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2-(3-Methoxyphenyl)-5-methyl-1,8-naphthyridin-4(*1H*)-one (HKL-1) induces G2/M arrest and mitotic catastrophe in human leukemia HL-60 cells

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ABSTRACT

2-(3-Methoxyphenyl)-5-methyl-1,8-naphthyridin-4(*1H*)-one (HKL-1), a 2-phenyl-1,8-naphthyridin-4-one (2-PN) derivative, was synthesized and evaluated as an effective antimitotic agent in our laboratory. However, the molecular mechanisms are uncertain. In this study, HKL-1 was demonstrated to induce multipolar spindles, sustain mitotic arrest and generate multinucleated cells, all of which indicate mitotic catastrophe, in human leukemia HL-60 cells. Western blotting showed that HKL-1 induces mitotic catastrophe in HL-60 cells through regulating mitotic phase-specific kinases (down-regulating CDK1, cyclin B1, CENP-E, and aurora B) and regulating the expression of Bcl-2 family proteins (down-regulating Bcl-2 and up-regulating Bax and Bak), followed by caspase-9/-3 cleavage. These findings suggest that HKL-1 appears to exert its cytotoxicity toward HL-60 cells in culture by inducing mitotic catastrophe.

Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (Jemal et al., 2011). A unifying feature of cancer is the uncontrolled proliferation of cells, which disrupts the normal function of surrounding tissues or even distant ones, ultimately leading to organ failure and death. Uncontrolled mitosis distinguishes tumor cells from normal cells, which makes targeting mitosis one of the most successful antitumor strategies (Jackson et al., 2007; Schmit and Ahmad, 2007; Sudakin and Yen, 2007). Disruption of the mitotic progression leads tumor cells to mitotic arrest, frequently followed by cell death. Mitotic catastrophe, a form of cell death resulting from abnormal mitosis, is a cytotoxic death pathway as well as an appealing mechanistic strategy for the development of anti-cancer drugs (Castedo et al., 2004). During mitosis, microtubules play a critical role, especially in the mitotic spindle formation. Interference with cellular microtubule dynamics results in mitotic arrest, prominent cytotoxicity, and apparent angiogenesis inhibition, all of which translates into an anticancer effect (Wood et al., 2001). Several anticancer drugs acting on microtubules have been widely used in clinics to treat various tumors, including hematological malignancies and breast, ovarian and non-small-cell lung carcinomas. However, direct inhibition of microtubule dynamics may also disrupt other essential physiologic processes, such as interphase and axonal transport (Gerdes and Katsanis, 2005a). Over the last decade, many unprecedented molecular and biochemical events involved in highly complex aspects of mitosis have been identified. Certain proteins appear to function solely in mitosis, which renders them ideal targets for the development of mitosis-specific cancer drugs (Schmit and Ahmad, 2007). Natural and synthetic compounds that selectively inhibit mitotic kinesins involved in spindle and kinetochore functions are currently in various stages of clinical trials (Bhat and Setaluri, 2007).

Our laboratory synthesized 2-(3-methoxyphenyl)-5-methyl-1,8-naphthyridin-4(*1H*)-one (HKL-1) and evaluated it as a potential antimitotic agent; Fig. 1A shows the structure of HKL-1 (Chen et al., 1997a, 1997b). Chemicals used to develop anticancer agents should have the highest tumorigenic potential, as even they comprise a relatively small fraction of the overall tumor cell composition. Antimitotic agents are the only mechanistic class showing high tumorigenic potential. The authors identified nine chemical agents that appear to strongly and selectively inhibit the growth of the most tumorigenic cell lines. HKL-1 was the one agent among these nine that potentially inhibits microtubule polymerization (Abdullah et al., 2009). The current research thus investigated HKL-1's anticancer mechanisms. Our

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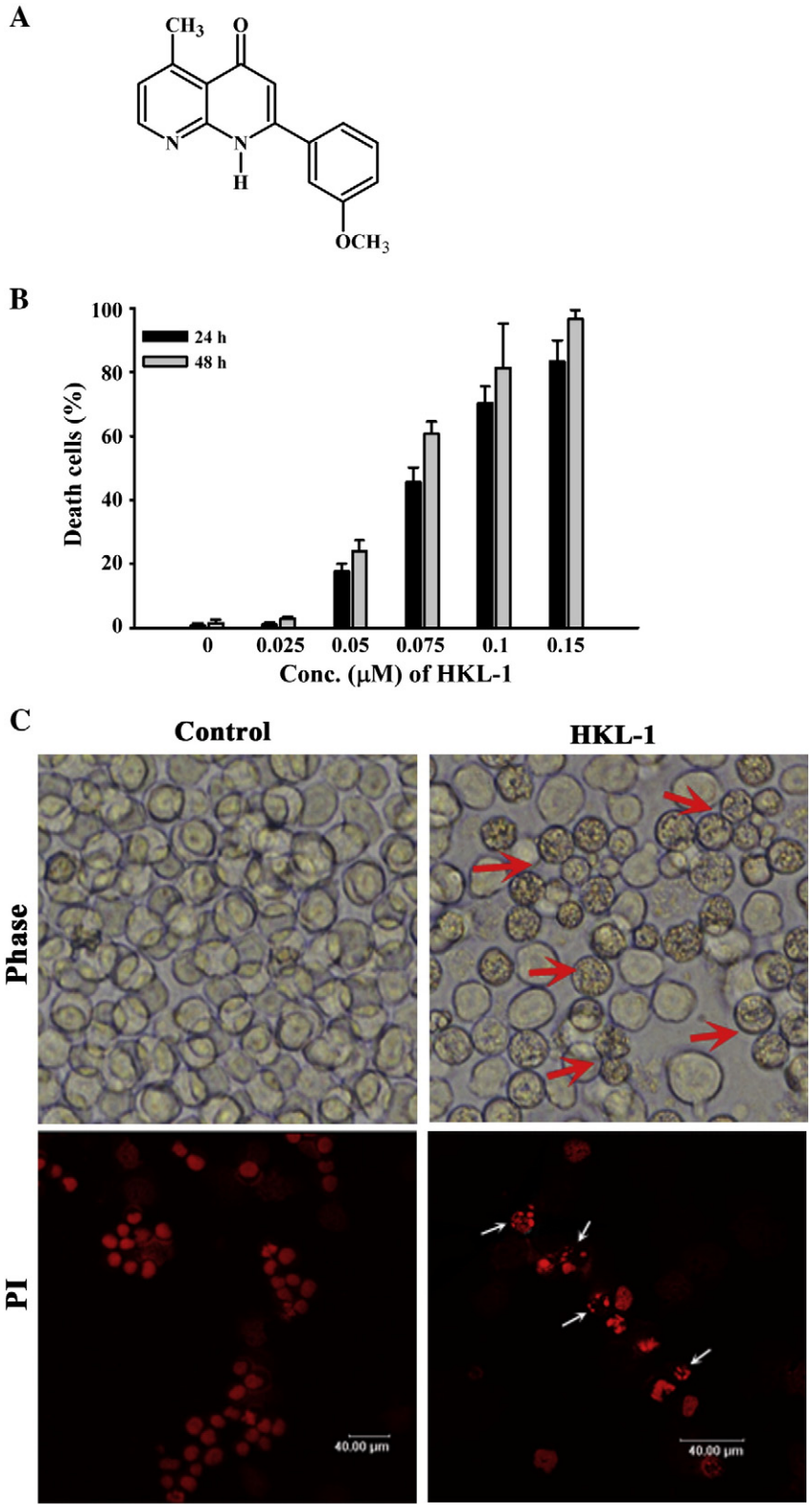

Fig. 1. HKL-1 induced cell death in HL-60 cells. (A) Chemical structure of HKL-1. (B) Viability. HL-60 cells were treated with the indicated concentrations of HKL-1 for 24 and 48 h. After treatment, the viability was counted using trypan blue exclusion. Data from four independent experiments are presented as mean ± SD. The cell death effects were observed in all conditions, except for 0.025 μM of HKL-1 ($P<0.001$ compared with the control). (C) Morphologic observation. Cells were treated with 0.1 μM HKL-1 for 24 h, then fixed and stained with PI. Morphological changes were observed under phase or fluorescence microscopy (magnification ×200). Red and white arrows indicate the apoptotic bodies.

results indicated that HKL-1 indeed promoted the arrest of the mitotic phase in leukemia HL-60 cells by altering the protein expression of mitosis-phase-regulated protein (cyclin B1, CDK1, and p21) and mitotic kinesins (centromeric protein E (CENP-E) and aurora B). The process continued thereafter, with mitotic catastrophe being induced to eliminate cancer cells.

Table 1
Inhibitory effect of HKL-1 on cell growth in a variety of human cancer cells.

Conc. (μM)	HL-60	NCI-H460	HCT116	Hep 3B	PBMCs	Detroit 551
0.025	85.0 ± 3.3*	–	–	–	–	–
0.05	37.1 ± 1.4*	–	83.4 ± 1.5*	70.5 ± 3.1*	–	–
0.075	8.1 ± 0.7*	–	–	–	–	–
0.1	2.4 ± 0.3*	83.9 ± 3.6*	60.1 ± 2.4*	59.1 ± 0.2*	95.9 ± 4.2	96.0 ± 2.2
0.15	2.1 ± 0.7*	59.1 ± 0.7*	44.4 ± 0.2*	45.8 ± 0.9*	–	–
0.2	1.4 ± 0.3*	43.1 ± 0.1*	37.0 ± 0.5*	22.7 ± 1.5*	–	–
0.25	–	–	30.1 ± 2.0*	13.8 ± 1.3*	–	–
0.5	3.1 ± 0.7*	25.8 ± 1.3*	23.8 ± 1.4*	6.7 ± 1.0*	–	–
1	2.4 ± 0.3*	18.1 ± 0.7*	18.1 ± 0.4*	5.8 ± 0.6*	94.2 ± 3.3	94.2 ± 2.3
5	1.1 ± 0.7*	12.4 ± 0.3*	11.9 ± 1.2*	3.1 ± 0.4*	93.9 ± 2.2	92.9 ± 4.2
IC_{50}	0.04 μM	0.18 μM	0.13 μM	0.13 μM	> 5 μM	> 5 μM

–: not determined.
* $P<0.001$ compared with the control at 48 h.

Materials and methods

Materials

HKL-1 was synthesized in our laboratory (Chen et al., 1997a, 1997b). In this study, HKL-1 was initially dissolved in DMSO as a stock concentration of 5 mM, and then was diluted to achieve the desired concentrations before each experiment. The final concentration of DMSO in the culture medium was kept below 0.1%. All of the cell culture materials were obtained from Gibco laboratories (Grand Island, NY). 0.4% Trypan blue solution, triton-X 100, RNase, phopidium iodide (PI), bovine serum albumin (BSA), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), and eithidium bromide (EtBr) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture. Human leukemia HL-60 cells, normal leukocytes (PBMCs), and non-small-cell-lung cancer NCI-H460 cells were maintained in RPMI-1640 medium. Human colon HCT116 cells were maintained in McCoy's 5A medium. Human hepatoma Hep 3B and normal skin Detroit 551 cells were maintained in DMEM medium. The aforementioned culture mediums were supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Logarithmically growing cancer cells were used for all experiments.

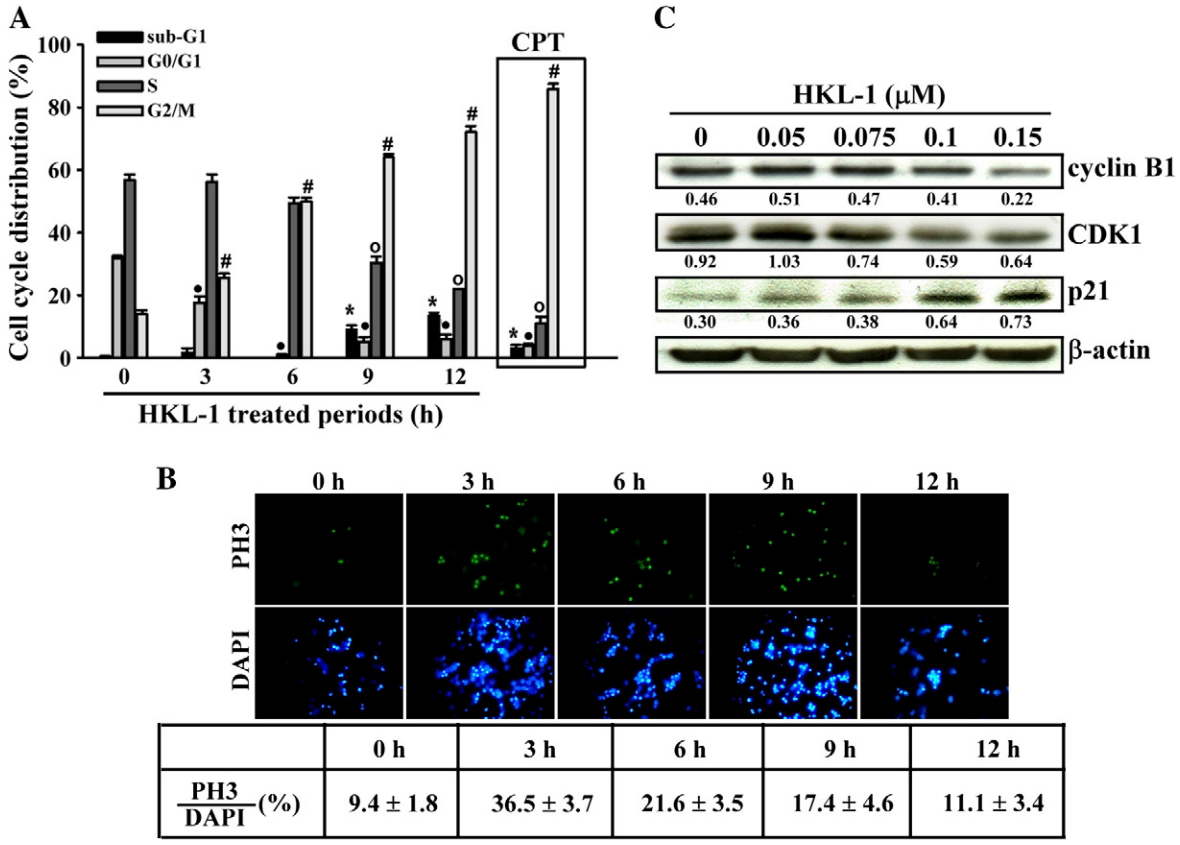

Fig. 2. HKL-1 affected cell cycle distributions in HL-60 cells. Cells were treated with HKL-1 (0.1 μM) for the indicated time periods. (A) The cells were harvested and subjected to cell cycle distribution analysis. Camptothecin (CPT, 20 nM, treated for 12 h) was used as a positive control. * indicates $P<0.001$ compared with the sub-G1 phase of the control; ● indicates $P<0.001$ compared with the G0/G1 phase of the control; **o** indicates $P<0.001$ compared with the S phase of the control; # indicates $P<0.001$ compared with the G2/M phase of the control. (B) The cells were harvested and fixed, and PH3 was assessed by immunocytochemistry. Morphological changes were observed under fluorescence microscopy (magnification ×200). Total cell population was visualized by DAPI staining. The percentage of pH3-positive cells is shown on the bottom line. (C) The cells were harvested and subjected to Western blot analysis. The relative protein amounts were quantified and normalized to the corresponding β-actin protein amount. The quantitative data are shown under each protein respectively.

Cytotoxicity. The cytotoxicity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (Hsu et al., 2005, 2007). Attached cells (NCI-H460, HCT116, Hep 3B, and Detroit 551) were pre-seeded into 96-well plates to allow for cell attachment, then HKL-1 was added to the plates for another 48 h. Distinct to attached cells, suspension cells (HL-60 and

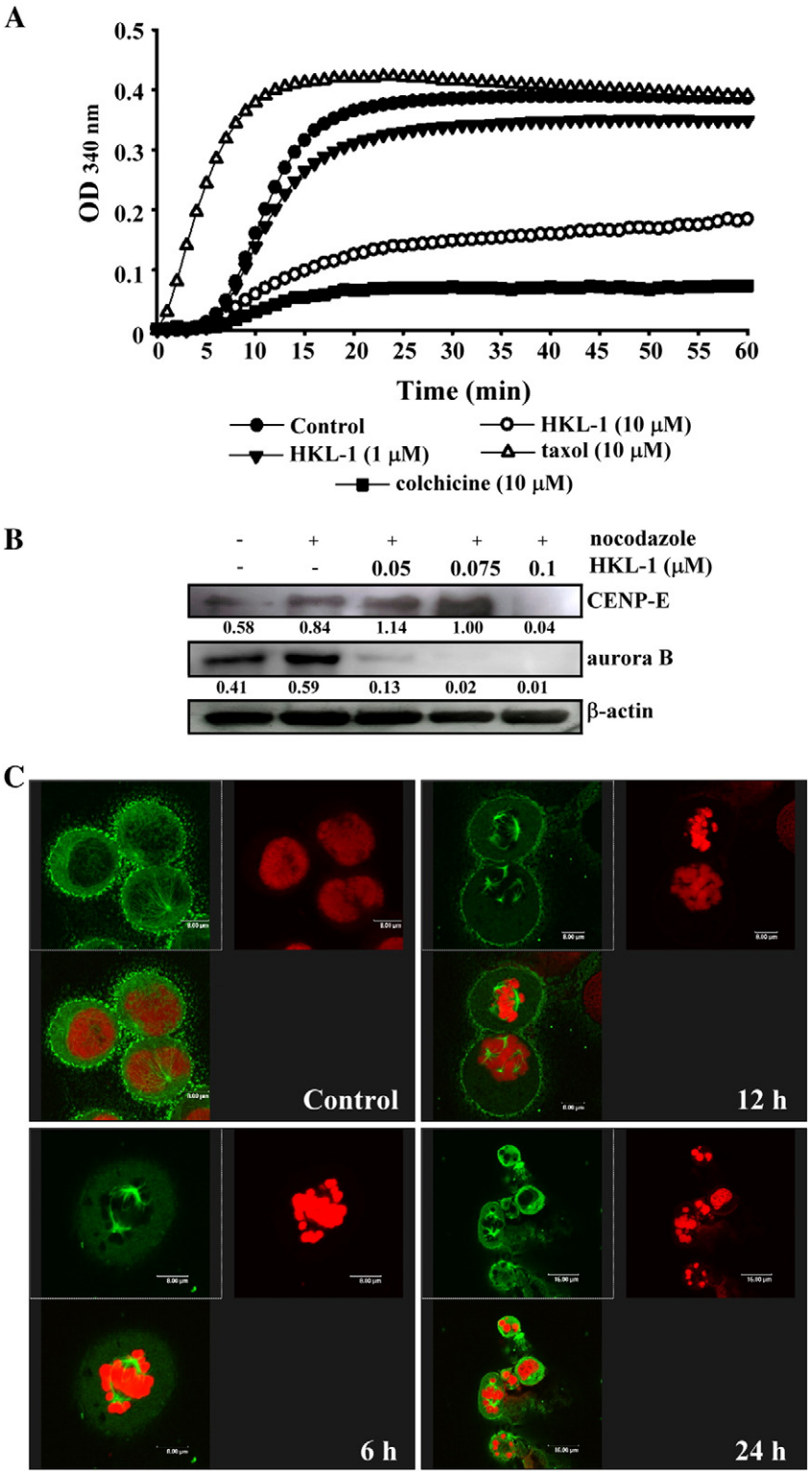

Fig. 3. HKL-1 suppressed cellular tubulin polymerization, disrupted mitotic spindle formation and affected the mitotic kinesin protein expression levels. (A) Purified tubulins were used to assay for microtubule formation *in vitro* in the presence of either taxol (10 μM), colchicine (10 μM), or HKL-1 (1, 10 μM). A shift of the curve above that of the control indicates an increase in polymerized microtubules. A shift of the curve below that of the control shows a decrease in the rate of polymerization. (B) HL-60 cells were treated with 0.1 μM HKL-1 for the indicated time periods, and then the cells were harvested, fixed and double-stained with anti-α-tubulin-FITC and PI. Morphological changes were observed under confocal fluorescence microscopy. (C) HL-60 cells were pre-treated with 1 nM nocodazole for 24 h, then various concentrations of HKL-1 were added for another 24 h. The cells were then harvested and subjected to Western blot analysis. The relative protein amounts were quantified and normalized to the corresponding β-actin protein amount. The quantitative data are shown under each protein respectively.

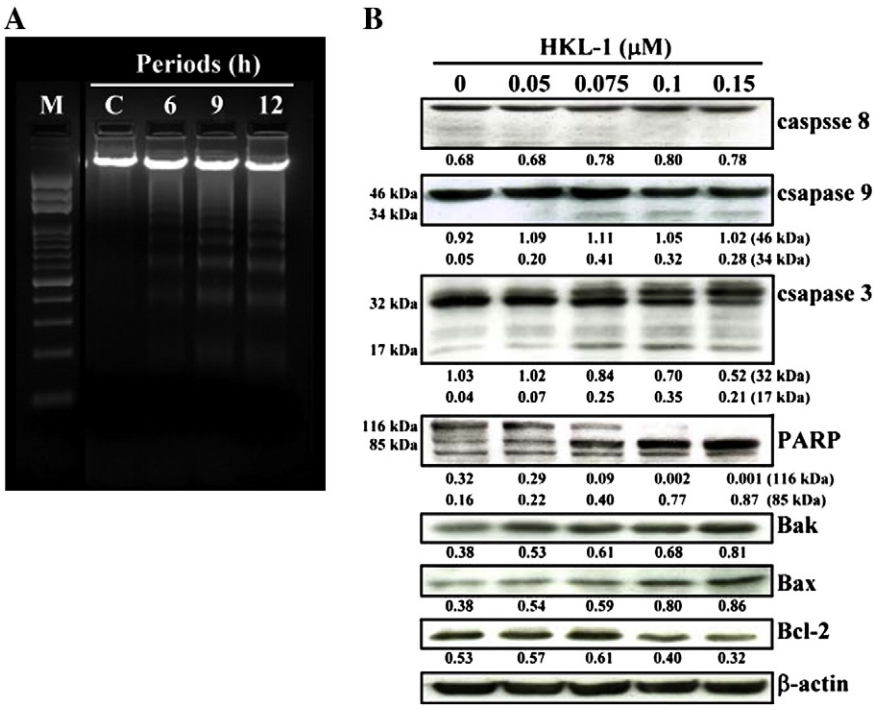

Fig. 4. HKL-1 induced apoptosis in HL-60 cells. (A) DNA fragmentation analysis. After treatment with 0.1 μM HKL-1 for the indicated time periods, DNA was isolated and separated on a 1.5% agarose gel. M: marker. (B) HL-60 cells were treated with various concentrations of HKL-1 for 24 h. The cells were then harvested and subjected to Western blot analysis. The relative protein amounts were quantified and normalized to the corresponding β-actin protein amount. The quantitative data are shown under each protein respectively.

PBMCs) were directly treated with HKL-1 for 48 h. Then the cytotoxicity was measured by scanning with an ELISA microplate reader (Bio-Rad Model 450) with a 570 nm filter, and the IC_{50} values of HKL-1 were calculated. IC_{50} value was defined as the drug concentration that inhibits 50% cell growth compared with controls and calculated by SigmaPlot 10.0 software.

Trypan blue exclusion experiment. Permeability assays involve staining damaged cells with a dye (i.e. trypan blue) and counting viable cells that exclude the dye. Thus the trypan blue exclusion experiment was performed to investigate whether HKL-1 induced cell death. Briefly, HL-60 cells were treated with HKL-1 (0.025–0.15 μM) for 24 and 48 h. At the end of treated period, 20 μl of cell suspension was gently mixed with 20 μl of trypan blue solution. Further the cell numbers were counted using a hemocytometer. The percentage of death cells means the numbers of trypan blue-stained cells over the numbers of all counting cells (including death and viable cells).

Cell cycle distribution analysis. Cell cycle analysis was performed by FACS® as described in a previous paper (Chen et al., 2007). HL-60 cells were treated with HKL-1 for the indicated time periods. After treatment, cells were washed once with PBS and then fixed overnight with 70% ice-cold ethanol at −20 °C. Thereafter, cells were stained with a solution containing 1% Triton-X 100, 0.1 mg/ml RNase, and 4 μg/ml PI, and were kept at room temperature in the dark for 30 min. Finally, cell cycle distributions were measured by FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and all histograms were analyzed using ModFit software.

Immunofluorescence microscopic examination. Immunofluorescence microscopy observations were performed as described in a previous paper (Hsu et al., 2007). Cells were treated with HKL-1 for the indicated time periods. After treatment, cells were fixed in methanol for 10 min, permeabilized with 0.3% triton X-100 for 10 min, and then blocked for non-specific binding sites with 2% BSA for 30 min. After blocking, fixed cells were incubated overnight with phosphohistone H3 (PH3, 1: 100 dilutions; Cell Signaling Technology) or anti-human α-tubulin antibody (1:100 dilutions; Santa Cruz). Thereafter, the cells were exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilutions) at room temperature for 60 min. This was followed by DNA staining with PI (20 μg/ml) immediately before the cells were photographed using a Leica TCS SP2 Confocal Spectral Microscope. The population of PH3-positive cells was determined by counting a total of at least 100 cells.

In vitro tubulin turbidity assay. The tubulin polymerization was detected using CytoDYNAMIX Screen 03 kit (Cytoskeleton Inc., Denver, CO) as described in a previous paper (Hsu et al., 2007). Tubulin proteins (of purity more than 99%) were suspended in G-PEM buffer containing 80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM GTP (pH 6.9), and 5% glycerol, either with or without test compounds. Then the mixture was transferred to a 96-well plate and the absorbance was measured at 340 nm at room temperature for a total of 60 min.

Fluorescence apoptotic morphological examination. Cellular apoptotic morphological observations were performed as described in a previous paper (Huang et al., 2011). HL-60 cells were treated with HKL-1 for the indicated time periods. After treatment, cells were pre-fixed in 3.7% paraformaldehyde at room temperature for 15 min, then fixed again in cold-ice methanol for 5 min. After being washed, cells were stained with PI (20 μg/ml) at room temperature for 5 min, and morphological changes observed under fluorescence microscopy.

DNA fragmentation electrophoresis analysis. Observations of DNA fragmentation were performed as described in a previous paper (Hsu et al., 2005). HL-60 cells were treated with HKL-1 for the indicated time periods. The DNA was prepared using the GNOME® DNA Isolation Kit (BIO 101, La Jolla, CA, USA). DNA electrophoresis was

performed in 1.5% agarose gels containing 0.5 μg/ml of EtBr at 70 V for 70 min, and DNA fragments were visualized by exposing the gel to UV light and photographing them.

Western blot analysis. HL-60 cells (2×10^5/ml) were treated with HKL-1 for the indicated time periods. After treatment, cells were washed once with PBS, then M-PER® Mammalian Protein Extraction Reagent (PIERCE) was added to prepare total cell lysates. Western blotting was conducted according to a previous paper (Hsu et al., 2007). Approximately 15–40 μg of total cellular proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were probed with target primary antibodies overnight, followed by probing with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. After being washed, the membranes were incubated in ECL Plus reagent before exposure to X-ray film. To quantify the relative protein amounts, the membranes were scanned and analyzed by Multi Gauge V3.0 software.

Statistical analysis. Values are presented as the means ± SD from four independent experiments. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Turkey's test. $P<0.05$ is considered statistically significant.

Results

HKL-1 non-selectively inhibited proliferation of cancer cells

The differential activity patterns for HKL-1 against a variety of human cancer cell lines are shown in Table 1. These results revealed that HKL-1 demonstrated non-selective *in vitro* anti-proliferative activity against all cancer cell lines. Among these cell lines, HL-60 cells exhibited relatively higher sensitivity to HKL-1, with an IC_{50} value of approximately 0.04 μM. Nevertheless, HKL-1 did not markedly affect the growth of human normal cells (PMBCs and Detroit 551) with IC_{50} values more than 5.0 μM. Thus we selected the leukemia HL-60 cell line for subsequent research into the mechanisms of HKL-1. First, we counted the cell numbers, using a trypan blue exclusion experiment, to examine whether HKL-1 causes cell death. As shown in Fig. 1B, HKL-1 induced cell death in a concentration- and a time-dependent manner, with the results being statistically significant. In addition, we found that HL-60 cells treated with HKL-1 displayed typical morphological features of apoptotic cells, with cell shrinkage and formation of apoptotic bodies (Fig. 1C). These results suggested that HKL-1 induced apoptosis in HL-60 cells.

HKL-1 inhibited cell growth via arresting cell cycle at mitotic phase

During the initial cytotoxicity test, we realized that treatment with HKL-1 evidently always caused the adhered cancer cells (NCI-H460, HCT116, and Hep 3B) to become detached and round as is frequently observed during the mitotic process (Boucrot and Kirchhausen, 2007). In particular, when HCT116 and Hep 3B cells were exposed to HKL-1, the cells became significantly enlarged (data not shown). To test the possibility that HKL-1 might affect cellular mitosis, we evaluated the effect of HKL-1 on the cell cycle distribution. As shown in Fig. 2A, for HL-60 cells exposed to HKL-1, the percentage of cells in the G1 phase was substantially lowered compared with the control cells. The decrease in the G1 phase was reflected in an apparently increased G2/M phase population, with approximately 72.1% of cells being in G2/M phase at 12 h, compared with 11.8% of the control cells. However, DNA cell cycle analysis cannot distinguish between cells in G2 or M phase, thus we used PH3 antibodies to examine whether HKL-1 arrests the cell cycle in the G2 or M phase. As shown in Fig. 2B, HKL-1-treated cells responded positively to PH3, which demonstrated that HKL-1 induced the HL-60 cells to enter the mitotic phase. To better understand the mechanisms of HKL-1-elicited mitotic arrest, we analyzed the effect of HKL-1 on key molecular events in the cell cycle. Cyclin B1 and CDK1 are intricately involved in the cell cycle progression through the G2/M phase transition (Stewart et al., 2003). As shown in Fig. 2C, a marked decrease in the expressions of cyclin B1 and CDK1 were detected within higher concentrations of HKL-1-treated cells (concentrations of 0.1 and 0.15 μM, respectively). In contrast, a significant increase in p21 was observed within the same concentrations of HKL-1-treated cells.

HKL-1 inhibits the polymerization of cellular microtubules

Microtubules are intracellular organelles formed from the protein tubulin. Tubulin inhibitors can change their dynamics by either promoting (i.e. taxol) or inhibiting (i.e. colchicine) polymerization (Hadfield et al., 2003). To investigate whether HKL-1 affected microtubule polymerization, we used an *in vitro* microtubule polymerization assay. As shown in Fig. 3A, an increase in the absorbance at 340 nm indicated an increase in tubulin polymerization. As expected, taxol increased the absorbance compared with that of the control tubulin, whereas colchicine strongly reduced it. This decreased absorbance due to HKL-1 indicated that HKL-1 caused microtubule depolymerization. Consistently, the immunofluorescence staining experiment showed that the microtubule networks of HL-60 cells were dramatically changed after treatment with HKL-1. As shown in Fig. 3B, the chromosomes did not properly align at the equator, although a bipolar spindle formed within 6 h after treatment. Some clusters of decondensed chromosomes were distributed randomly, and the bipolar spindle broke to form a multipolar spindle at 12 h after treatment. Finally, at 24 h after treatment the spindle formation had almost disappeared, and enlarged cells with multiple nuclei and micronuclei had appeared.

Besides tubulin, many non-structural protein factors have also been reported to regulate mitosis progression. Among them, CENP-E and aurora kinase B have been intensively investigated for the possibility that their inhibitors may be used as new anticancer agents (Abrieu et al., 2000; Fu et al., 2007). Because the protein expressions of CENP-E and aurora B were almost not detectable when HL-60 cells were treated with HKL-1 alone (data not shown), the cells were pretreated with 1 nM nocodazole for 24 h to amplify protein expressions. Thereafter, nocodazole-treated cells were then treated with various concentrations of HKL-1 for another 24 h. The protein expression of CENP-E and aurora B elicited by nocodazole was significantly suppressed by HKL-1 (Fig. 3C).

HKL-1 induces mitotic catastrophe

As illustrated in Figs. 1B, C, and 2A, the results of the experiment showed that HKL-1 induced apoptosis; this finding was confirmed using DNA electrophoretic analysis technique. As shown in Fig. 4A, we found that HKL-1 causes classic internucleosomal DNA fragmentation, and the intensity of the DNA ladder progressively increased in a time-dependent manner. Prior research had described one type of cell death (mitotic catastrophe) that consistently occurs from abnormal mitosis, which usually ends in the formation of large cells with multiple micronuclei and decondensed chromatin (Castedo et al., 2004). The results of our immunofluorescence staining test (Fig. 3B) confirmed that HKL-1 induced an arrest at metaphase in the HL-60 cells, followed by enlarged cells with multiple nuclei and micronuclei appearance. Thus we suggest that HKL-1 induces mitotic catastrophe in HL-60 cells.

Because mitotic catastrophe frequently shows cells with some phenotypic characteristic of apoptosis, we analyzed the effect of HKL-1 on key molecular events in apoptosis. As shown in Fig. 4B, caspase 9 and

caspase 3 were proteolytically processed into small active fragments, but the expression level of caspase 8 was not affected. PARP (caspase 3 substrate) was cleaved from 116 kDa to its characteristic 85 kDa fragment after HKL-1 treatment. We also examined the protein expression levels of Bcl-2 family proteins, including Bak, Bax, and Bcl-2. HKL-1 caused significant enhancement in the expressions of Bak and Bax, whereas Bcl-2 expression was markedly suppressed. These data suggests that regulation of Bcl-2 family proteins to induce caspase cascade activation is the plausible signal transduction pathway by which HKL-1 induced mitotic catastrophe in HL-60 cells.

Discussion

Microtubules are essential components of the cytoskeleton and play a critical role in many cellular processes, including cell division, cell motility, intracellular trafficking, and cell shape maintenance. Suppression of microtubule dynamics by microtubule-targeting drugs, such as the *vinca* alkaloids and taxol, can engage the mitotic spindle checkpoint, arresting the cell cycle progression at mitosis and eventually leading to apoptosis (Jordan and Wilson, 2004). However, direct inhibition of microtubule dynamics may disrupt a number of cellular processes including the transport of intracellular cargo or organelles across long distances (Gerdes and Katsanis, 2005b). Concerted efforts are ongoing to identify, design, and develop antimitotic agents that bind indirectly to tubulin and alter microtubule dynamics, but with minimal toxicity to normal tissues.

HKL-1, a 2-PN derivative, has been identified as an antimitotic agent that exerts a broad spectrum of activity against human leukemia, colon, CNS, prostate, and breast cancer cells *in vitro* (Chen et al., 1997a, 1997b). The current study showed that HKL-1 induced mitotic phase arrest followed by mitotic catastrophe in HL-60 cells, but without toxicity to normal cells. These findings suggest that HKL-1 may be viable as a novel antimitotic agent due to its ability to bind indirectly to the tubulin, and could avoid the neurotoxicity of traditional antimitotic agents.

Mitosis begins and ends with the activity of CDK1 and its binding partner cyclin B1. Proper control of CDK1/cyclin B1 activity is essential for appropriate cell cycle progression and exit, and in eukaryotic cells this complex activity is strictly regulated by p21 (a CDK inhibitor) in G2/M transition (Stewart et al., 2003). We found that HKL-1 induced p21 activity to inhibit CDK1 and cyclin B1, and then arrested HL-60 cells in the G2/M phase. In addition to analyzing cell cycle distribution, we used immunochemistry staining of PH3, which showed a positive response to HKL-1 treatment. These two findings confirmed that HKL-1 had induced the HL-60 cells to enter the mitotic phase. In addition, the PH3-staining data exposited another important signal, namely that HKL-1 decreased the percentage of PH3-positive cells in a time-dependent manner (Fig. 2B). This finding indicates that HKL-1 may be an aurora B kinase inhibitor, because aurora B kinase activity correlates closely with phosphorylation of histone H3 on ser-10; aurora B inhibitor treatment always induces a dramatic decrease in phosphorylation level of histone H3 on ser-10 (Pascreau et al., 2003).

Most antimitotic agents interact with microtubules to inhibit cell proliferation, while taxanes stabilize tubulin polymers, and *vinca* alkaloids and colchicines destabilize the polymerization (Jordan and Wilson, 2004). Our findings confirmed that HKL-1 inhibited tubulin polymerization and slowed mitosis at the metaphase/anaphase transition, inducing accumulation in a metaphase-like state. Prolonged exposure to HKL-1 caused more abnormal spindle organization in HL-60 cells, accompanied by the appearance of multiple nuclei and micronuclei. Mitotic kinesins have been found to regulate cell division by promoting the proper attachment of chromosomes to spindle microtubules. CENP-E is localized to kinetochores and is responsible for the proper alignment of chromosomes; it plays an essential role in capturing and positioning chromosomes to the mitotic spindle during metaphase (Abrieu et al., 2000; Yao et al., 2000). The depletion of the CENP-E motor leads to a failure of checkpoint activation and results in the appearance of some unaligned chromosomes (Tanudji et al., 2004; Yao et al., 2000). In HL-60 cells, HKL-1 inhibited CENP-E protein expression and led to a failure of chromosomal alignment, which indicates that HKL-1 may perhaps be a CENP-E inhibitor.

Aurora kinases (aurora A, B, and C) are only expressed in actively dividing cells, and also play important roles in spindle formation and the attachment of chromosomes to the spindle. Inhibition of aurora B induces premature mitotic arrest in cells, with the cells having failed in cytokinesis during a prior mitosis. The cells should contain diploid or tetraploid number of chromosomes (Ditchfield et al., 2003). The current study found that in HL-60 cells, HKL-1 inhibited aurora B protein expression, which caused chromosomes to decondense and resulted in the appearance of multipolar spindles, multiple nuclei, and micronuclei. This phenotype of enlarged cells with multiple nuclei was also observed

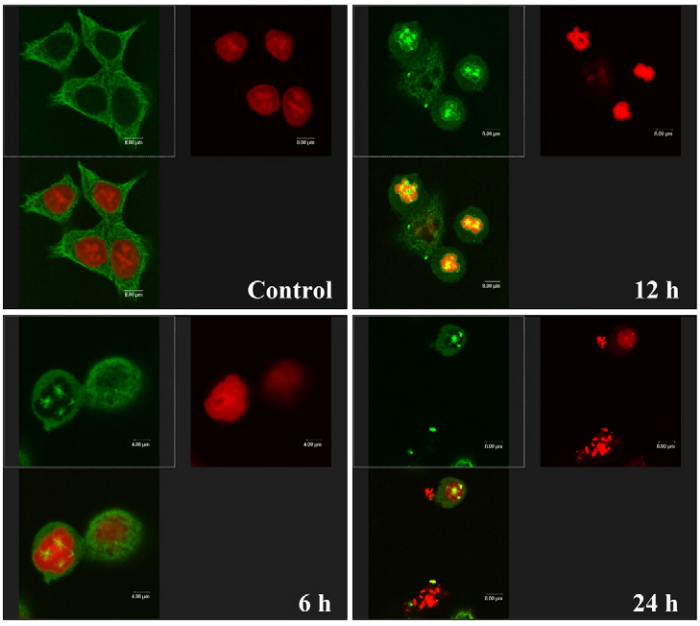

Fig. 5. HKL-1 disrupted mitotic spindle formation in HCT116 colon cancer cells. HCT116 colon cancer cells were treated with 0.5 μM HKL-1 for the indicated periods. Then the cells were then fixed and double-stained with anti-α-tubulin-FITC and PI, and morphological changes were observed under confocal fluorescence microscopy.

in HCT116 colon cancer cells that had been treated with HKL-1 (Fig. 5). Thus we suggest that in addition to being a CENP-E inhibitor, HKL-1 may perhaps also be an aurora B inhibitor.

The connection between mitosis arrest and apoptosis has not been clearly defined. However, a recent report described an alternative mechanism of cell death termed "mitotic catastrophe" (Castedo and Kroemer, 2004). Mitotic catastrophe has been described as cell death that occurs from abnormal mitosis, in response to agents that cause DNA or mitotic-spindle damage. Numerous molecular players controlled mitotic catastrophe, in particular cell-cycle-specific kinases, cell-cycle checkpoint proteins, caspases, and the Bcl-2 family members (Castedo and Kroemer, 2004; Castedo et al., 2004). HKL-1 induces mitotic catastrophe in HL-60 cells through regulating mitotic phase-specific kinases (down-regulating CDK1, cyclin B1, CENP-E, and aurora B) and the expression of the Bcl-2 protein family (down-regulating Bcl-2 and up-regulating Bax and Bak). This is followed by caspase-9/-3 cleavage, which causes the formation of distinct internucleosomal DNA fragments.

We here reported that HKL-1 may perhaps be a novel antimitotic agent that targets mitotic kinesins (i.e. CENP-E and aurora B). By overriding the spindle checkpoint, HKL-1 caused HL-60 cells to exit mitosis and rapidly become polyploidy, which induced mitotic catastrophe. Therefore, HKL-1 appears to be an attractive candidate for possible use as a novel antimitotic agent in the future.

Conflict of interest statement

No potential conflict of interests was disclosed.

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