A novel class of tubulin inhibitors with promising anticancer activities

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Abstract

We have developed a novel class (2-amino-4-phenyl-4H-chromene-3-carboxylate) of inhibitors of tubulin assembly by modifying HA14-1, which is a Bcl-2 inhibitor discovered by our group. Three of these compounds, mHA1, mHA6, and mHA11, showed \textit{in vitro} cytotoxicities against tumor cells that were more potent and more stable than authentic HA14-1, with IC\textsubscript{50} values in the nM range. In contrast, cytotoxic effects of these mHAs on normal cells were slight. Computational docking, colchicine-tubulin competitive binding, and tubulin polymerization studies demonstrated that these compounds bind at the colchicine binding site on tubulin and inhibit the formation of microtubules. Treatment of HL-60/Bcl-2 leukemia and CRL5908 lung cancer cells with these mHAs led to pronounced microtubule density decreases, G2/M cell cycle arrest, and apoptosis, as determined by immunofluorescence microscopy, flow cytometry, and DNA fragmentation analysis. These results support the continued development of these compounds as potential anticancer agents.
Introduction

Microtubules are components of cytoskeleton and are composed of α- and β-tubulin that form heterodimers (1). Microtubules are involved in many cellular processes, including intracellular transport, maintenance of cell shape, polarity, cell signaling, mitosis, and cytokinesis (2). Their role in mitosis makes them important cellular targets for anticancer drug developments. In the eukaryotic cell cycle, tubulin is polymerized into microtubules, which form the mitotic spindle. The spindle then moves the chromosomes to the opposite sides of the cell, in preparation for cell division into two daughter cells. Because of this important role in cell proliferation, microtubules have been recognized as one of the successful and efficacious drug targets for the development of novel anti-cancer chemotherapeutics (3-7).

Microtubule-inhibiting agents (MIAs) currently used in clinic therapies work through the suppression of the microtubule dynamics by misdirecting the formation of a functional mitotic spindle in fast dividing tumor cells. This arrests the cells in G2/M phase, thereby leading to apoptosis of the tumor cells. Based on their mechanism of action, MIAs are classified into two broad categories: microtubule stabilizing agents and destabilizing agents. Most MIAs bind to one of three sites on tubulin, the colchicine site, the vinblastine site, or the paclitaxel site (8). Due to the potent anti-cancer activity, these apoptotic therapeutic agents that target microtubules are among the most commonly prescribed antitumor agents. However, as with other anticancer drugs, intolerable toxicities and the emergence of drug resistance have limited the clinical use of the drugs targeting microtubules (9-11). Therefore,
a need still exists for discovery and development of novel chemotherapeutic agents that target microtubules, but that show lower drug resistance and fewer toxic side effects.

We have been focusing our efforts for many years on the research and development of novel tumor therapeutic agents that target different factors involved in apoptotic pathways. The small compound dubbed HA14-1, identified by our group, was the first reported Bcl-2 inhibitor (12, 13). In subsequent studies, we found that it can also bind tubulin in a manner that is competitive with colchicine, which binds at the interface within α/β-tubulin heterodimers. Colchicine is now a branded drug for treating several clinical diseases, including gout and Familial Mediterranean fever. Although the effectiveness and side effects of colchicine in cancer treatment need to be further characterized, the colchicine-binding site is a potential drug target that has attracted much attention for development of new anticancer agents (14); several such compounds are undergoing clinical trials (8, 15). In the present study, we used HA14-1 as the initial template compound and developed a new class (2-amino-4-phenyl-4H-chromene-3-carboxylate) of novel microtubule-targeting agents that show promising antitumor activities.

Materials and Methods

Chemical synthesis

A general synthetic procedure of mHA1, mHA6, and mHA11 is illustrated in Figure 1. These compounds were synthesized using a one-pot three-component reaction of substituted benzaldehyde, phenol analogs and ethyl cyanoacetate in the presence of piperidine. All of the
new compounds described were characterized by $^1$H NMR and mass spectrometry (MS) spectra.

**Preparation of mHA1**

A mixture of 3-Bromo-4,5-dimethoxybenzaldehyde (0.49 g, 0.002 mol), 3-(dimethylamino) phenol (0.27 g, 0.002 mol), ethyl cyanoacetate (0.21 mL, 0.002 mol) and piperidine (0.4 mL, 0.004 mol) was suspended in 15 mL anhydrous ethanol and stirred at room temperature for 4 h. After diluting with 80 mL CH$_2$Cl$_2$ and washing with water, the organic layer was dried over Na$_2$SO$_4$. The Na$_2$SO$_4$ was then removed by filtration and the solvent was evaporated. The crude product was purified by column chromatography (hexane/CH$_2$Cl$_2$) to give 0.6 g mHA1 at 63% yield.

**Preparation of mHA6**

Starting from 3-bromo-4,5-dimethoxybenzaldehyde, naphthalen-1-ol, and ethyl cyanoacetate, we then followed the procedure for the synthesis of mHA1, to give 0.45 g (46% yield) of mHA6.

**Preparation of mHA11**

Starting from 3-chlorobenzaldehyde, 3-(dimethylamino) phenol, and ethyl cyanoacetate, we then followed the procedure for the synthesis of mHA1, to give 0.32 g (43% yield) of mHA11.
Cell culture

The human leukemic HL-60/Bcl-2 cell line was obtained from Dr. Kapil N. Bhalla (University of Miami School of Medicine, Miami, FL) (13), which has been stably transfected with pZip-Bcl-2 plasmid. CRL5908 lung cancer cell line was kindly provided by Dr. Sandra G. Hudson. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin and 800 μg/mL genticin (G418, Invitrogen, San Diego, CA). Cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C.

Human bone marrow specimen collection

Human bone marrow specimens were obtained from three healthy adult donors. The patients were placed in the lateral decubitus position, with the top leg flexed and the lower leg straight. After sterilizing the chosen area of the posterior iliac crest skin, 10 mL of one percent lidocaine was infiltrated into the skin and subcutaneous tissue to anesthetize an area approximately 2-3 cm in diameter. An incision was made in the skin with a small surgical blade, through which the bone marrow aspiration needle, with a stylet locked in place, was inserted. Once the needle contacted the bone, it was advanced by slowly rotating clockwise and counterclockwise until the cortical bone was penetrated and the marrow cavity was entered. Once within the marrow cavity, the stylet was removed. Approximately one ml of bone marrow was aspirated using a 10 mL syringe. The sample was then transferred into an anticoagulant-containing (heparin) tube. The marrow needle was removed, and pressure was applied to the aspiration site with gauze until any bleeding had stopped. The bone marrow
specimen was then carefully diluted with 4 ml medium and layered over 5 mL Ficoll-Paque™ PLUS (GE Healthcare) in a tube, without disturbing the interface, and centrifuged at 3000 rpm for 30 min. The interface layer was aspirated to another tube, washed with medium and incubated with mHA1, mHA6, or mHA11 at different concentrations (as described below) for 72 h.

Measurement of cell viability

The cytotoxic effects of the HA14-1 analogs were measured by plating $3 \times 10^4$ cells/ per well in 96-well plates and determining cell survival. Cells were treated with various concentrations of these compounds (0.1 to 3 μM). The samples were then incubated at 37 °C for 72 h in a 5% CO₂ and 95% humidity incubator. After incubation, cell viability was measured using a CellTiter-Blue assay kit (Promega) according to the manufacturer’s instructions. Briefly, 20 μl of CellTiter-Blue reagent was added to 100 μl of culture media and cells were incubated for 2-4 h at 37 °C. Afterward, fluorescence at 540Ex/600Em was measured using a fluorescence plate reader (BioTek, Synergy 2). Each experimental data point was generated from at least three independent experiments.

Cell Morphological change

Cells were treated with mHAs at 1 μM of mHAs for 24 h and then examined for morphological changes by inverted fluorescence microscopy and photography.

Cell cycle analysis
HL-60/Bcl-2 cells (1x10^6) were treated with 1, 3, and 5 μM of mHA1, mHA6, mHA11, respectively, for 24 h at 37 °C. Cells were harvested at 1100 rpm and washed twice with PBS, then resuspended in 100 μl of PBS and 1ml of 75% cold ethanol and stored at -20 °C overnight. After centrifugation at 1100 rpm for 5 min, the supernatant was removed. A 500 μl PI staining buffer containing 80 μg/mL of propidium iodide, 100 μg/mL of RNase A, and 1% Triton was added to the samples. The cells were incubated for at least half an hour and then analyzed by flow cytometry with a FACScalibur system (LSRII, Becton Dickinson) using FlowJo7.5 analysis software.

**DNA fragmentation analysis**

HL-60/Bcl-2 cells were resuspended in fresh medium and added to 24-well plate wells at a density of 1x10^6 cells/well, in a final volume of 1 mL. Vehicle control (DMSO), positive control (colchicine at 0.1 and 1 μM), and mHA1, mHA6, or mHA11 (at 1 and 5 μM) compounds were then added to the appropriate wells. The plate was incubated for 24 h, and total DNA was extracted from the cells in each well using an Apoptotic DNA-ladder kit following the manufacturer’s instructions (Qiagen). The DNA samples were subjected to 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

**Tubulin polymerization assay**

Tubulin polymerization assays were conducted using the polymerization assay kit following the manufacturer's instructions (Cytoskeleton, Inc.). Briefly, 50 μl of 3 mg/ml tubulin (>99% pure) proteins in G-PEM buffer (80 mM PIPES, pH 6.9, 2 mM MgCl₂, 0.5
mM EGTA, 1 mM GTP, and 15% glycerol) was placed in 96-well microtiter plates in the presence of test agents. Polymerization was measured at every 60 sec intervals for 1 h using a Synergy 2 microplate reader (BioTek) (excitation at 360 nm and emission at 420 nm) at 37 °C. Tubulin levels were normalized to a value of 100 for the vehicle control.

[^]Colchicine-tubulin binding assay

One micromolar radiolabeled colchicine [ring C, Methoxy-[^3]H] (PerkinElmer, 1mCi/mL), 1% DMSO and various concentrations of test compounds in 50 μl G-PEM buffer containing 80 mM PIPES (pH 6.8), 1 mM EGTA, 1 mM MgCl₂ and 1 mM GTP, and 5% glycerol were incubated with 1 μM tubulin (>99% pure; Cytoskeleton, Inc.; 0.2 mg/mL) at 37 °C for 60 min. The binding solutions were filtered through a stack of two DEAE-cellulose filters and washed twice. The radioactivity in the filtrates was determined by liquid scintillation spectrometry (Perkin-Elmer Wallac). Nonlinear regression was used to analyze the data using GraphPad Prism.

Immunofluorescence staining

After treatment with 1 μM of mHA1, mHA6, and mHA11 for 24 h, the densities of microtubule and actin in adherent CRL5908 lung cancer cells were evaluated using immunobiochemistry. CRL 5908 cells at 5x10⁴ per well were cultured as described previously. One day after seeding, 1 μM mHA1, mHA6, or mHA11 were added to the cells and cultured at 37 °C for 24 h. Thereafter, cells were fixed for 30 min at 4 °C in 4% paraformaldehyde and incubated with 0.1% Triton X-100 permeabilizing buffer for 15 min at
room temperature (RT). After washing with PBS and blocking with 2% BSA in PBS for 30 min at RT, cells were incubated for 1 h protected from light at RT in 1:2000 anti-α-tubulin monoclonal antibody (Sigma) and 7:1000 Rhodamine-Phalloidin-labeled anti-actin antibody (Cytoskeleton) in PBS. Cells were then washed with PBS and incubated for 30 min protected from light at RT with 1:200 fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (Sigma) in PBS. Subsequently, all cells were stained with 1 μg/ml 4′,6-diamidino-2-phenyl-indole (Sigma). Samples were examined under a fluorescence microscope (Eclipse TE2000-U, Nikon, Japan) and photographed using a digital camera equipped with a fluorescein figure analysis system (NIS-Elements F3.0; Nikon, Tokyo, Japan).

**Molecular modeling**

The crystal structure of alpha-beta-tubulin heterodimers in a complex with DAMA-colchicine [PDB reference: 1SA0 (16)] was used to predict the binding models of tubulin bind with designed compounds. The binding modes of designed ligands 1, 6, 11 with tubulin were predicted by using the Autodock4 program. In preparation of receptor grid files, a 60x55x60 grid with a grid spacing of 0.375 Å was defined to cover the interface of αβ-tubulin heterodimer. Grid maps were then generated by Autogrid4 for docking simulations. Lamarkian genetic algorithm (LGA) was adopted in Autodock4 calculation, and 100 runs were performed for each ligand. The whole receptor structure was set to be rigid during the simulations. All other parameters for Autogrid and Autodock calculations were set to default values in AutoDockTools-1.5.2. For each ligand, the 100 conformations resulted from Autodock simulations were clustered and the representative binding mode to tubulin with
lowest predicted binding free energy.

**Statistical analysis**

Average values were expressed as mean ± SD. *P* values were calculated by one-way ANOVA (PSIPLLOT).

**Results**

**Cytotoxicity of new mHA agents toward leukemic HL-60/Bcl-2 cells and normal human bone marrow cells**

Of the series of HA14-1 analogs synthesized, mHA1, mHA6, and mHA11, showed the best anti-tumor activity. Human leukemia HL-60/Bcl-2 cells were treated with different concentrations of mHA1, mHA6, and mHA11 (0.1 to 3 µM) for 72 h. Cell viability, assessed by the CellTiter-Blue Cell Viability Assay, showed that the effects of mHA1, mHA6, and mHA11 were dose-dependent, with IC$_{50}$ values of 0.932±0.128, 0.958±0.045, and 0.648±0.049 µM, respectively (Figure 2. A-C), while IC$_{50}$ of the parent compound, HA14-1, was 9.394±0.18 µM (data not shown). Overall, these HA14-1 analogs were much more potent than the parent HA14-1 compound.

The usefulness of a potential anticancer compound depends not only on its cytotoxicity in malignant cells, but also on its relative lack of toxicity towards normal tissues. We therefore evaluated the effects of the HA14-1 analogs on normal human bone marrow cells (HBMCs). These cells were obtained from 3 normal adult donors, as previously described. After exposure to mHA1, mHA6, or mHA11 at different concentrations (0.1 - 3 µM) for 72 h, cell
viability was assessed by the CellTiter-Blue Cell Viability Assay. As shown in Figure 2 A-C, these compounds had almost no effects on normal HBMCs below 3 μM concentration, while 3 μM mHAs were able to kill almost all of the malignant HL-60/Bcl-2 cells \((P< 0.01)\). In contrast, treatment with colchicine, used here as the control colchicine site drug, resulted in the death of 30% of normal HBMCs at 25 nM (data not shown), even though its IC50 was only 33.5±3.5 nM on HL-60/Bcl-2 cells. The high toxicity of colchicine against normal cells limited its therapeutic value on cancer.

**Cell morphology changes in response to mHAs**

HL-60/Bcl-2 cells are suspension cells and typically have a spherical shape. After treatment with 1 μM of mHAs for 24 h, morphological changes including cell elongation, asymmetry, and formation of long pseudopodia were observed by fluorescence microscope (Figure 3A).

**DNA fragmentation analysis**

To confirm that these compounds could induce apoptosis in HL-60/Bcl-2 cells, we performed DNA fragmentation analysis which is a key feature of apoptosis (17). A clear DNA ladder was observed after 24 h treatment with 1 or 5 μM mHA1, mHA6, or mHA11 and 0.1 or 1 μM of colchicine, indicating that colchicine, and mHA1, mHA6, and mHA11 could induce apoptotic cell death in HL-60/Bcl-2 cells (Figure 3B).

**G2/M cell cycle arrest caused by mHAs**
To determine whether the inhibition of growth due to HA14-1 analogs is associated with cell cycle perturbation, the distribution of cells in different phases of the cell cycle was determined by flow cytometry, and analyzed using FlowJo7.5 analysis software. The HL-60/Bcl-2 control cell population profile is shown in Table 1 and indicates that most of the cells were in the G1 and S phase. The HA14-1 analogs all caused a statistically significant increase in the G2-M cell population. For example, increased from 14.5% to 70.9%, 55.8%, and 54.7% were observed for mHA1, mHA6, and mHA11 treatments, respectively, when administered at 1 μM for 24 h. A concomitant decrease in G1 and S cell population was observed. The distribution of cells in different phases of the cell cycle is summarized in Table 1. After exposure HL-60/Bcl-2 leukemia cells to 1, 3, and 5 μM of mHA1, mHA6, and mHA11, a sub-diploid peak was observed (Table 1). An increase in the sub-G0/G1 cell population from 5% to 13.3%, 13.9% to 20.3%, and 16.7% to 28.3% was occurred when cells were treated with 1 to 5 μM mHA1, mHA6, and mHA11, respectively, for 24 h.

All mHAs bind to colchicine-binding site and inhibit tubulin polymerization

In the crystal structure of of alpha-beta-tubulin heterodimers complex with DAMA-colchicine, colchicine binds at the interface of α/β-tubulin heterodimer and inhibits the assembly of tubulin by preventing curved tubulin from adopting a straight structure. The result of our docking study shows that mHA1, mHA6, and mHA11 could bind at the same site of colchicine on the tubulin protein. Radioligand binding assay confirms dose-dependent inhibition of tubulin polymerization by competing with the [3H]colchicine binding to tubulin for these three mHAs (Figure 4). In order to investigate the inhibitory effects of mHA1,
mHA6 and mHA11 on tubulin polymerization, >99% pure tubulin was treated with each of these compounds at 0.25–5 μM for 1 h at 37 °C (Figure 5). The contents of polymerized microtubules were measured every 60 sec for 1 h using 360 nm excitation and 420 nm emission filters. Results showed that mHA1, mHA6 and mHA11 exhibited strong inhibition of the microtubule formation comparable to the known microtubule polymerization inhibitor colchicine (IC50=25 nM), with IC50 values of 669 nM, 730 nM, and 755 nM, respectively.

To further determine whether the quantity and distribution of microtubule changes after treatment with these compounds, we performed an immunofluorescent staining study. Under normal culture condition, CRL 5908 cells were grown in spindle morphology and microtubules were arrayed along the long axes in the cytoplasm (Figure 6). However, 24 h after treatment with mHA11 (similar results were obtained after treatment with mHA1 or mHA6), the 5908 cells had shrunk, and the cell shape was changed from spindle to round, irregular, or multi-angular. The long microtubule structure in cytoplasm was disrupted and the microtubule fluorescence intensity was significantly reduced, suggesting the decrease in contents of microtubule.

**Docking study of HA14-1 analogs**

The crystal structure of alpha-beta-tubulin heterodimers in a complex with DAMA-colchicine reveals that colchicine binds at the interface of the α/β-tubulin heterodimer and inhibits the assembly of microtubules by preventing curved tubulin from adopting a straight structure. The results of our docking study show that mHA1, mHA6, and mHA11 could bind
at the same site as colchicine on the tubulin protein. mHA1 and mHA6 adopt a similar binding mode, which is slightly different from that adopted by mHA11 (Figure 7).

Discussion

HA14-1, the first reported Bcl-2 inhibitor identified by our group, can potently induce apoptosis in a wide variety of human cancers (12, 18-21). At concentrations above 10 μM, it also inhibits tubulin polymerization. We developed a series of HA14-1 analogs and identified a new class of 2-amino-4-phenyl-4H-chromene-3-carboxylate analogs: mHA1, mHA6 and mHA11 (Figure 1). These analogs showed a more stable and more potent anticancer growth activity than did authentic HA14-1, with IC_{50} values of less than 1 μM at 72 h in the HL-60/Bcl-2 cell line; while the IC_{50} value for HA14-1 of was about 10 μM. These new analogs also exhibited a mode of action that is different from that of HA14-1. HA14-1 is a fast-working agent: it induces reactive oxygen species (ROS) generation, cytochrome c release, and caspase-9/-3 activation (18, 22) within few hours after administration of treatment, which suggested that these responses are its main mechanism for killing tumor cells. Typical morphological changes associated with apoptotic cells, such as cell shrinkage, nuclear fragmentation, and chromatin condensation, occurred within 6 to 24 h after treatment with HA14-1. However, these new analogs induced different types of morphological changes, including cell elongation, asymmetry and formation of long pseudopodia (Figure 3A). All of these changes suggested that these new analogs may target microtubule functions, which is important for maintenance of cell shape and for cell division.
Our docking and protein-based binding studies provided further confirmed that these compounds bind at the same site as colchicine on the tubulin protein, causing disruption of tubulin polymerization (Figures 4-7). Analysis of DNA content by flow cytometry indicated that cells were blocked in the G2/M phase and this was accompanied with a decrease in mitotic cells and an increase in apoptotic cells (Table 1). The inductions of apoptosis in HL-60/Bcl-2 cells caused by these compounds (Figure 2, Table 1) were confirmed by DNA fragmentation analyses (Figure 3B). These novel compounds represent a new class of the emerging group of inhibitors of tubulin assembly that show promising anti-cancer activities and limited toxicity to normal bone marrow cells (Figures 2-7).

Microtubules are important molecular targets for anticancer therapy because of their critical role in mitosis. During mitosis, the microtubules of the interphase cytoskeleton depolymerize, and the tubulin repolymerizes to form the mitotic spindle (4). The duplicated chromosomes are then located and attached to the spindle for separation. This procedure requires highly coordinated microtubule dynamics. Therefore, agents such as taxanes, vinca alkaloids, and epothilones, and our newly synthesized HA14-1 analogs that disrupt microtubule dynamics also cause the arrest of cell cycle progression at mitosis and eventually lead to apoptosis (9, 23). Although many MIAs are actively used in the clinic against a wide variety of solid tumors and hematological malignancies, many limitations of currently approved agents are encountered, including variable sensitivity of different cancers, innate and acquired drug resistance, side effects of peripheral neuropathy and neutropenia, and poor solubility that necessitates their clinical use (4, 9). In addition to the development of
overexpression of drug efflux pumps (24), mutations in the genes encoding α- and β- subunits of tubulin and differential expression of tubulin isotypes that reduce the binding of a drug to tubulin also contribute to MIA resistance (25, 26). Therefore, discovery of new MIAs like our HA14-1 analogs may be helpful in overcoming these problems and might offer advantages over the current agents used for chemotherapy. In contrast to our mHA agents, which inhibit tubulin polymerization, paclitaxel enhances tubulin polymerization and does not compete with colchicine binding to tubulin (Figures 4-6). These data provide evidence that our mHA agents exerted their anti-microtubule effects via different mechanisms, including differences in the sites of tubulin binding (Figure 7). In addition, use of these MIAs in combination with other existing therapeutics could reduce the required doses of individual drugs, thereby lessening their toxicities and providing another way to overcome the shortcomings of currently used therapeutics (3, 27-29).

Bcl-2 is a proto-oncogene that prevents cells from undergoing apoptosis in response to cytotoxic stimuli. HL-60/Bcl-2 cells have been stably transfected with the pZip-Bcl-2 plasmid and they express higher levels of Bcl-2 protein than HL-60/neo cells do. Many rediscovered drug development strategies aim to affect multiple targets in a parallel fashion because multi-target drugs have a lower prevalence and reduced ranges of side effects than high-affinity, single-target drugs. mHA compounds were derived from HA-14-1, a known Bcl-2 binder and inhibitor. At concentrations lower than 5 μM, mHA compounds predominantly targeted microtubules. However, at concentrations around 5 μM, these mHA agents were also able to bind to Bcl-2 and inhibit Bcl-2 from binding with apoptotic protein-
derived probes (our unpublished data). The dual-target effects of these mHAs on both microtubule and Bcl-2, and their biological significance (such as whether this contributes to their lower cytotoxic effects on normal cells, Figure 2), need to be investigated further.

In summary, we have developed a novel class of anti-microtubule agents that show promising antitumor activity. These new HA14-1 analogs inhibit tubulin polymerization, thereby inducing cell cycle G2/M arrest and apoptosis. The promising cytotoxic effects on cancer cells with IC<sub>50</sub> value at nM ranges, coupled with the minor cytotoxic effects on normal bone marrow cells, indicate that these new analogs have a good therapeutic window and that their further development as therapeutics for human use will be worthwhile.

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FIGURE LEGENDS

Figure 1. Structures of HA14-1 and its analogs mHA1, mHA6, and mHA11 (A); and one-pot reaction for the synthesis of mHA1, mHA6, and mHA11 (B).

Figure 2. A-C: Cytotoxicity of mHA towards HL-60/Bcl-2 cell lines and normal human bone marrow cells (HBMCs). Cells were treated with different concentrations of mHA (A: mHA1, B: mHA6, C: mHA11) for 72 h, as described in the text. Cell viability was then evaluated with a CellTiter-Blue assay. Viability at each concentration was expressed as a percentage of the viability compared to the vehicle (DMSO) control. The experiments were performed three times and data were analyzed in Microsoft® Excel and plotted in Prism 5.

Figure 3. A: Morphological changes of HL-60/Bcl-2 cells in response to treatment with mHA1, mHA6, and mHA11. Cells were treated with 1 μM mHAs for 24 h and then examined using a fluorescence microscope. Most of the cells lost their normal spherical shape and morphological changes including cell elongation, asymmetry, and formation of long pseudopodia could be observed (Fig. 3A). Magnification is 200x. The result was representative of three independent experiments. B: Induction of DNA fragmentation. HL-60/Bcl-2 cells were treated for 24 h with mHA1, mHA6, mHA11 (1 or 5 μM) or colchicine (Colch, 0.1 or 1 μM). The DNA ladder was detected as described in “Materials and Methods.”
Figure 4. Effect of mHA1, mHA6, and mHA11 on tubulin binding of [3H]colchicine. Tubulin (>99% pure) was incubated with tritiated tubulin binders in the presence of mHA1 (x), mHA6 (◊), mHA11 (Δ), or Paclitaxel (○) at indicated concentrations for 1 h at 37 °C. Each point represents the mean of three independent experiments, and error bars indicate the standard error.

Figure 5. Inhibition of in-vitro polymerization of tubulin using mHA1, mHA6, or mHA11. The steady-state tubulin assembly level in the absence of an inhibitor was set 100%. IC50 values were determined by using the sigmoidal fitting method for plotting the steady-state levels of tubulin assembly (at 30 min after initiating the polymerization dynamic assay; excitation at 360 nm and emission at 420 nm) against drug concentrations and represent the concentrations of mHA1 (■), mHA6 (▲), mHA11 (▼) and colchicine (♦) for inhibition of the maximum tubulin polymerization level. Each point represents the mean value of three independent experiments.

Figure 6. Effects of mHAs on cellular microtubules. Upper panel: Expression of microtubules (A), actin (B) and both microtubules and actin (C, merged image) in nontreated CRL 5908 lung cancer cells. Lower panel: Expression of microtubules (D), actin (E) and both microtubules and actin (F, merged image) in CRL 5908 cells 24 h after treatment with 1 µM mHAs. The result was representative of three independent experiments.
Figure 7. Comparison of the predicted binding models of mHA1, 6, and 11 to the tubulin protein with that of DAMA-colchicine in the crystal structure. A. mHA1 and mHA6 bind to the colchicine-binding site in the same manner. B. The binding modes of mHA1 and mHA11 differ slightly. Carbon atoms in mHA1, mHA6, and mHA11 are shown as sticks; and DAMA-colchicine as lines. The carbon atoms are colored in magenta for mHA1, in orange for mHA6, in deep teal for mHA11, and in cyan for DAMA-colchicine. Nitrogen and oxygen atoms are colored in blue and red, respectively. These figures were rendered using Pymol.
Fig. 1.

A.

HA14-1  mHA1

mHA6  mHA11

B.

\[
\text{CHO} + \text{N} + \text{N} \rightarrow \text{N} \]

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Fig. 2.

A. Cell Viability (% of Control) of HL-60/Bcl-2 cells and Human bone marrow cells as a function of Concentrations (μM).

B. Cell Viability (% of Control) of HL-60/Bcl-2 cells and Human bone marrow cells as a function of Concentrations (μM).

C. Cell Viability (% of Control) of HL-60/Bcl-2 cells and Human bone marrow cells as a function of Concentrations (μM).
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.

A.  

B.  

C.  

D.  

E.  

F.
Fig. 7.
Table 1. Summary of the distribution of cells in different phases of the cell cycle.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration (μM)</th>
<th>Cell cycle</th>
<th>Sub-G1</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
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<tr>
<td>Control</td>
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Molecular Cancer Research

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Jingle Xi, Xuejun Zhu, Yongmei Feng, et al.

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