glycoprotein-expressing cells treated with epothilone A or B, the epothilones showed only a 4-12-fold drop in potency (EC50 values of 160 and 58 nM, respectively) compared to an approximately 20,000-

Table 2 Effects of taxol and epothilones on mitotic arrest and cytotoxicity

<table>
<thead>
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<th>Cell type</th>
<th>Treatment</th>
<th>Mitotic arrest (nM)</th>
<th>Cytotoxicity (nM)</th>
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<tbody>
<tr>
<td>HeLa</td>
<td>Taxol</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Epothilone A</td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Epothilone B</td>
<td>32</td>
<td>40</td>
</tr>
<tr>
<td>Hs578T</td>
<td>Taxol</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Epothilone A</td>
<td>7</td>
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<td></td>
<td>Epothilone B</td>
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values for mitotic arrest and cytotoxicity. The results demonstrate that the potencies of all three drugs are essentially identical in inducing mitotic arrest as well as cytotoxicity and are well below concentrations required for gross changes in interphase MT arrays (i.e., above 1 μM).

Taxol-induced cytotoxicity appears to proceed via an apoptotic pathway (33, 34). Since DNA fragmentation is a commonly used hallmark of apoptosis, we used DNA gel electrophoresis and in situ TUNEL labeling of human HeLa cells treated with taxol, epothilone A, or epothilone B. Agarose gel electrophoresis of 32P-end-labeled

Fig. 10. Autoradiogram of agarose gel containing genomic cellular DNA from cells treated with taxol or epothilone A. HeLa cells were treated with drugs for 24 h and processed as described in “Materials and Methods.” Lane 1, untreated control; Lane 2, 1 μM taxol; Lane 3, 1 μM epothilone A. Arrowheads, a 1-kb DNA ladder.
Table 3  Comparison of effects of taxol and epothilones on mitotic arrest and cytotoxicity in an MDR and parental cell line

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Mitotic arrest</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB3-1</td>
<td>Taxol</td>
<td>6 ± 1.2 ±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epothilone A</td>
<td>10 ± 13 ±</td>
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<td></td>
<td>Epothilone B</td>
<td>16 ± 15 ±</td>
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<tr>
<td>KBV-1</td>
<td>Taxol</td>
<td>17 ± 23 ±</td>
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<tr>
<td></td>
<td>Epothilone A</td>
<td>170 ± 160 ±</td>
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<tr>
<td></td>
<td>Epothilone B</td>
<td>92 ± 58 ±</td>
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fold drop in potency for taxol (23 μM EC_{50}). This suggests that epothilones are less effective substrates than taxol for the P-glycoprotein. As expected, the EC_{50} values for mitotic arrest by taxol, epothilone A, and epothilone B (17 μM, 170 μM, and 92 μM, respectively) were very similar to the cytotoxicity values.

DISCUSSION

Taxol and taxotere, related complex taxanes, have emerged as important new cancer therapeutics. Much of the recent enthusiasm for these agents is due to the fact that taxol has shown considerable efficacy against drug-refractory ovarian tumors (15) indicating that the mechanism of action of taxol can override at least certain aspects of acquired resistance to commonly used DNA-damaging antineoplastic agents. In addition, taxol has shown activity against breast cancer, also in patients who have undergone prior chemotherapy (16). Since the majority of clinical trials to date have been reduced in scope due to supply limitations and also have included a high percentage of patients who had undergone previous therapeutic regimens, there is hope that taxanes could have a broader application than the currently approved indications for breast and ovarian cancer.

Extended use of taxol suffers from a number of limitations. The first has been a limited taxol availability compounded by the inability to perform a large-scale synthetic reconstruction of the taxane ring. Fortunately, improved forestry of high-taxane yeasts and semisynthesis of taxotere from yeast needles partially address this issue. Secondly, the therapeutic index of taxol is narrow due to mechanism-based side effects of neutropenia, peripheral neuropathy, alopecia, and hypersensitivity reactions (20). To date, neutropenia and neuropathy have proved to be dose limiting in the clinic (35), although adjuvant cytokine therapy has been useful in reducing hematopoietic side effects. In addition, taxol is highly hydrophobic, necessitating delivery in Cremophor which itself can induce cardiac arrhythmias and extensive hypersensitivity reactions (3). Together, these features have resulted in the recommendation that taxol be administered in the 170-mg/m² dose range over a 24-h schedule to minimize hypersensitivity reactions. At this dosage, taxol levels in plasma reach steady state concentrations of more than 1 × 10^{-3} m (36). One complication of taxol therapy is that the plasma half-life is less than 5 h (37), indicating a relatively narrow window of treatment (see below). In vitro studies have highlighted additional limitations in the mechanism of cytotoxicity of taxol. Taxol cytotoxicity correlates with mitotic arrest but, as with other chemotherapeutics, taxol is effective only against rapidly dividing cells (14). Our studies have indicated that effective intracellular taxol concentrations can drop below threshold values required for arrest at the G2-M transition 48–72 h after addition of taxol to the culture medium. Thus, cells entering mitosis after this time are unaffected. Taken together, the in vitro data suggest that taxol will be effective only against cells with rapid doubling times, and a more effective dosing schedule might utilize lower doses over longer periods of time. However, the profile of taxol side effects might preclude such a regimen.

To address some of these issues, we sought to identify an alternative structural class of compounds which would mimic all the biological effects of taxol but with a structure more readily amenable to chemical manipulation. Using a novel, high throughput filtration-colorimetric assay for detecting MT-nucleating activity with a 10-fold greater sensitivity than traditional methods, we identified an active compound from an extract of the myxobacterium S. cellulosum. Electron microscope analysis confirmed that this extract promoted the assembly of bona fide microtubules. Purification by silica gel, gel filtration, and reverse phase chromatography revealed two related active components, epothilone A and B. Both epothilone A and epothilone B appeared to be equipotent to taxol in all of our in vitro assays. Most significantly, competition of [3H]taxol from preformed MTs revealed that taxol, epothilone A, and epothilone B possess similar half-maximal displacement values. This observation together with the fact that epothilones A and B had similar biological activity in our assays was consistent with the interpretation that epothilones A and B compete for the same MT binding site as taxol.

Many agents identified as potent agonists or antagonists of in vitro activity in drug screening can prove to have secondary activities that limit their usefulness in vivo. To ascertain whether epothilone A or B also exactly mimics the activity of taxol in cells, we compared their ability to induce MT bundling, to perturb mitosis, and to stabilize MTs against depolymerizing treatments. At 10^{-6}-10^{-4} m, epothilones induced formation of extensive short MT bundles throughout the cytoplasm of interphase cells over the course of 2–3 h, as has been shown for taxol. As noted by DeBrabander et al. (11) for taxol, these bundles were clearly not linked to the centriole, suggesting that epothilones, like taxol, not only stabilize intracellular MTs but can also override centrosome nucleating activity at high concentrations. At lower concentrations (5 × 10^{-9}-10^{-6} m), mitotic MT arrays were preferentially affected. Cells entering M phase developed multipolar spindles in the presence of either taxol or epothilones. Treatment with all three agents resulted in early prophase arrest in human cells and aberrant mitosis leading to multiminucleation in rodent cells, as described previously for taxol by Kung et al. (32). Finally, epothilones and taxol were equally effective at stabilizing the cellular MT network when exposed to prolonged cold (4°C) treatment. These activities confirm that the MT-stabilizing properties of epothilones and taxol affect cellular MT form and function in an indistinguishable manner.

Taxol-induced mitotic arrest has been shown to correlate with cytotoxicity by an apoptotic pathway (33). We have analyzed the kinetics of cell death relative to G2-M block and have demonstrated that DNA nicking is initiated rapidly after arrest at the G2-M transition. As shown in Fig. 9, epothiline- and taxol-induced cell death follows G2-M block with a similar time lag. Agarose gel electrophoresis of DNA isolated from these cells 24 h after drug treatment revealed the DNA ladder that is the hallmark of apoptosis. In situ TUNEL labeling of DNA nicks in individual cells confirmed that this process occurs selectively in G2-M blocked cells soon after mitotic arrest and, subsequently, in those multiminucleated G1-arrested cells that have succeeded in surviving an aberrant mitosis, as had been seen with taxol. From these observations, we conclude that epothilones exert equivalent biological effects to taxol on cultured cells. Since taxol has been shown to bind MTs in cells (38), we conclude that epothilones likewise mediate all these downstream effects by their MT-stabilizing activity observed in vitro and in cultured cells.

Epothilones A and B were found to differ from taxol in one important respect. Namely, the drop in potency exhibited by epothilones in MDR cells expressing P-glycoprotein was three orders of magnitude less than the drop in potency exhibited by taxol. Since
MDR-mediated resistance accounts for some aspects of acquired taxol resistance (21), this observation suggests that epothilones as antineoplastic agents could provide an important advantage over taxol.

Epothilones A and B have been patented recently as antifungal agents with possible cytotoxic or immunosuppressive properties (22). The epothilones are structurally homologous to a family of 16-member macroolide antibiotics including erythromycin. Interestingly, none of these agents possessed the MT-stabilizing properties of the epothilones, although rhizoxin is a known MT-destabilizing drug (30). The patent for epothilones A and B indicates that these compounds possess cytotoxic activity against a human T-24 bladder carcinoma line. Here we describe the mechanism of action underlying epothilone cytotoxic activity and reveal that epothilones represent an alternative structural class of MT-stabilizing agents to the taxanes. The epothilones furthermore appear to be competitive ligands for the taxol-binding site.

Both taxol and epothilones appear to bind tubulin $a/b$ heterodimers within MTs with a 1:1 stoichiometry. Whereas taxol EC$_{50}$ values for mitotic arrest and cytotoxicity of 5–20 nM have been observed here and elsewhere for human cell lines, these values refer to extracellular drug concentrations. However, the apparent differences in taxol sensitivity between human and rodent cell lines are caused by differences in intracellular accumulation, with the actual intracellular drug concentrations effecting these changes in human cell lines reaching much higher concentrations (39). Such findings predict that a high degree of occupancy of the MT polymer may be required for mitotic perturbation. Given that both epothilones and taxol effect MT stabilization through stoichiometric binding and that tubulin represents an abundant cellular protein, the biology of the system may preclude finding a drug which can improve on the stoichiometric potency of taxol for this mechanism of action. The simpler chemical structure of epothilones may instead provide a useful lead compound in the quest for a drug operating by the same MT-stabilizing mechanism as taxol but with an improved solubility profile and therapeutic index.

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Epothilones, a New Class of Microtubule-stabilizing Agents with a Taxol-like Mechanism of Action

Daniel M. Bollag, Patricia A. McQueney, Jian Zhu, et al.


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