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Brief Article

# Microtubule-Stabilizing Activity of Zampanolide, a Potent Macrolide Isolated from the Tongan Marine Sponge *Cacospongia mycofijiensis*

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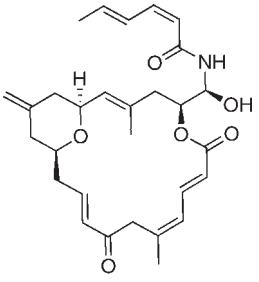
Zampanolide (**1**), a 20-membered macrolide from a Tongan marine sponge, stabilizes microtubules and blocks cells in $G_2$/M of the cell cycle. Zampanolide is cytotoxic in the low nanomolar range and induces microtubule bundles in cells. It leads to tubulin assembly in cells and in purified tubulin preparations and is not a substrate for the P-glycoprotein drug efflux pump. Zampanolide, with only four stereogenic centers, may be amenable to large-scale synthetic preparation.

## Introduction

Zampanolide (**1**)




**1**

was originally reported by Tanaka and Higa in 1996 from the marine sponge *Fasciospongia rimosa*, collected in Okinawa, Japan.[1] The compound has a 20-membered macrolide ring that is largely unsaturated and an *N*-acyl hemiaminal side chain. Zampanolide was isolated with another compound latrunculin A, an actin inhibitor. A previous collection of this sponge also produced laulimalide, a microtubule-stabilizing agent.[2] Synthetic preparations of zampanolide in its natural and unnatural forms have been reported;[3−5] however, because of the scarcity of the natural material, investigations on its mode of action have not been reported to date. In this paper, we report a potent microtubule-stabilizing activity for zampanolide that places it in an important group of anticancer compounds that includes the clinically valuable paclitaxel, docetaxel, ixabepilone (azaepothilone B), and a number of other potent microtubule-stabilizing agents, including laulimalide, peloruside A, discodermolide, and dictyostatin.[6,7] The addition of a new, highly potent microtubule-stabilizing compound with possible unique properties to the known microtubule-targeting agents may provide useful new information on structure−activity relationships within this important class of compounds.

## Results

**Isolation and Purification.** Zampanolide (**1**, $M_r$ = 495.26) was isolated from the marine sponge *Cacospongia mycofijiensis*, collected from 'Eua, Tonga. The methanolic extract was purified using a combination of reversed- (PSDVB, $Me_2CO/H_2O$, and $MeOH/H_2O$ gradients) and normal-phase (silica gel, $EtOAc/CH_2Cl_2$, DIOL,[a] IPA/*n*-hexane) chromatography. All NMR spectra and the optical rotation for zampanolide were in agreement with that of its original description.[1] In addition, the previously described compounds latrunculin A, laulimalide, isolaulimalide, mycothiazole, and dendrolasin were also isolated.

**Bioactivity.** $IC_{50}$ values in HL-60 cells (48 h of incubation) and 1A9 cells (72 h of incubation) were calculated from MTT dye reduction assays as previously described[8] (Figure 1A). The $IC_{50}$ values for zampanolide were 4.3 ± 1.1 nM (mean ± SEM) ($n$ = 5 preparations) in HL-60 cells and 14.3 ± 2.4 nM ($n$ = 6 preparations) in 1A9 cells. Trypan blue dye exclusion tests in HL-60 cells showed that concentrations of 2 nM zampanolide or greater were toxic to the cells after 24 h (Figure 1B). At 10 nM zampanolide, most of the cells were dead following 48 h of exposure.

**Cellular Morphology.** Micronuclei were present in HL-60 cells treated with 8 nM zampanolide for 48 h (Figure 1C). The average diameter of the cells was significantly increased in a dose-dependent manner by zampanolide compared to control cells (18.6 ± 0.4 $\mu$m) with mean diameters of

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[a] Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DIOL, 2,3-dihydroxypropoxypropyl-derivatized silica; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide; PBST, phosphate-buffered saline with 0.25% Triton X-100; P-gp, P-glycoprotein; PSDVB, polystyrenedivinylbenzene.

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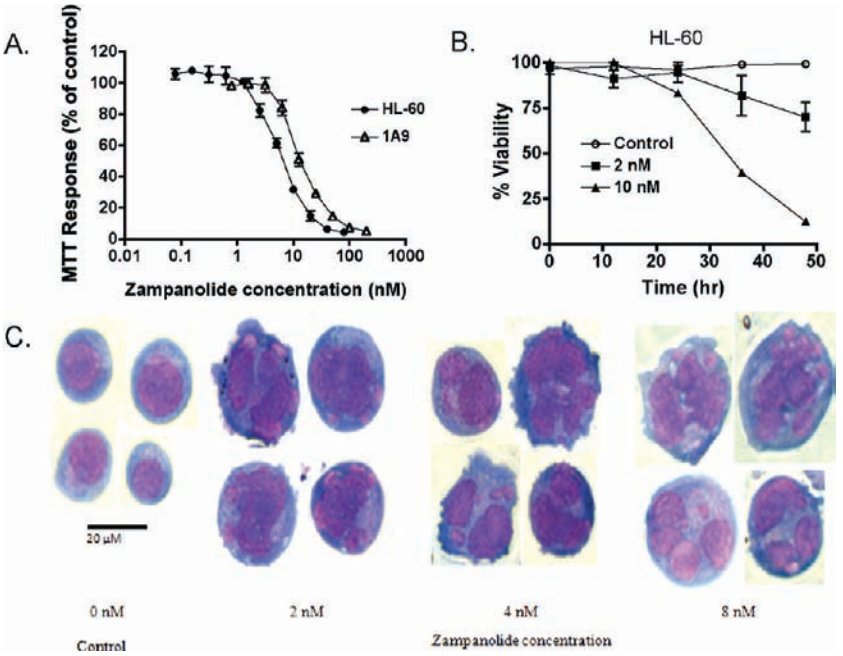


**Figure 1.** Cytotoxic effects of zampanolide. (A) Zampanolide dose−response curves for the MTT cell proliferation assay in human promyelocytic leukemia (HL-60) and ovarian cancer (1A9) cells after 48 and 72 h of treatment, respectively. Graphs are representative of five to six separate preparations. (B) Cytotoxicity of zampanolide in HL-60 cells measured by Trypan blue dye exclusion ($n = 2$ preparations). (C) HL-60 cells 48 h after addition of 2−8 nM zampanolide. Cells were cytospun onto slides, air-dried, fixed with 100% methanol, and stained with Giemsa stain.

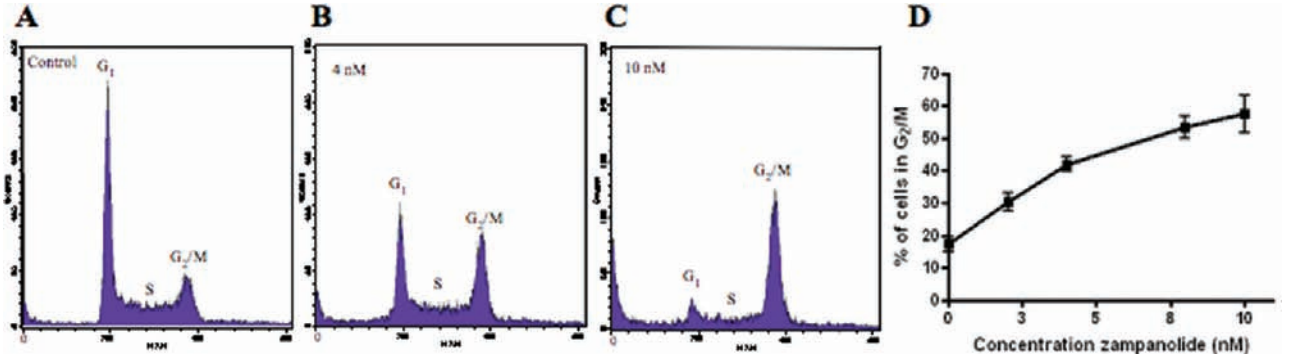


**Figure 2.** $G_2$/M cell cycle arrest. 1A9 cells were treated with zampanolide for 16 h and stained with PI, and cellular DNA content was determined by flow cytometry. Representative scans are presented for 0 nM (A), 4 nM (B), and 10 nM (C) zampanolide. A results summary is presented (D) (mean ± SEM; $n = 4$ wells from two preparations).

23.0 ± 1.0 nm at 2 nM zampanolide, 24.5 ± 0.9 nm at 4 nM, and 28.7 ± 1.1 $\mu$m at 8 nM.

**Cell Cycle Arrest.** Treatment of HL-60 cells with 2−10 nM zampanolide for 16 h led to a dose-dependent cell cycle arrest in the $G_2$/M phase of the cell cycle (Figure 2). At higher concentrations of drug, an increase in the number of cells in the subdiploid peak was also seen, indicating apoptosis or necrosis of the cells. The induction of $G_2$/M arrest by zampanolide suggested a possible antimitotic action of the compound.

**Microtubule Bundles and Multiple Asters.** To determine if zampanolide was targeting the microtubule, 1A9 cells were treated with 10 nM drug for 12 h and examined by immunocytochemistry following fluorescent staining for α-tubulin (Figure 3). Zampanolide at 10 nM induced significant microtubule bundles in interphase cells, and the percentage of cells with bundles was dependent on dose (Table 1). In mitotic cells, zampanolide caused the appearance of multiple asters. Asters are microtubule organelles that radiate out from the centrioles on each end of the spindle and help control the separation of the two daughter cells. Similar effects on microtubule structure were seen with paclitaxel.

**Cellular Tubulin Polymerization.** Tubulin in soluble and particulate fractions from 1A9 cells exposed to different concentrations of zampanolide or paclitaxel for 16 h was isolated and visualized by immunoblotting for α-tubulin (Figure 4). 1A9 cells typically showed only about 2% polymerized tubulin in the absence of stabilizing drug. Zampanolide caused a dose-dependent shift of soluble tubulin to the particulate fraction. At 100 nM zampanolide, approximately all cellular tubulin was in the polymerized form. Results were compared to paclitaxel, a known microtubule-stabilizing drug.

**Purified Tubulin Polymerization.** Zampanolide and paclitaxel at 10 $\mu$M caused isolated, purified bovine brain tubulin to polymerize in the presence of 1 mM GTP but in the absence of microtubule-associated proteins (Figure 5). Polymerization was assessed as an increase in light scattering

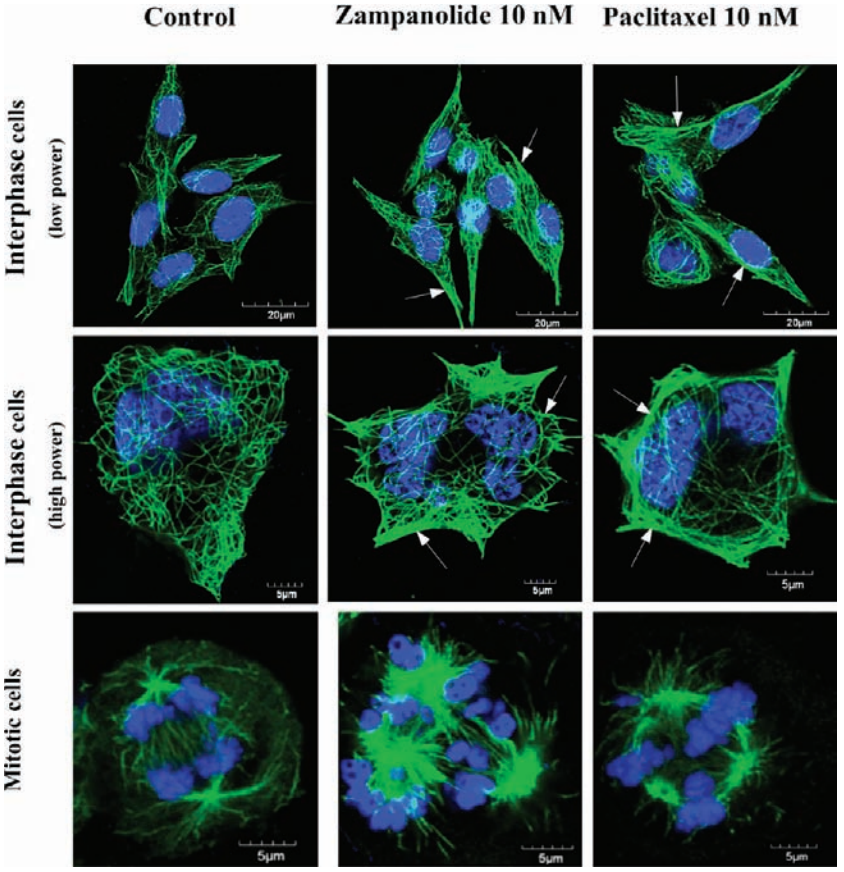


**Figure 3.** Microtubule bundles and multiple asters. 1A9 human ovarian carcinoma cells were treated with 10 nM zampanolide or 10 nM paclitaxel for 12 h, then stained for α-tubulin to visualize the microtubules. Both zampanolide and paclitaxel induced the formation of bundles of microtubules in interphase cells (arrows) and multiple asters in mitotic cells. Results are representative of three independent preparations.

(absorbance) at 340 nm wavelength. In the absence of drug, polymerization still occurred but at a much slower rate.

**MDR Susceptibility.** A2780 and A2780AD (MDR phenotype) human ovarian cancer cells were tested with paclitaxel and zampanolide using the MTT cell proliferation assay. The P-gp overexpressing cell line A2780AD was many-fold resistant to paclitaxel compared to the parental cell line A2780 (208 ± 64, $IC_{50}$ ratio A2780AD/A2780) ($n$ = 3 independent preparations). The paclitaxel $IC_{50}$ values were 2.8 ± 1.4 nM (A2780) and 415 ± 48 nM (A2780AD). In contrast, zampanolide was equally potent in both cell lines (1.4 ± 0.7 $IC_{50}$ ratio) ($n$ = 3 preparations). The zampanolide $IC_{50}$ values were 7.1 ± 2.0 nM (A2780) and 7.5 ± 0.6 nM (A2780AD).

**Table 1.** Dose−Response for Microtubule Bundles and Asters[a]

| agent conc (nM) | % interphase cells with microtubule bundles | % mitotic cells with multiple asters |
|---|---|---|
| | Zampanolide (**1**) | |
| 0 | 1.6 ± 0.6 | 0 |
| 5 | 9.6 ± 2.3 | 4.3 ± 1.7 |
| 8 | 28.6 ± 3.0 | 21.6 ± 1.8 |
| 10 | 55 ± 4.7 | 51.4 ± 3.2 |
| 20 | 74.3 ± 4.4 | 57.3 ± 1.8 |
| Paclitaxel | | |
| 0 | 1.1 ± 1.2 | 0 |
| 5 | 13.7 ± 3.2 | 6.6 ± 3.6 |
| 10 | 76.5 ± 10.2 | 43.3 ± 3.3 |
| 20 | 81.9 ± 4.6 | 52.2 ± 4.0 |

[a] Following treatment of 1A9 cells with zampanolide or paclitaxel, the proportion of cells with microtubule bundles or asters is presented as the mean ± SEM ($n$ = 3 separate preparations).

## Discussion and Conclusions

Secondary metabolites isolated from marine organisms that have potent microtubule-stabilizing activity continue to attract considerable attention. Recently, we reported the antimitotic behavior of peloruside A, a potent cytotoxin from the New Zealand marine sponge *Mycale hentscheli*.[9] Other compounds with a similar mode of action, such as the epothilones,[10] discodermolide,[11] eleutherobin,[12] laulimalide,[13] peloruside A,[9] and dictyostatin,[14] have been presented as possible therapeutic replacements for the taxoid drugs paclitaxel[15] and docetaxel,[16] but only a derivative of epothilone, ixabepilone, has made it into the clinic at this time.[17] Our recent efforts to identify new compounds from the Tongan marine sponge *Cacospongia mycofijiensis* resulted in the isolation of zampanolide, along with other known compounds latrunculin A, dendrolasin, mycothiazole, laulimalide, and isolaulimalide. It is interesting that this sponge (and possibly its symbiotic bacteria) seems to have concentrated its secondary metabolite production on the eukaryotic cell cytoskeleton with tubulin (zampanolide, laulimalide, isolaulimalide) and actin (latrunculin A) targets predominating. Recent synthetic preparations of natural and unnatural zampanolide have been reported.[3−5] Although the macrolide showed potent bioactivity in a number of cancer cell lines ($IC_{50}$ = 2−10 nM for growth inhibition), no information was available on the mode of action of zampanolide.

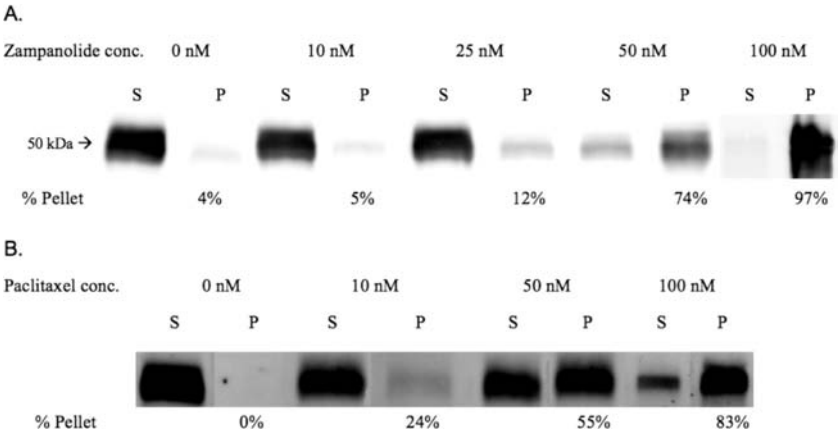


**Figure 4.** Tubulin polymerization in 1A9 cells. Immunoblots are presented for α-tubulin following electrophoresis of soluble (S) and particulate (P) fractions of 1A9 cells treated with (A) zampanolide or (B) paclitaxel for 16 h. The percentage of tubulin in the cellular pellet is presented below the band. Results are representative of four independent experiments.

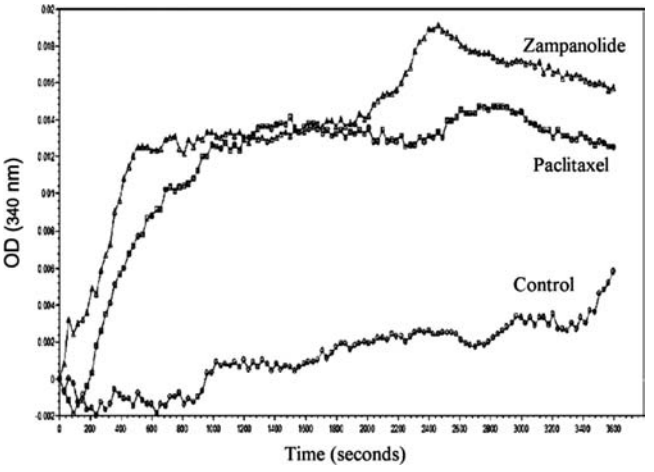


**Figure 5.** Purified bovine brain tubulin polymerization. Purified bovine brain tubulin was treated with 10 $\mu$M zampanolide or 10 $\mu$M paclitaxel. The reaction was initiated by addition of drug to 2 $\mu$M tubulin at $t = 0$ at 37 °C in a 96-well plate. Control wells were treated with an equivalent volume of ethanol (1% final concentration). Absorbance at 340 nm was monitored at 30 s intervals for 1 h. The graph is representative of three independent experiments.

We now show that zampanolide is a novel and potent microtubule-stabilizing compound with properties similar to those of other drugs in this class. It arrests cells in $G_2$/M of the cell cycle, induces microtubule bundles in interphase cells and multiple asters in dividing cells, and causes a dose-dependent shift of soluble tubulin to polymerized tubulin inside cells and in the test tube, similar to what is seen with paclitaxel. Cells treated with zampanolide develop micronuclei and increase their volume by up to 2.7-fold, consistent with mitotic slippage and induction of apoptotsis.[18] Ovarian cancer cells that overexpress the P-gp multiple drug resistance (MDR) pump are resistant to paclitaxel[19] but in our study retained 100% of their sensitivity to zampanolide. Thus, zampanolide has potential as a chemotherapy replacement for treating tumors that develop MDR-mediated resistance to paclitaxel and docetaxel. The overall functionality of zampanolide is relatively hydrophobic in comparison to other macrolide microtubule-stabilizing agents and indeed paclitaxel, as evidenced by TLC. The low number of stereogenic centers in the molecule makes it an attractive compound for large-scale synthetic preparations for further study or clinical applications.

## Experimental Section

NMR spectra (DMSO-$d_6$, 600 MHz) were obtained using a Varian DirectDrive spectrometer equipped with a triple resonance HCN cryogenic probe. High-resolution positive-ion mass spectra were recorded on a Waters Q-TOF Premier tandem mass spectrometer. Optical rotations were performed using a Perkin-Elmer 241 polarimeter. Normal-phase column chromatography was carried out using YMC Co. Ltd. 2,3-dihydroxypropoxypropyl-derivatized silica (DIOL) and silica gel. Reversed-phase column chromatography was achieved using Supelco Diaion HP20 poly(styrene divinylbenzene) (PSDVB) chromatographic resin. HPLC was performed using a Rainin Dynamax SD-200 solvent delivery system, with UV detection using a Varian ProStar 335 photodiode array detector. Solvents used for flash normal- and reversed-phase column chromatography were of HPLC or analytical grade quality. All other solvents were purified by glass distillation. Solvent mixtures are reported as % v/v unless otherwise stated. Specimens were stored at −20 °C until required for extraction. Zampanolide was stored at −80 °C as a 1 mM solution in absolute ethanol. Paclitaxel (Taxol) was purchased from LC Laboratories (Woburn, MA).

**Extraction and Isolation.** Methanolic extracts (2 × 1 L) of frozen *Cacospongia mycofijiensis* (341.0 g) were loaded onto PSDVB beads (HP20) and eluted with increasing concentrations of $Me_2CO$ in $H_2O$ (20−100% v/v). A portion (264.6 mg) of the 80% $Me_2CO/H_2O$ fraction was purified on silica gel and eluted with increasing concentrations of EtOAc in $CH_2Cl_2$ (0−100% v/v). A 5.6 mg portion of the 50% EtOAc/$CH_2Cl_2$ silica gel fraction was finally purified using HPLC (DIOL, 5 $\mu$m, 250 mm × 10 mm, 5% IPA/*n*-hexane), yielding zampanolide (**1**, 1.7 mg, $t_R$ = 26.1 min). The isolated zampanolide was confirmed as >95% purity by HPLC and NMR analysis.

**Zampanolide (1).** Colorless glasslike solid; $[\alpha]_D^{25}$ −165.8° (*c* 0.53, $CH_2Cl_2$); HRESIMS $[M + Na]^+$, observed *m*/*z* 518.2515, calculated *m*/*z* 518.2519 for $C_{29}H_{37}NO_6Na$, $\Delta$ = −0.8 ppm; all other data as previously published.[1]

**TLC Analysis.** Silica gel, 2% MeOH/$CH_2Cl_2$: paclitaxel $R_f$ = 0.14; zampanolide $R_f$ = 0.24.

**Cytotoxicity and Cell Cycle Block.** The human promyelocytic leukemic HL-60 cell line and the human ovarian carcinoma 1A9, A2780, and A2780AD cell lines were cultured in RPMI-1640 medium with 10% fetal calf serum and penicillin−streptomycin as previously described.[8,20] 1A9, A2780, and A2780AD cells were also supplemented with 0.25 units/mL insulin. HL-60 cells were obtained from Professor Mike Berridge, Malaghan Institute of Medical Research, Wellington, NZ. 1A9 cells were obtained from Dr. Paraskevi Giannakakou, Weill Medical

College of Cornell University, New York, NY, and A2780 and A2780AD cells were obtained from Dr. Steven Williams, Fox Chase Cancer Center, Philadelphia, PA. The A2780AD cell line demonstrated its MDR phenotype by overexpressing the P-glycoprotein (P-gp) drug efflux pump[19] and being highly resistant to paclitaxel treatment.

Effects of zampanolide on cell growth and viability were assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide) cell proliferation assay, as previously described[8] and a Trypan blue dye exclusion assay.[21] Cell cycle analysis was carried out by flow cytometry using a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ) as previously described.[8]

**Immunocytochemistry.** Cells were plated onto 12 mm glass coverslips and allowed to attach for 24 h, then treated with drug for 12 h. The cells were fixed with ice-cold methanol/acetone (1:1) for 5 min, blocked for 30 min with 5% BSA in PBST (0.25% Triton X-100 in phosphate-buffered saline), and incubated at room temperature for 1 h with the rabbit polyclonal primary antibody to human α-tubulin (1:500, ab18251; Abcam). After being washed three times with PBST, the cells were incubated with Alexa Fluor 488 conjugated goat antirabbit secondary antibody (1:1000, A11008; Molecular Probes, Invitrogen) for 1 h in the dark. The cells were washed three times and the coverslips mounted onto glass slides using Prolong Gold Antifade with DAPI (Molecular Probes, Invitrogen). Fluorescent staining was examined with an Olympus FluoView FV1000 confocal laser scanning microscope using a 100× oil-immersion objective.

**Cellular Tubulin Polymerization in 1A9 Cells.** A previously described in situ cellular assay[8] was used to quantify drug-induced tubulin polymerization by electrophoresis of soluble and particulate fractions (14000*g* for 10 min) and immunoblotting for tubulin. 1A9 cells were treated with different concentrations of zampanolide or paclitaxel for 16 h and then processed as described. For Western blotting, the electrophoresed proteins were transferred to Immobilon FL membrane (Millipore Corp, Billerica, MA) and labeled with rabbit polyclonal primary antibody to α-tubulin (1:1000, ab18251; Abcam) and Cy5 conjugated goat antirabbit secondary antibody (1:2500, PA45011V, Amersham, GE Healthcare). Tubulin bands on the membrane were scanned using a Fujifilm FLA-5100 imaging system (Fuji Photo Film Co. Ltd., Japan) and quantified by densitometry with ImageJ (NIH). The percentage of polymerized tubulin was calculated by dividing the band density of polymerized tubulin by the sum of the densities of the soluble and polymerized tubulin.

**Purified Tubulin Polymerization.** The polymerization of purified bovine brain tubulin (Cytoskeleton Inc., Denver, CO) was measured by changes in the optical density of tubulin solutions at 340 nm using a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA). The purified tubulin was reconstituted in buffer consisting of 1 mM GTP, 80 mM PIPES, 0.5 mM EGTA, 2 mM $MgCl_2$, pH 6.9, according to the manufacturer's instructions. The assembly reaction was performed at 37 °C in a 96-well plate. The concentration of tubulin was 2 $\mu$M, and zampanolide and paclitaxel were added at 10 $\mu$M in buffer to start the reaction.

A2780 and A2780AD (MDR phenotype) human ovarian cancer cells were tested with paclitaxel and zampanolide, using the MTT cell proliferation assay.

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**Supporting Information Available:** $^{13}C$ and $^{1}H$ NMR spectra for zampanolide (**1**); more complete results for trypan blue and $G_2/M$ block in HL-60 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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