Abstract. The anticancer properties of MHY-449, a novel dihydrobenzofuro[4,5-b][1,8]naphthyridin-6-one derivative, in various human cancer cell lines have been previously reported. The aim of the present study was to investigate the activities of MHY-449 on human lung cancer cells in order to elucidate its underlying molecular mechanisms of action. The result showed that MHY-449 treatment inhibited cell growth in a time- and concentration-dependent manner. Specifically, MHY-449 induced cell cycle arrest at the S phase, and the resulting increased sub-G1 fraction led to the induction of apoptosis, as determined by flow cytometric analysis and DNA fragmentation. In addition, MHY-449 was shown to induce alterations in the ratio of Bax/Bcl-2 protein expression, and contribute to the loss of mitochondrial membrane potential. These cellular events then triggered the caspase cascade and subsequent poly(ADP-ribose) polymerase cleavage. The apoptotic cell death induced by MHY-449 was inhibited by pretreatment with Z-VAD-FMK, a pan-caspase inhibitor. Moreover, MHY-449 downregulated the phosphorylation of Akt, and the phosphatidylinositol-3 kinase/Akt inhibitor LY294002 was found to enhance its induction of apoptosis. Taken together, the results suggested that MHY-449 exerts anticancer effects by promoting cell cycle arrest and apoptosis via the downregulation of Akt. Based on these data, MHY-449 serves as a potential candidate in the chemoprevention and/or treatment of lung cancer.

Introduction

Lung cancer is characterized by uncontrolled cell growth in tissues of the lung, trachea, or bronchi. This malignancy is currently the leading cause of cancer-related mortality in the world (1,2). In Korea, the incidence of lung cancer increases gradually with age (3). Recently, Jung et al (1) reported that lung cancer is the fourth most prevalent type of malignancy, and the most common cause of cancer-related mortalities in Korea (1). Most (~85%) patients with lung cancer have the non-small cell lung cancer (NSCLC) subtype, and the majority of these patients have advanced disease (defined as stage IIIB or IV at the time of diagnosis) (4). The rapid developments in oncogene-directed targeted therapies have significantly changed the treatment paradigm of NSCLC during the last decade. However, tumors exposed to targeted therapies may develop acquired resistance against these treatments over time. Therefore, the development of novel agents to combat NSCLC, and associated resistance, is imperative.

The (±)-(R*)-5-methoxy-11-methyl-2-(((R*)-2-methyloxiran-2-yl)-1,2-dihydrobenzofuro[4,5-b][1,8]naphthyridin-6(11H)-one, MHY-449, is a novel synthetic compound containing xanthone and acridone components, derived from psorospermin and acronycin templates, respectively (5). MHY-449 has been shown to induce apoptosis in various human cancer cell lines, including breast (5), colon (6) and prostate (5,7). While its precise mechanism of anticancer activity has not been fully elucidated, we have previously reported that MHY-449 induces G2/M phase cell cycle arrest (5,6), inhibits Akt/forkhead box O1 (7), activates caspases (6) and extracellular signal-regulated kinase (ERK) (7), modulates cell cycle regulatory proteins (6), and downregulates the anti-apoptotic protein Bcl-2 (6,7). These anticancer activities make MHY-449 an attractive candidate for potential pharmaceutical development. In this study, we evaluated the effect of MHY-449 on NSCLC cells in vitro. The results showed that MHY-449 exhibits a
potent anticancer effect in NSCLC cells, attributable to its ability to induce apoptosis. It was also found that Akt is essential to MHY-449-induced apoptosis in these cells.

Materials and methods

Chemicals. The simplified code name and chemical structure of MHY-449 used in this study are shown in Fig. 1. The methods used for the design and synthesis of this compound have been previously described (ref1). MHY-449 was dissolved in dimethyl sulfoxide (DMSO) to yield a 10-mM stock solution and stored at -20°C until use. The maximal concentration of DMSO did not exceed 0.1% (v/v) in the treatment range, where there was no influence on cell growth. DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Amresco LLC (Solon, OH, USA). Antibodies specific for pro-caspase-3, -8, and -9, poly(ADP-ribose) polymerases (PARP), BH3-interacting domain death agonist (Bid), Bcl-2-associated X protein (Bax), B-cell CLL/lymphoma 2 (Bcl-2), phospho-Akt, Akt, β-actin and Z-VAD-FMK, were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and cell viability assay. The human A549 and NCI-H460 lung cancer cell lines were cultured at 37°C in humidified 5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (all from GE Healthcare Life Sciences, Logan, UT, USA). The non-transformed human Hs27 foreskin fibroblast cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences) supplemented with 10% FBS. To determine cell viability, the MTT assay was performed. The cells were seeded in 24-well culture plates, cultured for 24 h, treated with or without various reagents at the indicated concentrations, and then incubated in the dark with MTT (0.5 mg/ml) at 37°C for 2 h. The formazan granules generated by the live cells were dissolved in DMSO, and the absorbance at 540 nm was monitored using a multi-well reader (Thermo Fisher Scientific, Vantaa, Finland).

Annexin V/PI staining. To quantitatively determine the percentage of apoptotic cells, we used the BD Pharmingen FITC Annexin V Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The cells were stained with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) solution at room temperature for 15 min in the dark. The stained cells were then analyzed using flow cytometry within 1 h.

DNA fragmentation assay. The cells were lysed in a buffer containing 5 mM Tris- HCl (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100 for 30 min on ice. After centrifugation at 27,000 x g for 15 min, the fragmented DNA in the supernatant was treated with RNase, followed by proteinase K digestion, extraction with a phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v/v), and isopropanol precipitation. DNA was separated using a 1.6% agarose gel, stained with ethidium bromide (0.1 µg/ml), and visualized using an ultraviolet source.

Western blot analysis. The cells were harvested, lysed, and equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes for immunoblotting. Blots were probed with the desired primary antibodies overnight, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), and then visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Measurement of mitochondrial membrane potential (MMP, ΔΨm). MMP was determined using a flow cytometer and the lipophilic cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetra-ethylbenzimidazolylcarbocyanine iodide (JC-1; Calbiochem, San Diego, CA, USA). JC-1 is a dye that stains the mitochondria of living cells in a membrane potential-dependent manner. The cells were treated with various concentrations of JC-1, harvested, and washed with cold PBS. The cells were stained with 10 µM JC-1 for 20 min at 37°C in the dark. The cells were subsequently washed with cold PBS and then analyzed using an Accuri C6 flow cytometer (BD Biosciences, Ann Arbor, MI, USA).

Flow cytometric analysis of sub-G1 phase and cell cycle population. The DNA content was measured following the staining of the cells with PI (Sigma-Aldrich Co., LLC, St. Louis, MO, USA). After treatment with various concentrations of MHY-449, the cells were collected, washed with cold PBS, and then fixed in 70% ethanol at -20°C overnight. The fixed cells were washed with cold PBS and then stained with cold PI solution (50 µg/ml in PBS) at 37°C for 30 min in the dark. Analysis was performed using an Accuri C6 flow cytometer.

Statistical analysis. Results were presented as the mean ± standard deviation (SD) of three separate experiments and analyzed using the Student's t-test. The acceptable level of significance was established at P<0.05.

Results

MHY-449 inhibits the proliferation of lung cancer cells. The anti-proliferative activities of MHY-449 on NSCLC cell lines A549 and NCI-H460, were first evaluated using the MTT cell viability assay. As shown in Fig. 2, MHY-449 reduced cell viability in a concentration- and time-dependent manner in
each of the cell lines. The A549 cell line was more sensitive to the effects of MHY-449 in comparison to the NCI-H460 cell line. The IC₅₀ of MHY-449 was found to be 0.66 µM for A549 cells (Fig. 2A) and 1.57 µM for NCI-H460 cells (Fig. 2B) at 48 h. In subsequent experiments, we used MHY-449 at concentrations up to 1 and 2 µM for use with A549 and NCI-H460 cells, respectively.

The effects of MHY-449 on the normal Hs27 cell line were also analyzed (Fig. 2C). MHY-449 (2 µM) inhibited >40% of the cell growth in A549 and NCI-H460 lung cancer cell lines, whereas little growth inhibition was observed in the non-transformed human Hs27 foreskin fibroblast cell line.

**MHY-449 induces apoptotic cell population in NSCLC cells.** To examine the mechanisms responsible for MHY-449-mediated cell growth inhibition, its effect on apoptosis induction was examined in NSCLC cells. The A549 and NCI-H460 NSCLC cell lines were treated with different concentrations of MHY-449 for 24 h. An increased proportion of A549 cells was found to be in the sub-G1 phase (29.6%) 24 h after treatment with MHY-449 (1 µM) compared to the vehicle-treated control cells (1.7%). However, the ability of MHY-449 to promote apoptosis in NCI-H460 cells was modest compared to this effect in A549 cells. As shown in Fig. 3, MHY-449 only slightly altered the percentage of NCI-H460 cells in the sub-G1 phase. Specifically, MHY-449 (2 µM) treatment increased the sub-G1 phase cell population from 2.4% (vehicle-treated control) to 10%. These results suggested that MHY-449 induces apoptosis in NSCLC cells in a concentration-dependent manner.

**MHY-449 treatment induces apoptosis in NSCLC cells.** To confirm that the effects of MHY-449 in NSCLC cells are associated with the induction of apoptosis, we performed flow cytometric analyses using cells stained with Annexin V/PI. Exposure of A549 and NCI-H460 cells to increasing concentrations of MHY-449 for 24 h resulted in a gradual increase in the apoptotic cell population. Fig. 4A shows that MHY-449 increased the proportions of early (lower-right quadrant) and late (upper-right quadrant) apoptotic cells with increasing concentration in the two cell lines. For example, 33.9% of cells advanced to late apoptosis-induced death following treatment with MHY-449 (1 µM) as compared to the number of apoptotic cells in the vehicle-treated control (4.5%).

We determined the effect of MHY-449 on DNA fragmentation in A549 and NCI-H460 cells, another hallmark event of apoptosis. As shown in Fig. 4B, the ladder pattern of DNA was observed in the NSCLC cells in a concentration-dependent manner with respect to MHY-449. In A549 cells, this typical ladder pattern of internucleosomal fragmentation resulted from exposure to 1 µM MHY-449, whereas in NCI-H460 cells, 2 µM concentrations were required (Fig. 4B).

These results indicated that MHY-449 induces apoptosis in lung cancer cells. To determine the possible origin of this effect, we investigated the influence of MHY-449 on apoptosis-regulated gene expression. In particular, we assessed the potential of MHY-449 to alter the expression levels of caspases in NSCLC cells. We found that treating cells with MHY-449 resulted in a concentration-dependent decrease of pro-caspase-8, -9, and -3 levels, and also cleavage of their substrate PARP (Fig. 4C).

As mitochondria play a critical role in apoptosis triggered by a variety of stimuli (8), we investigated the effect of MHY-449 on signaling molecules known to be involved in these pathways. Exposure of NSCLC cells to MHY-449 triggered a downregulation of the whole form of pro-apoptotic protein Bid, resulting from Bid cleavage and activation. MHY-449 was also found to increase the levels of the pro-apoptotic protein Bax in A549 cells. No analogous alterations of Bax expression were observed in NCI-H460 cells because of MHY-449 treatment. A clear decrease in (anti-apoptotic protein) Bcl-2 expression was observed in the two NSCLC cell lines (Fig. 4C) following treatment with MHY-449. These results suggested that, MHY-449 induces the apoptosis of human lung cancer cells via activation of the caspase cascade and through the mitochondrial pathway.
Caspases are involved in MHY-449-induced apoptosis in NSCLC cells. To investigate the significance of caspase activation in MHY-449-induced apoptosis, we determined the viability of NSCLC cells pretreated with Z-VAD-FMK, a broad-spectrum caspase inhibitor, for 1 h followed by treatment with MHY-449 for 24 h. As shown in Fig. 4D, pretreatment of A549 cells with Z-VAD-FMK promoted their viability as compared to cells treated with MHY-449 only. Similar results were obtained using NCI-H460 cells (Fig. 4D). To verify the role of caspase activation in MHY-449-induced apoptosis, we determined apoptotic cell death by analyzing the progressive proteolytic cleavage product of PARP, an activated caspase-3 substrate protein. Pretreatment of the cells with Z-VAD-FMK was found to abolish MHY-449-induced cleavage of PARP (Fig. 4E). These results suggested that activation of the caspase cascade is involved in the induction of apoptosis by MHY-449.

MHY-449 promotes loss of the mitochondrial membrane potential. The alteration of Bax, Bid, and Bcl-2 proteins are thought to contribute to apoptotic cell death by promoting the release of apoptogenic molecules from the mitochondria into the cytosol. As the expression levels of Bax, Bid, and Bcl-2 in NSCLC cells were found to be influenced by MHY-449 (Fig. 4C), we determined whether the resulting death of these cells was associated with disruption of the mitochondrial membrane potential (MMP). The effect of MHY-449 on MMP was determined by flow cytometric analyses following staining of the examined cells with JC-1, a lipophilic cationic dye that selectively enters mitochondria. As shown in Fig. 5A and B, JC-1 in the control cells exhibited red fluorescence due to the accumulation of J-aggregates indicating intact MMP (upper panel). By contrast, treating the cells with MHY-449 resulted in a concentration-dependent green fluorescence (lower panel) resulting from the cytosolic accumulation of monomeric JC-1. The populations of A549 cells with a disrupted membrane potential were 20.2, 29.2, 41.2 and 63.7% at 0, 0.25, 0.5, and 1 µM MHY-449 concentrations, respectively (Fig. 5C). MHY-449 (1 µM) also increased the population of NCI-H460 cells with a disrupted membrane potential to 41.6% compared to that observed in the control (17%; Fig. 5C). Collectively, these results indicated that MHY-449 induces a loss of MMP in NSCLC cells.

Akt inhibitor enhances apoptosis induced by MHY-449. Recent evidence has determined that Akt signaling is essential for NSCLC cell survival (9). Activation of the Akt pathway renders cells resistant to apoptosis via the regulation of pro- and anti-apoptotic proteins (10,11). It was also reported that inhibition of the Akt pathway served to arrest cancer cell
proliferation and significantly delay tumor growth (12,13). Thus, we examined the involvement of Akt signaling in mediating apoptosis induced by MHY-449. Treatment of A549 cells with the Akt inhibitor LY294002 alone had a minimal effect on inducing apoptosis (Fig. 6A). We also observed no notable induction of apoptosis in NCI-H460 cells treated with LY294002 (Fig. 6A). We also determined the influence of Akt inhibition on MHY-449-induced apoptosis in NSCLC cells. As shown in Fig. 6A, LY294002 enhanced the proportion of cells that underwent MHY-449-induced apoptosis in the NSCLC cell lines evaluated. Specifically, this proportion increased from 18.1 to 35.9% in A549 cells and from 9.8 to 21.6% in NCI-H460 cells. These results indicated that Akt inhibition enhances the apoptotic effect of MHY-449 in NSCLC cells.
To confirm this result, we performed western blot analysis to investigate the effect of LY294002, MHY-449, and their co-administration on Akt activation and PARP cleavage. LY294002 was found to inhibit Akt activation in the two lung cancer cell lines evaluated. Of note, the concentrations of phosphorylated Akt (active form) were markedly reduced after these cells were treated with MHY-449 (Fig. 6B). Treatment of the NSCLC cells with LY294002 and MHY-449 was found to significantly suppress Akt activation (Fig. 6B). As such, the apoptogenic effect of MHY-449 on NSCLC cells was markedly enhanced by LY294002 (Fig. 6B). These results suggested that the Akt pathway is likely involved in MHY-449-induced apoptosis of NSCLC cells.

**Discussion**

In the present study, we have determined that synthetic MHY-449 exerts potent antitumor effects in NSCLC cells. To the best of our knowledge, this is the first study describing this effect of MHY-449 on NSCLC cells, despite its documented cytotoxicity in numerous other cancer cell lines (5-7). MHY-449 can effectively induce NSCLC cells to undergo apoptosis. When the underlying mechanism for this effect was examined, we found that MHY-449 triggered the caspase cascade, and also induced apoptosis through the mitochondrial pathway. Therefore, a resulting loss of mitochondrial membrane potential is likely a primary mechanism by which MHY-449 exerts antitumor effects in NSCLC cells.
We also demonstrated that concentrations of phosphorylated Akt were markedly reduced in MHY-449-treated cells. Furthermore, the pharmacologic inhibition of Akt was found to markedly enhance MHY-449-induced NSCLC cell apoptosis. Constitutive activation of the Akt pathway is frequently observed in NSCLC cells, which acts to sustain their proliferation and survival. MHY-449 may be considered in the treatment of lung cancer owing to its apoptogenic properties in these cells, and ability to effectively suppress the Akt pathway.

Apoptosis is an important event leading to programmed cell death, which is often essential for the physiologic development and maintenance of organisms (14). The present study demonstrates that MHY-449 exerts a pronounced cytotoxic effect in two different NSCLC cell lines. MHY-449 was shown to effectively decrease the proliferation of A549 cells, which were more sensitive to this agent than NCI-H460 cells. MHY-449 also produced clear increases in sub-G1 populations and DNA fragmentation patterns in these cell lines. Phosphatidylserine exposure on the external plasma membrane leaflet in MHY-449-treated NSCLC cells was confirmed by Annexin V staining. Collectively, these results suggest that MHY-449 effectively inhibits NSCLC cell proliferation and induces apoptosis in a concentration-dependent manner.

The caspase family of cysteine proteases plays a central role in regulating apoptosis. It is has been well-established that certain caspases (e.g., caspase-8 and -9) play upstream initiator roles in apoptosis by coupling cell death to stimuli from the downstream effector caspases (e.g., caspase-3, the most significant promotor of apoptosis) (15). Of note, the downregulation of caspases-3, -9, and -8, as well as cleavage of PARP, was observed in present study. The observed caspase-mediated properties of MHY-449 are in agreement with previous studies using colon (6) and prostate (7) cancer cells. We also demonstrated that MHY-449, in the presence of Z-VAD-FMK, prevented the degradation of PARP in these cells. However, Z-VAD-FMK did not effectively rescue NSCLC cells from MHY-449-induced apoptosis. Thus, these results suggest that MHY-449-triggered apoptosis is likely mediated, at least in part, by the caspase cascade.

Since mitochondria play a crucial role in the extrinsic and intrinsic pathways of apoptosis (16), we also examined the effect of MHY-449 on mitochondrial function. Members of the Bcl-2 family of proteins are known to govern apoptotic cell death either as activators (e.g., Bad, Bax, and Bid) or inhibitors (e.g., Bcl-2, Bcl-XL, and Bcl-W) of mitochondrial outer membrane permeabilization (16). For example, Bid is readily cleaved by caspase-8 following stimulation of the extrinsic apoptotic pathway (17). Our results indicate that the expression of full-length Bid decreased following exposure to MHY-449, which then led to its cleavage. The results also demonstrated that MHY-449 served to simultaneously decrease Bcl-2 expression and increase Bax expression. This resulting decrease in the Bcl-2/Bax ratio is known to be a critical factor in determining which cells undergo apoptosis (18). In addition, the level of MMP disruption was enhanced by MHY-449 in NSCLC cells. Taken together, these results suggest that MHY-449-induced apoptosis is associated with alteration of the Bcl-2/Bax ratio and downregulation of the full-length Bid, which collectively induce outer mitochondrial membrane permeabilization and the loss of MMP.

We observed that MHY-449 represses phosphorylated Akt expression in NSCLC cells. In agreement with this finding, Akt activation has been shown to be negatively regulated by MHY-449 in human prostate cancer cells (7). In addition, we demonstrated that exposing NSCLC cells to an Akt inhibitor (LY294002, 20 µM) alone only minimally induces cell death. However, concomitant treatment of cells with LY294002 and MHY-449 resulted in enhanced apoptotic cell death. Given the established role of the phosphatidylinositol-3 kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling cascade as a pivotal pathways in cancer cell growth and survival (19-21), an agent that can perturb this signaling sequence may serve to prevent and/or treat tumor progression. Previous studies have indicated that NSCLC cells highly express phosphorylated and activated Akt (22,23). Additionally, the tumor-suppressor gene phosphatase and tensin homolog (PTEN) is involved in the regulation of cell survival as well as apoptosis through the PI3K/Akt pathway. Specifically, the downregulation of PTEN is accompanied by a corresponding upregulation of phospho-Akt levels (19). We found that A549 cells were more sensitive to the combination of LY294002 and MHY-449 than were NCI-H460 cells. The phosphorylation of Akt is known to occur more readily in A549 cells than in NCI-H460 cells, since A549 cells harbor more methylated promoter CpG sites in PTEN (11,24). Although we have yet to determine the precise effect(s) of MHY-449 on PTEN, the known discrepancy in Akt phosphorylation between A549 and H460 cells may explain their difference in susceptibility to concomitant LY294002/MHY-449 treatment.

In conclusion, the results of the present study have provided evidence that MHY-449 induces human NSCLC cell death stemming from activation of the caspase family and PARP cleavage. In addition, MHY-449-induced apoptosis proceeds via mitochondrial-mediated pathways involving activation and inhibition of Akt. These documented anticancer properties make MHY-449 an attractive candidate for further study in the prevention and therapy of lung cancer.

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References


