2-(Naphthalene-1-yl)-6-pyrrolidinyl-4-quinazolinone Inhibits Skin Cancer M21 Cell Proliferation through Aberrant Expression of Microtubules and the Cell Cycle

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ABSTRACT

Microtubules are a proven target for anticancer drug development because they are critical for mitotic spindle formation and the separation of chromosomes at mitosis. 2-(Naphthalene-1-yl)-6-pyrrolidinyl-4-quinazolinone (HL66) induced cell death with the large cells and multiple micronuclei in M21 skin cancer cells. We demonstrated that HL66-induced cell death is caspase-independent and accompanied by the failure of cell cycle progression. Therefore, HL66-induced cell death may be a mitotic catastrophe. HL66 inhibits the dephosphorylation on Thr14 or Tyr15 of cyclin-dependent kinase (Cdks) 1 and the formation of Cdk1/cyclin B1 complex, which might be associated with cell cycle arrest at the S and G2/M phases. HL66 is an antimicrotubule agent by molecular modeling on the basis of ligand binding to tubulin molecule. Furthermore, we also demonstrated that HL66, like vinblastine, is a tubulin-destabilizing agent via microtubule disruption in M21 cells. These results describe a novel pharmacological property of HL66 as a microtubule inhibitor, which may make it an attractive new agent for the treatment of skin cancer.

Introduction

Quinazolinones have been demonstrated to be antimitotic agents and exert their antitumor activity through inhibition of the DNA repair enzyme system or dysregulation of cell cycle progression of cancer cells (Hamel et al., 1996; Yang et al., 2004). However, the reasons the molecular mechanisms of quinazolinones produced their biological effects remain unknown. The efficacy of antitumor drugs has been estimated based on their capacity to induce apoptosis in tumor cells. It is now evident that apoptosis may not be the primary mechanism of cell death in tumors (Roninson et al., 2001; Castedo et al., 2004; Brown and Attardi, 2005). The term “mitotic catastrophe” is used to describe cell death occurring during mitosis, as a result of DNA damage or deranged spindle formation coupled to the debility of different checkpoints of the cell cycle that would normally arrest progression into mitosis. Therefore, mitotic catastrophe is a type of cell death resulting from abnormal mitosis, which usually ends in the formation of large cells with multiple micronuclei and decondensed chromatin (Molz et al., 1989; Swanson et al., 1995). It has been argued that mitotic catastrophe would be fundamentally different from apoptosis (Roninson et al., 2001). However, reports describing mitotic catastrophe frequently show cells with some phenotypic characteristic of apoptosis, such as chromatin condensation or the expression of apoptosis-related proteins (Chakrabarti and Chakrabarti, 1987; Heald et al., 1993).

The modulated expression of cell cycle regulatory molecules on antiproliferation or mitotic catastrophe has been investigated in many cancer cells. Many reports have indicated that antimicrotubule drugs, such as taxanes and Vinca alkaloids, may disturb G2/M transition and induce cell cycle arrest and apoptosis or mitotic catastrophe in tumor cells (Wang et al., 1999; Roninson et al., 2001). Activation of cyclin-cyclin-dependent kinase (Cdks) complexes is required for cell cycle progression. Distinct pairs of cyclins and Cdks regulate progression through different stages of the cell cycle.
However, progression of the cell cycle from the G2 to M phase is driven by the activation of the Cdk1/cyclin B1 complex. The abnormal activations of cyclin B1 and Cdk1, resulting in deficient cell cycle checkpoints, have been demonstrated to induce mitotic catastrophe (Castedo et al., 2004).

Paclitaxel and vinblastine belong to the class of antimicrotubule agents and widely used chemotherapeutic drugs against a number of malignancies, but the development of drug resistance limits its usefulness (Gottesman et al., 2002; Doyle and Ross, 2003; Cheung et al., 2010). Higher doses are then required to achieve the same efficacy with consequent increases in systemic toxicity to normal tissues. Thus, many investigators concentrated their efforts on understanding the mechanisms of drug resistance or identifying novel and highly specific anticancer drugs to overcome the disease (Kartalou and Essigmann, 2001; Agarwal and Kaye, 2003). For overcoming paclitaxel- or vinblastine-resistant tumor cells and developing a more potent antitumor agent, we designed a series of quinazolinone derivatives by molecular modeling on the basis of ligand binding to tubulin molecule. In this study, the screening test for antitumor activity of 2-(naphthalene-1-yl)-6-pyrrolidinyl-4-quinazolinone (HL66) has demonstrated that HL66 displayed antiproliferation in several cancer cell types, especially M21 skin cancer cells. The HL66-induced M21 cell mitotic catastrophe, via microtubule disarray, was caspase-independent and accompanied by the characteristics of large cells with multiple micronuclei, Bcl-2 phosphorylation, and cell cycle arrest at the S and G2/M phases. The aberrant expression of the activity of the Cdk1/cyclin B1 complex was involved in the HL66-induced M21 cell death in this study. We have also demonstrated that HL66, like vinblastine, is a microtubule-depolymerizing agent in M21 cells.

**Materials and Methods**

**Materials.** HL66 was designed and synthesized as described previously with a modification of the starting materials (Hour et al., 2007). Antipain, aprotinin, dithiothreitol, EGTA, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and Tris were purchased from Sigma (St. Louis, MO). An annexin V-FITC apoptosis detection kit was purchased from BioVision (Mountain View, CA). Antibodies to Bcl-2, cyclin D, cyclin E, Cdk1, Cdk2, Cdk4, Cdk1(pY15), and p21 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and caspase-4, caspase-9, Bcl-2/pS70), and Cdk1(pT14) from Abcam Inc. (Cambridge, MA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and -rabbit IgG were from Jackson ImmunoResearch Laboratories Inc. (Hamburg, Germany).

**Cell Culture.** The M21 cell line (human malignant melanoma) was kindly provided by Dr. Feng-Yao Tang (Department of Nutrition, China Medical University, Taiwan). A375.S2 (human malignant melanoma) and A431 (human epidermoid carcinoma) cell lines were from the American Type Culture Collection (Manassas, VA). Cells were grown in monolayer culture in RPMI medium 1640, Eagle’s minimum essential medium, or Dulbecco’s modified Eagles medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere comprised of 95% air and 5% CO2. When cells were treated with HL66, the culture medium containing 1% fetal bovine serum was used. All data presented in this study are from at least three independent experiments.

**Trypan Blue Exclusion Assay.** Cells were seeded at a density of 5 × 10^5 cells per well onto 12-well plates 48 h before being treated with drugs. The cells were incubated with various concentrations of HL66 for 4, 8, 16, and 24 h. The control cultures were treated with 0.1% dimethyl sulfoxide (DMSO). After incubation, cells were washed with phosphate-buffered saline (PBS). The number of viable cells was determined by staining the cell population with Trypan blue. One part of 0.2% Trypan blue dissolved in PBS was added to one part of the cell suspension, and the number of unstained (viable) cells was counted.

**Annexin V-FITC/PI Double-Staining Assay.** Annexin V/propidium iodide (PI) staining assay was used to further classify M21 cells in the early apoptosis or late apoptosis stages. Annexin V-FITC/PI double-staining of the cells was determined using the annexin V-FITC apoptosis detection kit (BioVision, Inc., San Francisco, CA). This test uses the property of annexin V-FITC to bind to the membrane phospholipid phosphatidylserine in the presence of Ca2+. Cells were seeded at a density of 3.5 × 10^5 cells onto 6-cm dishes 48 h before treatment with drugs. Cells were incubated with 0.033 μM HL66. For annexin-based fluorescence-activated cell sorting analysis, cells were trypsinized, washed twice in ice-cold PBS, and resuspended in 500 μl of binding buffer. Approximately 10^5 cells were then stained for 5 min at room temperature with annexin V-FITC and PI in a Ca2+-enriched binding buffer (Annexin V-FITC kit) and analyzed with a FACSscan flow cytometer (BD Biosciences). Annexin V-FITC and PI emissions were detected in the FL1 and FL2 channels of the FACSscan flow cytometer, using emission filters of 520 and 623 nm, respectively. Approximately 10,000 counts were made for each sample. The annexin V-FITC−/PI(−) population was regarded as control cells (Q3), whereas annexin V-FITC(+)/PI(−) cells were taken as a measure of early apoptosis (Q4), annexin V-FITC(+)/PI(+) as late apoptosis (Q2), and annexin V-FITC−/PI(+) as necrosis (Q1).

**4′,6-Diamidino-2-phenylindole Dihydrochloride Staining.** M21 cells were seeded onto 12-well plates 48 h before treatment with drugs. The cells were incubated with vehicle alone or 0.033 μM HL66. After treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and stained with 1 μg/ml DAPI for 5 min at 37°C. The cells were then washed with PBS and examined by fluorescence microscopy (Olympus IX 70; Olympus, Tokyo, Japan).

**Morphological Investigation.** Cells were seeded at a density of 5 × 10^5 cells per well onto 12-well plates 48 h before treatment with drugs. The cells were incubated with vehicle alone or 0.033 μM HL66. The control cultures were treated with 0.1% DMSO. After treatment, the cells were immediately photographed with an Olympus IX 70 phase-contrast microscope. A field was chosen in the center of each well at approximately the same location for photography.

**Protein Preparation.** Cells were seeded at a density of 1 × 10^6 cells onto 10-cm dishes 48 h before treatment with drugs. Cells were incubated with 0.033 μM HL66 for 4, 8, 16, or 24 h. After treatment, adherent and floating cells were collected at the indicated time intervals and washed twice in ice-cold PBS. Cell pellets were resuspended in cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml antipain) for 30 min at 4°C. Lysates were clarified by centrifugation at 13,000 rpm for 30 min at 4°C, and the resulting supernatant was collected, aliquoted, and stored at −80°C until assay. To determine the mitochondrial and cytosolic cytochrome c, Bax, and AIF levels, the cells were harvested, and mitochondrial and cytosolic fractions were isolated with the ProteoExtract Cytosol/Mitochondria Fractionation Kit (Calbiochem, San Diego, CA) according to the manufacturer’s instructions. The protein concentrations were estimated using the Bradford method (Bradford, 1976).
Western Blot Analysis. Samples were separated by various concentrations of SDS-polyacrylamide gel electrophoresis (PAGE; Bio-Rad Laboratories, Hercules, CA). The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris-HCl, pH 9.0–9.4, 40 mM glycine, 0.375% SDS, and 20% methanol) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA). The blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl) with 0.05% Tween 20 (TBST) for 1 h, washed, and incubated with antibodies to β-actin (1:5000), AIF (1:1000), Bax (1:500), Bel-2 (1:1000), Bel-2p570 (1:1000), procaspase-2 (1:1000), procaspase-3 (1:1000), procaspase-4 (1:12,000), procaspase-8 (1:8000), procaspase-9 (1:200), cytochrome c (1:5000), cyclin A (1:500), cyclin B1 (1:500), cyclin D (1:500), cyclin E (1:500), Cdk1 (1:2500), Cdk2 (1:2500), Cdk4 (1:500), Cdk3/p114 (1:2000), Cdk1/p15 (1:500), p21 (1:500), and p53 (1:1000). Secondary antibody consisted of a 1:20,000 dilution of HRP-conjugated goat anti-mouse IgG [for p53 (1:1000). Secondary antibody consisted of a 1:20,000 dilution of (1:500), Cdk1(pT14) (1:2000), Cdk1(pY15) (1:500), p21 (1:500), and cyclin D (1:500), cyclin E (1:500), Cdk1 (1:2500), Cdk2 (1:2500), Cdk4 (1:500), Cdk3/p114 (1:2000), Cdk1/p15 (1:500), p21 (1:500), and p53 (1:1000). Secondary antibody consisted of a 1:20,000 dilution of HRP-conjugated goat anti-mouse IgG.

Localization of Microtubules. In this study, the detection of α- and β-tubulin was used to examine the microtubule formation in M21 cells. Cells grown on coverslips were treated with vehicle alone, 0.033 μM HL66, 0.006 μM vinblastine, or 0.07 μM paclitaxel for 8, 12, and 24 h. To visualize α-tubulin, the cells were incubated with mouse anti-α-tubulin antibody, washed, and subsequently stained with FITC-conjugated goat anti-mouse IgG. To detect β-tubulin, the cells were incubated for 30 min at 37°C with 250 nM Tubulin Tracker Green reagent. TubulinTracker Green reagent is an uncharged, non-fluorescent compound that easily passes through the plasma membrane of live cells. Once inside the cell, the lipophilic blocking group is cleaved by nonspecific esterases, resulting in a green fluorescent, charged form. After three washings in PBS, the cells were observed by fluorescence microscopy (H600L; Nikon, Tokyo, Japan).

Cell Cycle Analysis. In brief, 2 × 10⁶ cells were trypsinized, washed twice with PBS, and fixed in 80% ethanol. Fixed cells were washed with PBS, incubated with 100 μg/ml RNase A for 30 min at 37°C, stained with propidium iodide (50 μg/ml), and analyzed on a FACScan flow cytometer (BD Biosciences). Approximately 10,000 counts were made for each sample. ModFit LT3.0 software (Verity Software House, Topsham, ME) was used for cell cycle distribution analysis.

Immunostaining. Cells grown on coverslips were treated with vehicle alone or 0.033 μM HL66 for 12 h. After treatment, cells were washed with PBS, fixed with formaldehyde for 10 min, and then permeabilized with 1% Triton X-100 in PBS for 10 min. Fixed cells were subsequently incubated with a blocking solution (2.5% bovine serum albumin) for 1 h at room temperature. Cells were then incubated 1 h at 37°C with protein-specific antibodies diluted 1:50 in TBST solution. The cells were washed three times with TBST and incubated for 30 min at 37°C with fluorescein-conjugated anti-mouse IgG antibody diluted 1:50 in TBST. After washing with TBST, the specimens were mounted in glycerin and observed by fluorescence microscopy (H600L; Nikon).

Molecular Modeling. The three-dimensional crystal structures of the appropriate proteins were downloaded from the RCSB Protein Data Bank website (http://www.rcsb.org/pdb). Automated docking was then carried out. The LigandFit within the software package Discovery Studio 2.5 (Accelrys, San Diego, CA) was used to evaluate and predict the in silico binding free energy of the inhibitors within the macromolecules.

Statistical Analysis. Statistically significant differences from the control group were identified by Student’s t test for paired data. A P value less than 0.05 was considered significant for all tests.

Results

The Effect of HL66 on Cell Proliferation in M21 Cells. Because HL66 is a quinazolinone analog, we attempted to evaluate the effect of HL66 on cell proliferation in M21 cells. Figure 1 shows the results of Trypan blue exclusion assay on M21 cells after treatment with HL66. Twenty-four hours of continuous exposure to various indicated concentrations of HL66 (0.01, 0.033, and 0.1 μM) on M21 cells resulted in dose- and time-dependent decreases in cell number relative to control cultures (Fig. 1). The IC₅₀ (inhibitory concentration) of HL66 was approximately 0.033 μM. Therefore, 0.033 μM HL66 was chosen for further experiments. We also demonstrated that HL66 had the same antitumor effects on CH27 (human lung cancer), HSC-3 (human oral cancer), Hep3B (human liver cancer), A431 (human epidermoid carcinoma), and A375.S2 (human malignant melanoma) cell lines. As shown in Table 1, HL66 had an excellent anticancer effect on M21 cells.

Effects of HL66 on the Apoptotic Characteristics in M21 Cells. M21 cells failing to maintain cell proliferation may be destined to apoptosis by HL66. To further investigate whether the induction of cell death by HL66 was a typical

![Fig. 1.](http://example.com/f1.png) Evaluation of cytotoxicity after incubation of M21 cells with HL66. Cells were incubated with vehicle alone or 0.01, 0.033, or 0.1 μM HL66 for 4, 8, 16, and 24 h. After incubation, the viable cells were measured by Trypan blue exclusion assay. The data are presented as proportional viability (%) by comparing the treated group with the untreated group, the viability of which was assumed to be 100%. All results are expressed as the mean percentage of control ± S.D. of triplicate determinations from four independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with the corresponding control values.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Effects of HL66 on cytotoxicity of M21, A375.S2, A431, CH27, HSC-3, and Hep3B cells</th>
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</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>IC₅₀ (μM)</td>
</tr>
<tr>
<td>M21 (human malignant melanoma)</td>
<td>0.033 ± 0.005</td>
</tr>
<tr>
<td>A375.S2 (human malignant melanoma)</td>
<td>0.043 ± 0.004</td>
</tr>
<tr>
<td>A431 (human epidermoid carcinoma)</td>
<td>0.083 ± 0.010</td>
</tr>
<tr>
<td>CH27 (human lung cancer cell line)</td>
<td>0.053 ± 0.006</td>
</tr>
<tr>
<td>HSC-3 (human oral cancer cell line)</td>
<td>0.041 ± 0.003</td>
</tr>
<tr>
<td>Hep3B (human liver cancer cell line)</td>
<td>1.350 ± 0.122</td>
</tr>
</tbody>
</table>
apoptosis of M21 cells, the analysis of phosphatidylserine externalization was performed. PI is a nonspecific DNA intercalating agent, which is excluded by the plasma membrane of living cells, and thus can be used to distinguish necrotic cells from apoptotic. Therefore, annexin V/propidium iodide staining and flow cytometry analysis were used to confirm M21 cell apoptosis in this study. The typical features of apoptosis (the cell populations of early and late apoptosis) were not observed after treatment with 0.033 μM HL66 (Fig. 2A). The phenotypic characteristics of HL66-treated M21 cells were evaluated by microscopic inspection of overall morphology and DAPI staining of nuclear morphology. The nuclei feature of control cells was round, whereas shrunk cells with several micronuclei and even some blebbing and condensed nucleus were observed after treatment with 0.033 μM HL66 for 16 h (Fig. 2B). We also demonstrated that paclitaxel (0.07 μM)- or vinblastine (0.006 μM)-treated cells had a similar appearance to those in the HL66-treated cells (data not shown). The IC\textsubscript{50} value of paclitaxel and vinblastine was 0.07 and 0.006 μM, respectively. In this study, the ratio of cells with multiple micronuclei was estimated. As shown in Table 2, there were significant differences between HL66-treated and control cells after the treatment of cells with 0.033 μM HL66 for 16 and 24 h. Because many of the HL66-treated cells possessed multiple micronuclei, the proportion of cells in the sub-G\textsubscript{1} phase was also examined by flow cytometric analysis. Cells treated with HL66 for 16 and 24 h displayed a significant increase in the percentage of cells at the sub-G\textsubscript{1} phase (Table 3). Based on the above data, HL66-induced M21 cell death might be a mitotic catastrophe, which usually ends in the formation of large cells with multiple micronuclei.

![Fig. 2. Effects of HL66 on apoptotic characteristics in M21 cells. A, annexin V-FITC/PI staining of M21 cells. Cells were incubated with 0.1% DMSO or 0.033 μM HL66 for 4, 8, 16, and 24 h. The cells were then processed for annexin V-FITC/PI staining and analyzed by flow cytometry. B, HL66 induced phenotypic changes in cell nucleus. M21 cells were incubated with 0.033 μM HL66 for 4, 8, 16, and 24 h. After being treated with HL66, the cells were fixed with formaldehyde, permeabilized with Triton X-100, and stained with 1 μg/ml DAPI for 5 min. The cells were then examined by fluorescence microscopy (300×). A phase-contrast image of the cells was also taken. The arrows indicate the large cells with multiple micronuclei. All results are representative of three independent experiments.]
Effects of HL66 on the phenotypic changes in cell nucleus of M21 cells

Cells were incubated with 0.1% DMSO or 0.033 μM HL66 for 4, 8, 16, and 24 h. For quantitative image analysis samples were stained with DAPI, followed by examination with an Olympus IX 70 microscope at magnification of 10×. The field of cells was examined and photographed. For each sample four fields of view were selected randomly. All cells, which contained single or multiple micronuclei, were counted in the entire field. All results are expressed as the mean percentage of total population ± S.D. of triplicate determinations from three independent experiments.

<table>
<thead>
<tr>
<th>Hour</th>
<th>Multiple Micronuclei Cell Numbers (of Total Population)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (0.1% DMSO)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

### TABLE 3

Effect of HL66 on the distribution of cells in phases of the cell cycle of M21 cells

Cells were incubated with 0.1% DMSO or 0.033 μM HL66 for 4, 8, 16, and 24 h. After treatment, cells were stained with propidium iodide and subjected to cytometric analysis. All results of cell cycle distribution are expressed as the mean percentage of control ± S.D. of three independent experiments.

<table>
<thead>
<tr>
<th>Hour</th>
<th>Sub-G1 (% of Total Population)</th>
<th>Cell Cycle Distribution (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>G0/G1</td>
</tr>
<tr>
<td>4</td>
<td>1.4 ± 0.3</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>1.0 ± 0.1</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>16</td>
<td>1.1 ± 0.1</td>
<td>17.8 ± 3.5</td>
</tr>
<tr>
<td>24</td>
<td>1.5 ± 0.5</td>
<td>21.6 ± 3.1</td>
</tr>
</tbody>
</table>

The Effect of HL66 on the Expression of Apoptosis-Related Proteins in M21 Cells

In this study, HL66 had no effect on the proform of caspase-2, -3, -4, -8, and -9 proteins (Fig. 3A). AIF and caspase-3 are the key indicators of intracellular signaling of caspase-independent and -dependent apoptosis, respectively. Therefore, the protein expression of AIF was examined in this study. After M21 cells were treated with 0.033 μM HL66 for the indicated time intervals, there were no changes in the expression of AIF protein (Fig. 3A). Based on the above data, M21 cells resistant to HL66-induced apoptosis can be killed via mitotic catastrophe, an alternative cell death mechanism. The expression of apoptosis-related proteins, such as cytochrome c, Bcl-2, and Bax, was also examined in this study. HL66 had no effect on the protein expression of cytochrome c (Fig. 3A). The amount of Bax protein significantly increased after treatment with HL66 for 4 h (Fig. 3A). Exposure of M21 cells to 0.033 μM HL66 for 8 h resulted in significant decreases in Bcl-2 protein levels (Fig. 3A). However, Bcl-2 phosphorylation was induced after treatment with HL66 for 4 h. This study also investigated the distribution of Bax, cytochrome c, and AIF in cytosolic and mitochondrial fractions. Bax, cytochrome c, and AIF were clearly detected in cytosolic and mitochondrial fractions (Fig. 3B). When M21 cells were treated with 0.033 μM HL66 for 16 and 24 h, HL66 induced cytochrome c translocation from mitochondrial fraction to cytosolic fraction (Fig. 3B). There was a significant increase in the protein amount of Bax in either the cytosolic or the mitochondrial fraction after HL66 stimulation (Fig. 3B). It is worth noting that AIF was found largely in the mitochondrial fraction after treatment with HL66 (Fig. 3B).

**Effects of HL66 on Microtubule Polymerization in M21 Cells.** To further examine whether the microtubules were injured by HL66 in M21 cells, the effect of HL66 on microtubule localization was examined by the detection of α- and β-tubulin. We demonstrated that HL66 caused severe disruption and breakage of M21 cell microtubules compared with that in control cells after treatment with HL66 for 12 h (Fig. 4). In this study, vinblastine and paclitaxel were used as reference compounds of the damage effect on microtubule. In M21 cells treated with 0.07 μM paclitaxel, tubulins were denser and concentrated prominently on the nuclear margins, suggesting that paclitaxel promoted the formation of microtubule (Fig. 4). When M21 cells were treated with 0.006 μM vinblastine for 12 h, cells had a similar appearance to those in the HL66-treated cells (Fig. 4). Based on the above findings, these results are consistent with the result of our molecular modeling study in which HL66 was identified as an antimicrotubule agent and could bind to the vinblastine binding site of tubulin molecule.

**HL66 Induced Cell Cycle Arrest at the S and G2/M Phases of M21 Cells.** Because microtubules are essential for mitosis, we analyzed the changes in cell cycle distribution...
demonstrated that vinblastine (0.006 μM) induced S- and G2/M-phase arrest was accompanied by cell death by HL66 and a mitotic cell death is associated with S-phase arrest of M21 cells (Fig. 5). These findings indicate that M21 cells failing to progress in cell cycle may be destined to cell death upon treatment with HL66. Flow cytometric analysis was performed on cells treated with 0.033 μM HL66 for 4, 8, 16, and 24 h. Cell cycle analysis revealed time-dependent S- and G2/M-phase arrest after treatment with HL66. Significant S- and G2/M-phase arrest was indicated by decreasing proportions of cells in the G0/G1 phase (Fig. 5 and Table 3). We also demonstrated that vinblastine (0.006 μM) and paclitaxel (0.07 μM) caused a marked increase in S- and G2/M-phase arrest of M21 cells (Fig. 5). These findings indicate that M21 cells failing to progress in cell cycle may be destined to cell death by HL66 and a mitotic cell death is associated with S- and G2/M-phase arrest.

Effects of HL66 on the Protein Expression of Cell Cycle Regulatory Molecules in M21 Cells. Because HL66-induced S- and G2/M-phase arrest was accompanied by a significant decrease in the G0/G1 phase, the protein expression of cell cycle regulators, cyclin D/Cdk4 (G1-phase progression), cyclin A or E/Cdk2 (G1/S transition and S-phase progression), and cyclin B1/Cdk1 (G2/M transition), was detected during treatment with HL66 for 4, 8, 16, and 24 h. As shown by immunoblotting, the levels of cyclin B1 and Cdk2 protein significantly increased during 0.033 μM HL66 treatment over 24 h (Fig. 6A). HL66 had no effect, however, on the protein expression of Cdk1 (Fig. 6A). It is well known that cyclin D1 levels begin to rise early in G1, and continue to accumulate until the G1/S-phase boundary when levels rapidly decline. The degradation of the cyclin is essential for the replication of DNA. From cell cycle analysis data, we have demonstrated that HL66 induced an arrest of the cell cycle in S phase. As expected, cyclin D expression decreased markedly with decreases in Cdk4 in the HL66-treated cells in this study. Exposure of M21 cells to HL66 resulted in the decrease in cyclin A protein levels (Fig. 6A). Based on the above data, the expression of cell cycle regulatory molecules is involved in the HL66-induced changes in cell cycle progression. We also demonstrated that the dephosphorylation of Cdk1 on Thr14 and Tyr15 was inhibited, although the protein levels of Cdk1 were not changed after treatment with HL66 (Fig. 6A). The levels of p53 and p21 protein were also examined during HL66-induced M21 cell death. After treatment with HL66, p53 and p21 levels decreased to comparable levels in control cells (Fig. 6A), indicating that p53 and p21 protein expression were involved in HL66-induced M21 cell death. Densitometric analysis of the cell cycle-related protein expression is shown in Table 4.

Effects of HL66 on the Localization of Cyclin B1 and Cdk1. To further evaluate the involvement of specific regulators of cell cycle such as cyclin B1 and Cdk1 in HL66-induced S- and G2/M-phase arrest, the localization of cyclin B1 and Cdk1 was analyzed by immunofluorescent staining in M21 cells. In this study, HL66 induced cyclin B1 translocation from the cytosol into the nucleus, and nuclear cyclin B1 levels increased significantly in M21 cells (Fig. 6B). As shown in Fig. 5B, the Cdk1 distributed throughout the cells in mainly punctuate areas and more staining was observed in the nucleus in M21 cells. After treatment with HL66, the immunostaining patterns of Cdk1 were similar to those seen in control cells (Fig. 6B). This study also found that the distribution of phosphorylation of Cdk1 at Tyr15 (pY15) was observed throughout the cells, and after treatment with HL66, most of the Cdk1 (pY15) shifted to peripheral regions of the extranucleus and concentrated prominently on the nuclear margins (Fig. 6B). The dotted staining and bright green fluorescence of phosphorylation of Cdk1 at Thr14 (pT14) was most intense in the cell nucleus after treatment with HL66 (Fig. 6B). Based on the above findings, we demonstrated that the dephosphorylation of Cdk1 at Thr14 and Tyr15 was not observed during HL66-induced M21 cell death even though HL66 significantly triggered the increase in the translocation of cyclin B1. Therefore, we guess that HL66 inhibits the formation of cyclin B1/Cdk1 complex through failure to dephosphorylation of Cdk1 at Thr14 and Tyr15, leading to cycle arrest at the S and G2/M phases.

Molecular Modeling of the HL66 and Tubulin Interaction. The three-dimensional crystal structures of the tubulin domain complexed with a tubulin-binding agent were downloaded from the RCSB Protein Data Bank website (http://www.rcsb.org/pdb). The Protein Data Bank code 1sa0 is complexed with colchicine, 1z2b is complexed with vinblastine, and 1jff is complexed with paclitaxel. The computational modeling of HL66 and these three tubulin crystal structures indicated that HL66 can bind excellently to the vinblastine binding site, however, moderately to the colchicine binding site and not to the paclitaxel binding site at all.

![Fig. 4.](image-url) HL66 induced the change in microtubule formation of M21 cells. Cells were incubated with vehicle alone, 0.033 μM HL66, 0.07 μM paclitaxel, or 0.006 μM vinblastine for 8, 12, and 24 h. A, to visualize α-tubulin the immunostain of cells was performed with mouse monoclonal anti-α-tubulin antibody as described under Materials and Methods. B, to detect β-tubulin cells were incubated for 30 min with 250 nM Tubulin Tracker Green reagent. The specimens were observed by fluorescence microscopy. All results are representative of three independent experiments.
From the result of docking simulation, we suppose that HL66 is a tubulin-binding agent as vinblastine acts. As shown in Fig. 7, the interaction between HL66 and 1z2b involved the hydrogen binding of N3-H with Asp179, van der Waals interactions of C7-H with Pro222, van der Waals interactions of 6-pyrrolidinyl with Pro222, Thr223, Tyr224, and Leu248.

Fig. 5. HL66 induced cell cycle arrest of M21 cells. Cells were treated with 0.1% DMSO, 0.033 μM HL66, 0.07 μM paclitaxel, or 0.006 μM vinblastine for 4, 8, 16, and 24 h. After treatment, cells were stained with propidium iodide and subjected to cytometric analysis. Results are representative of three independent experiments.

Fig. 6. Effects of HL66 on the expression of cell cycle regulatory molecules in M21 cells. A, the effect of HL66 on the protein levels of cell cycle regulatory molecules were detected by Western blot analysis. M21 cells were incubated with 0.1% DMSO or 0.033 μM HL66 for 4, 8, 16, and 24 h. Cell lysates were subjected to SDS-PAGE [10% for p53, cyclin A, cyclin B1, and cyclin E; 13% for cyclin D, Cdk1(pT14), Cdk1(pY15), Cdk1, Cdk2, and Cdk4; 14% for p21], and then probed with primary antibodies as described under Materials and Methods. –, control cells; +, HL66-treated cells. B, the effect of HL66 on the localization of cyclin B1 and Cdk1 protein in M21 cells. Cells were incubated with 0.1% DMSO or 0.033 μM HL66 for 12 h. After treatment, cells were fixed and stained with isozyme-specific antibodies as described under Materials and Methods. The specimens were observed by fluorescence microscopy. All results are representative of three independent experiments.
TABLE 4
Densitometric analysis of the Western blot results of cell cycle-related proteins

The degree of protein expression was quantified by AlphaEase image software. Data are plotted as the mean percentage of the relative control ± S.D. All results are representative of three independent experiments.

<table>
<thead>
<tr>
<th>Hour</th>
<th>Cdk1</th>
<th>Cdk2</th>
<th>Cdk4</th>
<th>Cdk1(pT14)</th>
<th>Cdk1(pY15)</th>
<th>Cyclin B1</th>
<th>Cyclin A</th>
<th>Cyclin D</th>
<th>Cyclin E</th>
<th>p53</th>
<th>p21</th>
<th>β-Actin</th>
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<tr>
<td>4</td>
<td>107 ± 8</td>
<td>129 ± 6</td>
<td>78 ± 5</td>
<td>211 ± 9</td>
<td>317 ± 12</td>
<td>168 ± 7</td>
<td>91 ± 5</td>
<td>86 ± 8</td>
<td>124 ± 9</td>
<td>111 ± 4</td>
<td>61 ± 5</td>
<td>97 ± 7</td>
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<tr>
<td>8</td>
<td>96 ± 10</td>
<td>161 ± 9</td>
<td>80 ± 7</td>
<td>249 ± 6</td>
<td>464 ± 8</td>
<td>137 ± 5</td>
<td>69 ± 6</td>
<td>60 ± 9</td>
<td>60 ± 8</td>
<td>113 ± 3</td>
<td>59 ± 7</td>
<td>98 ± 5</td>
</tr>
<tr>
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<td>104 ± 9</td>
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</table>

and van der Waals interactions of 2-(naphthalene-1-yl) with Pro175, Ser178, Asn329, and Ile332. These interactions made HL66 bind readily to tubulin with low potential energy.

**Discussion**

This study has shown that HL66-induced M21 cell death may be a mitotic catastrophe, which was accompanied by the characteristic of a large cell with multiple micronuclei. Moreover, M21 cells displayed S- and G2/M-phase arrest of the cell cycle after treatment with HL66. Consistent with such results, other studies have suggested that mitotic catastrophe is a type of cell death resulting from abnormal mitosis, which usually ends in the formation of large cells with multiple micronuclei (Roninson et al., 2001; Castedo et al., 2004). In this study, we demonstrated that the cleavage in caspase family members, such as caspase-2, -3, -4, -8, and -9, was limited after treatment with HL66. This phenomenon was also demonstrated by other studies in which mitotic catastrophe would be unrelated to apoptosis (Roninson et al., 2001; Nabha et al., 2002), assuming that apoptosis must be mediated by mitochondria. Furthermore, the phosphorylation on Thr14 and Tyr15 of Cdk1 was not induced by HL66 during HL66-induced cell death. We also found that HL66-induced cyclin B1 (pY15) immunolocalization was more intense than that found in control cells, especially in the peripheral regions of the extranuclear. Based on the above data, mitotic arrest induced by HL66 may be associated with nuclear accumulation of cyclin B1 and the inhibition of the dephosphorylation in Thr14 and Tyr15 of Cdk1. Therefore, we guess HL66 inhibits the formation of cyclin B1/Cdk1 complex through failure to dephosphorylate of cyclin B1 at prophase and Tyr15 of Cdk1.

Because HL66 induced the mitotic arrest of cells that preceded cell death, cell cycle regulators, in particular the expression of cyclin B1 and Cdk1, were examined. Cyclin B1 translocates from the cytosol to the nucleus during the prophase, and cyclin B1 must be destroyed by the anaphase-promoting complex to allow mitosis to proceed at the end of metaphase (Peters, 2002). However, the activity of Cdk1 and cyclin B1 complex is regulated by the dephosphorylation of Cdk1 on Thr14 and Tyr15 and nuclear accumulation of cyclin B1 protein. Furthermore, the nuclear accumulation of cyclin B1 and Cdk1 has been reported to correlate with mitotic catastrophe (Nigg, 2001; Yoshikawa et al., 2001). In this study, HL66 induced a marked increase in the protein level of cyclin B1 and significant cyclin B1 translocation from cytosol into nucleus in M21 cells. Furthermore, the dephosphorylation on Thr14 and Tyr15 of Cdk1 was not induced by HL66 during HL66-induced cell death. We also found that HL66-induced cyclin B1 complex through failure to dephosphorylate of cyclin B1 at prophase and Tyr15 of Cdk1. Therefore, we guess HL66 inhibits the formation of cyclin B1/Cdk1 complex through failure to dephosphorylation of cyclin B1 at prophase and Tyr15, leading to the cycle arrest at the S and G2/M phases. Moreover, HL66 could induce the sustained activation of cyclin B1 at prophase, leading to the failure of the progression of mitosis from prophase to metaphase and formation of cytokinesis-arrested cells with multiple micronuclei.

Many reports indicated that Cdk1 phosphorylates Bcl-2 on Ser-70 during the G2/M phase of the cell cycle and the phosphorylation of Bcl-2 is correlated with the accumulation of cells in the G2/M phase (Furukawa et al., 2000; Pathan et al., 2001). It has been also reported that the phosphorylation of Bcl-2 is not proportional to the extent of apoptosis (Furukawa et al., 2000), and Bcl-2 phosphorylation is only a marker of M-phase events (Ling et al., 1998). Furthermore, drugs affecting the integrity of microtubules could induce Bcl-2 phosphorylation (Haldar et al., 1997). In this study, exposure of M21 cells to HL66 resulted in significant decreases in Bcl-2 protein levels, which were accompanied by a significant increase in Bcl-2 phosphorylation. We also identified that HL66 is an antimicrotubule agent using molecular docking.
study on the basis of ligand binding to tubulin molecule and was able to inhibit the tubulin polymerization as vinblastine did. These results seemed reasonable to suggest that HL66 is a tubulin-distabilizing agent and is able to induce cell cycle arrest at the G2/M phase, leading to M21 cell death with the characteristic of the phosphorylation of Bcl-2, which was correlated with the HL66-induced inhibition of the dephosphorylation of Cdk1 on Thr14 and Tyr15.

In this study, the decrease of p21 protein levels was observed after the addition of HL66. This result is not consistent with previous observations in which drugs induced G1/M arrest by the induction of p21 (Chang et al., 2004; Huang et al., 2011). Taylor et al. (1999) have suggested that p53 negatively regulates the transcription of cyclin B1 by enhancing the transcription of Cdk1 inhibitors, such as p21. However, this study demonstrated that HL66 caused a significant increase in cyclin B1 protein expression. Waldman et al. (1996) have suggested that p21 is required for the precise coordination of the S and M phases. Because of the absence of p21, cells arrest in a G2-like state and then undergo additional S phases without intervening normal mitosis (Waldman et al., 1996). Furthermore, the down-regulation of the p21 gene expression and the corresponding protein might induce the brief accumulation of cells in the G2 phase (Mansilla et al., 2006; Su et al., 2011). In our study, p53 and p21 protein expression was inhibited by HL66 in M21 cells, suggesting that the HL66-induced changes in the transition from G2/M to S phase, which results in an increasing proportion of cells in S phase, might be associated with the down-regulation of p53 and p21 protein expression. The down-regulation of Cdk4 and cyclin D1 protein level were also observed after treatment with HL66 in this study. This result is consistent with previous observations in which cyclin D1 levels are the major regulator involved in the progression of G2 phase and continue to accumulate until the G2/M phase boundary when levels rapidly decline (Choi and Kim, 2008; Onumah et al., 2009). In the G1 checkpoint, cyclin E and its partner Cdk2 regulate cell cycle progression. No significant change in protein expression pattern of cyclins E was observed in this study. However, expression of Cdk2 markedly increased in a time-dependent manner in M21 cells at 0.033 μM HL66. This result also suggested that HL66 is able to induce the degradation of cyclin D1 and increase in Cdk2 expression, leading to the cell cycle arrest in S phase.

In conclusion, HL66, like vinblastine, is able to inhibit tubulin polymerization in M21 cells. From our observations of the HL66-induced M21 cell death that was resistant to apoptosis, we identified mitotic catastrophe as having the characteristics of a large cell with multiple micronuclei, being caspase-independent, and experiencing cell cycle arrest at the S and G2/M phases. Furthermore, the aberrant expression of the regulatory proteins of the cell cycle was involved in HL66-induced M21 cell death in this study.

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Authorship Contributions
Participated in research design: Y. C. Wu, Hour, and Lee.

Contributed new reagents or analytic tools: Hour, C. Y. Wu, and Lee.
Wrote or contributed to the writing of the manuscript: Lee.

References


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