A thalidomide analogue with *in vitro* antiproliferative, antimitotic, and microtubule-stabilizing activities

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Abstract

We discovered a thalidomide analogue [5-hydroxy-(2,6-diisopropylphenyl)-1H-isindole-1,3-dione (5HPP-33)] with antiproliferative activity against nine cancer cell lines *in vitro*. Flow cytometric analyses showed that the compound caused G2-M arrest, which occurred mainly at the mitotic phase. In addition, immunofluorescence microscopy and *in vitro* tubulin polymerization studies showed that 5HPP-33 has antimitotic activity with a paclitaxel-like mode of action. It is effective against four different paclitaxel-resistant cell lines. Thus, 5HPP-33 represents a potential antitumor agent. [Mol Cancer Ther 2006;5(2):450–6]

Introduction

Thalidomide, a drug discovered in the mid-1950s in a search for better antiepileptic agents, was found to possess little antiepileptic effect but was marketed as a safe, nonaddictive sedative-hypnotic drug with good antiemetic activity. The drug was used extensively for morning sickness during pregnancy until it was found to cause significant fetal abnormalities (1). Thalidomide was never approved in the United States initially because of concerns of peripheral neuropathy. Recently, several novel thalidomide analogues have been reported (21–24). These analogues can be classified into two groups: the selective cytokine inhibitory drugs, which possess phosphodiesterase type 4 inhibitory activities, and the immunomodulatory drugs. Both selective cytokine inhibitory drugs and immunomodulatory drugs possess potent tumor necrosis factor-α inhibitory and antiangiogenic activities. In addition, immunomodulatory drugs also possess T-cell costimulatory activity (23). Furthermore, a subset of selective cytokine inhibitory drugs has been reported to exhibit direct *in vitro* and *in vivo* antitumor activity (25). Currently, two immunomodulatory drugs are in clinical trials for the treatment of cancers. Revlimid (Fig. 1) is now in phase III clinical trials for multiple myeloma and metastatic melanoma, whereas Actimid (Fig. 1) has entered a phase I/II trial for multiple myeloma and a phase II trial for prostate cancer.

Recently, we reported the antiangiogenic activities of several putative metabolites of thalidomide (26). Our ongoing search for potent antiangiogenic thalidomide analogues revealed a compound, 5-hydroxy-(2,6-diisopropylphenyl)-1H-isindole-1,3-dione (5HPP-33; Fig. 1), with distinct biological activities. 5HPP-33 has been reported to have tumor necrosis factor-α inhibitory activity *in vitro* (27, 28). In addition, the molecule was also recently reported to have tubulin polymerization inhibitory activity (29). In the evaluation of 5HPP-33 described here, we determined that 5HPP-33 possesses antiproliferative activity *in vitro* against nine different cancer cell lines and induces cell cycle arrest at G2-M phase as determined by flow cytometric analyses. In addition, through immunofluorescence microscopy and *in vitro* tubulin polymerization studies, we show that 5HPP-33 has antimitotic activity with a paclitaxel-like mode of action yet is effective against four different paclitaxel-resistant cell lines.

Materials and Methods

Chemicals and Reagents

Chemicals (except 4-hydroxyphthalic acid, which was purchased from Fisher Scientific, Pittsburgh, PA) and silica gel were purchased from Sigma-Aldrich Chemical Co.
2 mmol/L L-glutamine, and 1% gentamicin. NIH3T3 cells and its transfectants were cultured in DMEM supplemented with 10% FBS. Breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured in DMEM F-12 plus 10% FBS, and its transfectants were cultured in complete RPMI 1640 (Life Technologies Invitrogen, Grand Island, NY) plus 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 2 mmol/L L-glutamine (Life Technologies), and 1% gentamicin (Life Technologies). TCCSUP was grown in MEM (Eagle) in Earle’s balanced salt solution containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and nonessential amino acids supplemented with 10% FBS. HT-29 was grown in McCoy’s 5A containing 2 mmol/L L-glutamine supplemented with 10% FBS, 5 mmol/L glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin (Life Technologies), and 1% gentamicin (Life Technologies). TCCSUP was grown in MEM (Eagle) in Earle’s balanced salt solution containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and nonessential amino acids supplemented with 10% FBS. HT-29 was grown in McCoy’s 5A containing 2 mmol/L L-glutamine supplemented with 10% FBS. Breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured in DMEM F-12 plus 10% FBS, 2 mmol/L L-glutamine, and 1% gentamicin. NIH3T3 cells and its transfectants were cultured in DMEM supplemented with 10% FBS, 5 mmol/L L-glutamine, 50 units/mL penicillin (Life Technologies), and 50 μg/mL streptomycin (Life Technologies). I9A cells and its mutants (I9APTIX10 and I9APTIX22) were grown in RPMI 1640 with 10% FBS.

**Cell Lines and Culture**

Tumor cell lines [MCF-7 and MDA-MB-231 (breast), PC-3, DU-145, and LNCaP (prostate), HT-29 (colon), TCCSUP (bladder), and Hs Sultan (Burkitt’s lymphoma)] were originally obtained from American Type Culture Collection (Rockville, MD). PC-3, DU-145, LNCaP, and Hs Sultan cells were cultured in complete RPMI 1640 (Life Technologies Invitrogen, Grand Island, NY) plus 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 2 mmol/L L-glutamine (Life Technologies), and 1% gentamicin (Life Technologies). TCCSUP was grown in MEM (Eagle) in Earle’s balanced salt solution containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and nonessential amino acids supplemented with 10% FBS. HT-29 was grown in McCoy’s 5A containing 2 mmol/L L-glutamine supplemented with 10% FBS. Breast cancer cell lines (MCF-7 and MDA-MB-231) were cultured in DMEM F-12 plus 10% FBS, 2 mmol/L L-glutamine, and 1% gentamicin. NIH3T3 cells and its transfectants were cultured in DMEM supplemented with 10% FBS, 5 mmol/L L-glutamine, 50 units/mL penicillin (Life Technologies), and 50 μg/mL streptomycin (Life Technologies). I9A cells and its mutants (I9APTIX10 and I9APTIX22) were grown in RPMI 1640 with 10% FBS.

**Antiproliferative Assays**

Cells were grown as attached cultures at 37°C in a humidified 5% CO2 atmosphere. Cells were plated into 96-well plates at a cell density of 1,000 per well in 100 μL medium and allowed to attach overnight. Varying concentrations of compounds were added to the cells with a total final volume per well of 200 μL. The treated cells were incubated at 37°C, 5% CO2 for 72 hours. Cell viability was determined by CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay (Promega, Madison, WI). IC50 values were calculated using the SOFTmax Pro plate reader program. Each compound was run at least thrice in triplicate. The antiproliferative assays for the I9A cells and its mutants and the NIH3T3 cells and its transfectants with multidrug-resistant pumps (NIH3T3-MDR-G185 and NIH3T3-MDR-V185) were done as described in the literature (30).

**Cell Cycle Analyses**

Mitotic cells were quantitated by two-variable flow analysis using propidium iodide and the mitotic antibody TG3 (a kind gift of Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY). Trypsinized, washed cells (1A9) were resuspended in 500 μL RPMI 1640; 500 μL FCS and 3 mL ice-cold 70% ethanol were added dropwise. The cells were kept at 4°C for at least 30 minutes, after which they were centrifuged and resuspended in blocking solution (2% bovine serum albumin in PBS) and incubated at 4°C overnight. The cells were then centrifuged and resuspended in blocking solution with TG3 antibody (hybridoma cell culture medium), diluted 1:10, and allowed to incubate for 30 minutes. After washing with blocking solution, the cells were stained with FITC-conjugated goat anti-mouse secondary antibody for 30 minutes. The cells were then centrifuged and resuspended in 450 μL PBS, 450 μL propidium iodide solution (50 μg/mL in PBS), and 50 μL RNase solution (5 mg/mL in PBS) and allowed to incubate for 30 minutes. The suspension was then passed through a nylon mesh filter and analyzed on a FACScount flow cytometer (Becton Dickinson, San Jose, CA). FITC fluorescence was monitored with a 525 nm bandpass filter and propidium iodide with a 585 nm bandpass filter.

**Tubulin Polymerization Assay**

Porcine brain tubulin was purified according to the reported procedure (31, 32). Assembly reaction was done at 37°C in PME buffer [0.1 mol/L PIPES (pH 6.9) 1 mmol/L MgCl2, 1 mmol/L EGTA] at a protein concentration of 1 mg/mL (10 μmol/L) in a 96-well half-area plate. With the plate kept on ice, PME was added to each well followed by GTP to a 1 mmol/L final concentration. This was followed by various drug dilutions and controls into different wells. Tubulin was added to the wells and assembly was initiated by simultaneous addition of glutamate (final concentration, 0.4 mol/L) at 37°C. Polymerization was monitored by the increase in absorbance at 351 nm using Spectra Plus microplate reader (Molecular Devices Corp., Sunnyvale, CA).

**Tubulin Depolymerization Assay**

Porcine brain tubulin was purified according to the reported procedure (31, 32). The assay was carried out on a 96-well half-area (Costar, Acton, MA) plate in PME buffer [0.1 mol/L PIPES (pH 6.9) 1 mmol/L MgCl2, 1 mmol/L EGTA] at a protein concentration of 1.5 mg/mL (15 μmol/L). The components of disassembly assay, including PME buffer, various drug dilutions, DMSO [final concentration, 12% (v/v)], and tubulin, were added to 96-well half-area plate kept on ice. Reaction was initiated by simultaneous addition of GTP to a 1 mmol/L final concentration to all of the wells. Polymerization was monitored by the increase in absorbance at 351 nm using Spectra Plus microplate reader (Molecular Devices Corp., Sunnyvale, CA).

**Immunofluorescence Microscopy**

MCF-7 cells were cultured in chamber slides and treated with compounds as indicated in the figure legends. After treatment, cells were fixed by immersion in −20°C absolute methanol for 10 minutes. Slides were then washed with PBS and blocked with blocking buffer consisting of 4% bovine serum albumin in PBS for 10 minutes at room temperature. Slides were then incubated with monoclonal anti-α-tubulin antibody (DM1A, Sigma Chemical Co., Milwaukee, WI). The chemicals were checked for purity by TLC and nuclear magnetic resonance. Melting points were determined on a Thomas Hoover capillary melting point apparatus and were uncorrected. Proton nuclear magnetic resonance spectra were obtained with a Bruker WH-250 (250 MHz) spectrophotometer.

**Figure 1.** Structures of thalidomide, its analogues, and 5HPP-33.
St. Louis, MO), diluted 1:200 in blocking buffer for 1 hour, washed thrice with PBS, and then incubated for 1 hour with FITC-labeled anti-mouse antibody (Vector, Burlingame, CA) diluted 1:500 in blocking buffer. The slides were washed again with PBS, DNA was stained with 4',6-diamidino-2-phenylindole, and slides were mounted with Fluoromount G. Slides were observed with a Zeiss LSM 410 scanning confocal microscope.

Results

Synthesis of 5HPP-33

The synthesis of 5HPP-33 was carried out according to the procedure described in the literature (33). Briefly, A mixture of 4-hydroxyphthalic acid (1 g, 5.5 mmol) and 2,6-diisopropylaniline (1.12 mL, 6 mmol) were stirred and refluxed in acetic acid for 12 hours. The solution was then poured in water and the resulting suspension was filtered to obtain the white crude product. The product was purified by silica gel chromatography and eluted with ethyl acetate/petroleum ether (1:4) to yield 1.53 g 5HPP-33 (86% yield). 1H nuclear magnetic resonance (250 MHz, CDCl₃) δ 1.07 (12H, d, J = 6.82 Hz), 2.63 (2H, hept, J = 6.82 Hz), 7.24 (1H, dd, J = 8.17, 2.10 Hz), 7.30 (1H, d, J = 2.10 Hz), 7.32 (2H, d, J = 7.68 Hz), 7.47 (1H, t, J = 7.68 Hz), 7.47 (1H, d, J = 8.17 Hz), and 11.16 (1H, br s); melting point 200°C to 202°C (lit. 200–201°C; ref. 33).

5HPP-33 Inhibits Growth in a Variety of Tumor Cell Types In vitro

5HPP-33 and thalidomide were examined for their antiproliferative activities against nine human cancer cell lines derived from six different tissues. The cancer cells were subjected to 72 hours of continuous exposure to the drugs. The test compounds were dissolved in DMSO and evaluated using six concentrations at 3-fold dilutions, the highest being 3 × 10⁻⁴ mol/L. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay was then done after 72 hours. The IC₅₀ values, which are the concentrations of the compounds producing 50% growth inhibition, are listed in Table 1. 5HPP-33 showed potent antiproliferative activities against all nine cell lines with IC₅₀s in the low micromolar range (Table 1). Thalidomide, on the other hand, had little antiproliferative activity with IC₅₀s of >300 μmol/L in all cell lines tested.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>5HPP-33 [IC₅₀ (μmol/L)]</th>
<th>Thalidomide (μmol/L)</th>
<th>Paclitaxel (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>6.2 ± 0.4</td>
<td>&gt;300</td>
<td>0.031 ± 0.0005</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>5.2 ± 0.3</td>
<td>&gt;300</td>
<td>Not tested</td>
</tr>
<tr>
<td>HT-29</td>
<td>5.1 ± 1.4</td>
<td>&gt;300</td>
<td>Not tested</td>
</tr>
<tr>
<td>TCCSUP</td>
<td>6.5 ± 1.0</td>
<td>&gt;300</td>
<td>Not tested</td>
</tr>
<tr>
<td>LNCaP</td>
<td>1.7 ± 0.2</td>
<td>&gt;300</td>
<td>Not tested</td>
</tr>
<tr>
<td>DU-145</td>
<td>5.2 ± 0.2</td>
<td>&gt;300</td>
<td>0.024 ± 0.001</td>
</tr>
<tr>
<td>PC-3</td>
<td>8.3 ± 0.8</td>
<td>&gt;300</td>
<td>0.017 ± 0.004</td>
</tr>
<tr>
<td>1A9</td>
<td>2.1 ± 0.1</td>
<td>&gt;300</td>
<td>Not tested</td>
</tr>
<tr>
<td>Hs Sultan</td>
<td>11.1 ± 1.9</td>
<td>&gt;300</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Table 1. 5HPP-33 showed inhibition of cell proliferation of breast, prostate, ovarian, colon, bladder and lymphoma cancer cells

NOTE: 5HPP-33 showed inhibition of cell proliferation of both estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) breast cancer cells, colon cancer cells (HT-29), bladder cancer cells (TCCSUP), androgen-dependent (LNCaP) and androgen-independent prostate cancer cells (DU-145 and PC-3), ovarian carcinoma cells (1A9), and Burkitt’s lymphoma (Hs Sultan). Cells (1,000 per well) were treated with varying concentration of 5HPP-33 and cell-associated protein was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. IC₅₀s represent the means of three experiments in triplicate.
Effectsof5HPP-33onCellCycle
To determine if the 5HPP-33-induced reductions in cell viability shown in Table 1 involved effects on cell cycle progression, two-variable flow cytometric analyses were done on drug-treated 1A9 cells. For each drug concentration, two plots were produced (Fig. 2A–F), arranged as vertical pairs. The bottom plots present single-variable DNA content data, which allowed calculation of cell distribution among G1, S, and G2-M phases of the cell cycle. The top plots show the number of cells reacting with a mitosis-specific antibody (Y axis) as a function of DNA content (X axis), which permitted quantitation of M-phase cells separately from those in G2. The results were tabulated in Table 2 and clearly show that 5HPP-33 caused mitotic arrest in the concentration range (10 \( \mu \)mol/L) consistent with that shown to be antiproliferative (Table 1). At higher concentrations (30–100 \( \mu \)mol/L), cells appeared to be arrested at additional points in the cell cycle.

Stimulation of TubulinPolymerization In vitro by 5HPP-33and Paclitaxel
The incubation of purified porcine tubulin with various concentrations of 5HPP-33 induced tubulin polymerization as determined by spectrophotometric measurements of increases in turbidity under conditions that do not promote abundant control polymerization. 5HPP-33 caused a dose-dependent increase in the rate and extent of microtubule assembly (Fig. 4). In addition, the polymers formed were resistant to cold. The control (no drug) microtubules depolymerized by 80% within 10 minutes of incubation on ice, whereas both 5HPP-33-treated samples showed no cold-induced depolymerization (data not shown). Hence, 5HPP-33 seemed to stabilize microtubules (like paclitaxel) rather than destabilize them (like colchicine).

5HPP-33 Does NotInhibitTubulinPolymerization
It was reported previously that 5HPP-33 inhibited tubulin polymerization (29). Using GTP-induced assembly of purified porcine brain tubulin (without microtubule-associated protein) as our assay, under conditions that favor control polymerization, we showed that 5HPP-33 did not inhibit tubulin in the drug concentration we used (10–0.625 \( \mu \)mol/L; Fig. 5).

Effects of 5HPP-33 on Paclitaxel-ResistantCell Lines
To determine if 5HPP-33 is a substrate for a common drug efflux pump, P-glycoprotein (MDR1), the effects of 5HPP-33 on NIH3T3 cells transfected with the wild-type pHaMDR1/A (NIH3T3-MDR-G185) and mutant pHaMDR1/A (NIH3T3-V185) MDR1 retroviral vectors were evaluated (34). As expected, both of the transfected cell lines, NIH3T3-G185 and NIH3T3-V185, were more resistant to paclitaxel.

### Table 2. % G2-M and % M cells in paclitaxel and 5HPP-33-treated cells

<table>
<thead>
<tr>
<th></th>
<th>% G2-M</th>
<th>% M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.5</td>
<td>4.7</td>
</tr>
<tr>
<td>10 nmol/L Paclitaxel</td>
<td>81</td>
<td>64</td>
</tr>
<tr>
<td>3 ( \mu )mol/L 5HPP-33</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>10 ( \mu )mol/L 5HPP-33</td>
<td>96</td>
<td>70</td>
</tr>
<tr>
<td>30 ( \mu )mol/L 5HPP-33</td>
<td>68</td>
<td>18</td>
</tr>
<tr>
<td>100 ( \mu )mol/L 5HPP-33</td>
<td>47</td>
<td>19</td>
</tr>
</tbody>
</table>

**Immunofluorescence Microscopy of 5HPP-33-Treated Cells**
To investigate the mechanism for the mitotic arrest induced by 5HPP-33, the effects of drug treatment on microtubules were assessed by two approaches. First, the integrity of microtubules in cells treated with 5HPP-33 was examined by immunofluorescence microscopy. Secondly, the effects of 5HPP-33 on tubulin polymerization in vitro were assessed spectrophotometrically. For the immunofluorescence study, cells were grown in chamber slides and exposed to drugs for 3 hours before fixation and staining for microtubules with a monoclonal anti-\( \alpha \)-tubulin antibody. Interphase cells are presented in Fig. 3 because their well-spread microtubule arrays are easily visualized. In the control cells, microtubules were clearly seen, and individual microtubules often appeared long and relatively straight (Fig. 3). Paclitaxel treatment seemed to result in a larger number of microtubules, which appeared shorter and not as straight as in the control cells (Fig. 3). Nocodazole treatment caused the loss of most microtubules (Fig. 3). 5HPP-33 treatment caused changes similar to those of paclitaxel, in that microtubules seemed more abundant and appeared shorter than in the control cells. However, in 5HPP-33-treated cells, the short microtubules appeared to be straighter than those observed following paclitaxel treatment. Thus, we concluded that 5HPP-33 likely stabilized microtubules and favored their polymerization.

**Figure 3.** Effect of 5HPP-33, paclitaxel, and nocodazole exposure on microtubules in MCF-7 cells. Cells were exposed to drug for 3 h before fixation and staining with an anti-tubulin antibody as described in Materials and Methods.
and colchicine than the untransfected cells (relative resistance (RR), 6.7–87; Table 3). In addition, the G185 cells were more resistant to paclitaxel than to colchicine (RR, 57 versus 6.7, respectively), whereas the converse was true for the V185 cells (RR, 25 versus 87 for paclitaxel and colchicine, respectively). In contrast, both transfected cell lines containing functional MDR efflux pumps exhibited sensitivities to 5HPP-33 that were nearly identical to that displayed by the untransfected cells (RR, 1.2 and 1.5 for G185 and V185 cells, respectively).

To determine if 5HPP-33 is effective against cells that possess tubulin point mutations, two paclitaxel-resistant human ovarian cancer cell lines with mutant β-tubulins that exhibit impaired paclitaxel-driven polymerization were used (35). 1A9PTX10 and 1A9PTX22 cells contain point mutations at nucleotides 810 (T-to-G; Phe270Val) and 1,092 (G-to-A; Ala364Thr), respectively, of β-tubulin. These cell lines do not express MDR (35). The cytotoxicities of 5HPP-33 on these two cancer cell lines were compared with that of the parental 1A9 cells (no mutations; Table 4). The mutated sublines were reported to be 24-fold more resistant to paclitaxel and collateral sensitive to vinblastine (Table 4). When we tested them, the resistance to paclitaxel was even greater than the reported value. In contrast, 5HPP-33 was equally effective against the parental 1A9 cells and the two paclitaxel-resistant cell lines, with RRs of 1.1 and 0.7 for PTX10 and PTX22, respectively.

Discussion

Thalidomide and its analogues have generated excitement for the treatment of inflammatory and autoimmune diseases as well as cancers. We have identified a thalidomide analogue (5HPP-33) with potent in vitro activity against a variety of tumor cell lines (both solid tumor and lymphoma). The IC_{50} values of the antiproliferative activities on these cell lines ranged from 1.7 μmol/L (LNCaP) to 11.1 μmol/L (Hs Sultan; Table 1). Interestingly, comparison of the IC_{50}s of 5HPP-33 on the prostate cancer cell lines suggests that 5HPP-33 is effective against prostate cancer cell lines irrespective of their p53 status (LNCaP, wild-type p53; PC-3 and DU-145, mutant p53). In addition, the antiproliferative activities of 5HPP-33 on many cancer cell lines were independent of their hormone dependencies. Both androgen-dependent (LNCaP) and androgen-independent (PC-3 and DU-145) prostate cancer cell lines and estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) breast cancer cell lines were equally susceptible to 5HPP-33. However, 5HPP-33 is not the first thalidomide analogue reported to have broad in vitro antiproliferative activities on cancer cells. A subset of the selective cytokine inhibitory drugs (structures have not been released) were shown to exhibit direct in vitro and in vivo antitumor activities (25). Selective cytokine inhibitory drug-3 was the most effective analogue in that series at inhibiting tumor cell growth. The antiproliferative IC_{50}s of selective cytokine inhibitory drug-3 on PC-3 and DU-145 cancer cells were reported to be ~40 and 20 μg/mL, respectively (25). Thus, 5HPP-33 seems to be considerably more potent than selective cytokine inhibitory drug-3 against these prostate cancer cell lines.

Mechanistically, the antiproliferative effect of 5HPP-33 was attributed to cell cycle arrest in the G2-M phase. Flow cytometric analyses showed that 5HPP-33 concentrations up to 10 μmol/L induced a dose-dependent G2-M arrest in 1A9 cells (Table 2). At 10 μmol/L, 96% of the cells were in

Figure 5. Effect of 5HPP-33 on the assembly of purified porcine brain tubulin. The assembly of 1.5 mg/mL (15 μmol/L) purified bovine tubulin was measured by a change in the absorbance at 351 nm at 37°C in the absence (control; ) or presence of 10 μmol/L ( ), 5 μmol/L ( ), 2.5 μmol/L ( ), and 0.625 μmol/L ( ). Podophyllotoxin at 10 μmol/L ( ) was used as a positive control.

Figure 4. Effect of 5HPP-33 and paclitaxel on tubulin polymerization. The polymerization of porcine brain tubulin was monitored in the presence of 5HPP-33 and paclitaxel (A) and 5HPP-33 (B). , 40 μmol/L 5HPP-33; , 5 μmol/L paclitaxel; , control; , 20 μmol/L 5HPP-33.

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G2-M phase. At higher concentrations, however, cells appeared to be arrested at other points in the cell cycle, because the percentage of cells in G1-S phase increased at concentrations >10 μmol/L. Two-variable flow cytometry using a mitosis-specific antibody was employed to quantitatively distinguish between cells in the G2 and M phases. This analysis clearly showed that the majority of the cells arrested at G2-M are in the mitotic phase and that this accumulation peaks at 10 μmol/L 5HPP-33 (Table 2).

Many antimitotic agents exert their effects by perturbing microtubules by either destabilizing them (such as colchicine or nocodazole) or hyperstabilizing them (such as paclitaxel). Effects of 5HPP-33 on microtubules were investigated by two approaches. In the first, immunofluorescent visualization of microtubules in drug-treated 1A9 cells revealed that 5HPP-33 induced changes similar to those caused by paclitaxel (i.e., production of more abundant and shorter microtubules) but opposite to those induced by nocodazole, a microtubule destabilizer, which caused an apparent loss of microtubule content (Fig. 3). In the second approach, the ability of 5HPP-33 to stabilize microtubules was confirmed by a cell-free tubulin polymerization assay using conditions that do not promote polymerization in the control. 5HPP-33 induced a dose-dependent increase in the rate and extent of purified porcine tubulin polymerization in the absence of microtubule-associated proteins (Fig. 4). Moreover, the microtubules formed were resistant to cold.

Thus, we concluded that 5HPP-33 likely stabilized microtubules and favored their polymerization. Recently, 5HPP-33 was reported to have tubulin polymerization inhibitory activity. It was reported that, at 10 μmol/L, 5HPP-33 was reported to inhibit tubulin polymerization by 83% compared with control (29). However, using a widely used tubulin depolymerization assay in which the conditions favor polymerization in the control (36), we did not observe any inhibition of tubulin polymerization by 5HPP-33 in a wide range of concentration (10–0.625 μmol/L). When comparing the two depolymerization assays, the only major difference is that we conduct the depolymerization assay in the absence of microtubule-associated protein.

5HPP-33 is not the first totally synthetic small molecule shown to induce tubulin polymerization. Three other small molecules have been reported to have a similar paclitaxel-like effect (37–39). However, in comparison with these compounds, 5HPP-33 has the simplest structure and is the first thalidomide analogue reported to have such activity.

Currently, paclitaxel is approved for the treatment of ovarian, breast, and non–small cell lung cancers and AIDS-related Kaposi’s sarcoma (40). Despite the clinical success of paclitaxel, drug resistance has been shown in the laboratory and in the clinic (41–44). Mechanisms of paclitaxel resistance include the overexpression of the drug efflux pump P-glycoprotein (MDR1; ref. 41) and diminished affinity of the drug for tubulin resulting from mutation. The effects of these drug resistance mechanisms on the antitumor efficacy of 5HPP-33 were investigated in two model systems. First, the sensitivities to 5HPP-33 of two transfected cell lines overexpressing two forms of MDR were evaluated. Although the cells expressing either form of MDR were substantially more resistant to paclitaxel than the nontransfected counterpart, all of the cells, regardless of MDR status, were equally sensitive to the antiproliferative effects of 5HPP-33 (Table 3). The results indicate that 5HPP-33 is not affected by the P-glycoprotein pump. In the second model, paclitaxel-resistant 1A9 ovarian cancer cell sublines, which possess mutant β-tubulin and exhibit impaired paclitaxel-driven tubulin polymerization, were evaluated for their sensitivities to 5HPP-33. 5HPP-33 was equally effective against the parental 1A9 cells and the two paclitaxel-resistant cell lines with RRIs of 1.1 and 0.7 (Table 4). These results suggest that 5HPP-33 is a potentially effective microtubule-targeted agent for the treatment of paclitaxel-resistant tumors. A possible explanation for the retained sensitivity to 5HPP-33 in these mutant lines is that 5HPP-33 does not bind to the same site on tubulin as paclitaxel. Currently, displacement studies are under way to address this phenomenon.

In conclusion, we have identified a small molecule (5HPP-33) with antiproliferative activity against nine different cancer cell lines in the low micromolar range. 5HPP-33 causes G2-M arrest and can induce paclitaxel-like tubulin polymerization. In addition, it is active against four paclitaxel-resistant cell lines characterized by differing mechanisms of drug resistance. Preliminary findings from our ongoing work shows that 5HPP-33 causes apoptosis in

### Table 3. Cytotoxicity of 5HPP-33, paclitaxel, and colchicine on wild-type and MDR transfected NIH3T3 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>NIH3T3 IC50 (μmol/L)</th>
<th>NIH3T3-G185 IC50 (μmol/L)</th>
<th>NIH3T3-V185 IC50 (μmol/L)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HPP-33</td>
<td>3</td>
<td>3.6</td>
<td>4.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.04</td>
<td>2.28</td>
<td>5.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.06</td>
<td>0.4</td>
<td>6.7</td>
<td>16.7</td>
</tr>
</tbody>
</table>

**NOTE:** Cytotoxicity of 5HPP-33, paclitaxel, and colchicine on NIH3T3 cells alone or transfected with retroviral vectors expressing either of two forms of MDR1: wild-type (G185) or a point mutant (V185) that alters MDR selectivity. Average of two separate experiments. RR = (ratio of the IC50 of transfected cells) / (IC50 of the nontransfected cells).

### Table 4. Cytotoxicity of 5HPP-33 in paclitaxel-resistant 1A9 ovarian carcinoma cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>1A9 Parental IC50 (μmol/L)</th>
<th>PTX10 IC50 (μmol/L)</th>
<th>PTX22 IC50 (μmol/L)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HPP-33</td>
<td>2.1 ± 0.1</td>
<td>1.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.001</td>
<td>150</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel*</td>
<td>0.002</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.0035</td>
<td>0.5</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** All 5HPP-33 data are means of triplicate determination. RR = (ratio of the IC50 of the resistant cells) / (IC50 of the parental cells).

*The cytotoxicity of paclitaxel was tested at the same time and is shown compared with published data from ref. 35 for paclitaxel and vinblastine.
PC-3 prostate cancer cells and can suppress PC-3 prostate tumor xenograft growth in nude mice. These findings indicate that 5HPP-33 has potential value as a novel microtubule-targeted small molecule for cancer chemotherapy and warrants further investigation in this regard.

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4 H. Xu, unpublished data.

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