The novel synthesized 2-(3-(methylamino)phenyl)-6-(pyrrolidin-1-yl)quinolin-4-one (Smh-3) compound induces G2/M phase arrest and mitochondrial-dependent apoptotic cell death through inhibition of CDK1 and AKT activity in HL-60 human leukemia cells

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Abstract. 2-Phenyl-4-quinolone series compounds have exhibited growth inhibitory influence on several human cancer cell lines. In this study, we investigated the effects of 2-(3-(methylamino)phenyl)-6-(pyrrolidin-1-yl)quinolin-4-one (Smh-3) on viability, cell cycle and apoptotic cell death which occurred in different leukemia cell lines (HL-60, U937 and K562) in a dose- and time-dependent manner, but which did not obviously impair the viability of normal human umbilical vein endothelial cells (HUVEC) in vitro. The approximate IC50 was 103.26±4.59 nM for a 48 h treatment in HL-60 cells. Cell cycle analysis showed that 100 nM Smh-3 induced significant G2/M arrest in examined cells. Within 0, 12, 24 and 48 h of treatment, Smh-3 inhibited CDK1 activity and decreased protein levels of CDK1, cyclin A and cyclin B. Smh-3-induced chromatin condensation and DNA fragmentation were determined by DAPI and TUNEL staining. Cell apoptosis was significantly reduced after pretreatment with a pan-caspase inhibitor (Z-VAD-fmk) and results indicated that Smh-3-induced apoptosis was mainly mediated by activation of the caspase cascade in HL-60 cells. Results from colorimetric assays and Western blot analysis indicated that activities of caspase-9, -7 and -3 were promoted in Smh-3-treated HL-60 cells during cell apoptosis. Smh-3-induced apoptosis in HL-60 cells was accompanied by an apparent increase in ROS production, and protein levels of cytosolic cytochrome c, apoptotic protease activating factor-1 (Apaf-1) and apoptosis-inducing factor (AIF). Strikingly, Smh-3 induced apoptosis in HL-60 cells by simultaneously suppressing protein levels of AKT, p-AKT, p-mTOR and p-BAD and inducing BAD protein levels. Taken together, we conclude that Smh-3 acts against leukemia cells in vitro via G2/M phase arrest, down-regulation of AKT activity and induction of mitochondrial-dependent apoptotic pathways.

Introduction

Leukemia is one of the hematologic malignancies in human population. About 4.0 per 100,000 people die of leukemia each year in Taiwan based on the report of the Department of Health, R.O.C. (Taiwan) in the 2009 report (http://www.doh.gov.tw/EN2006/index_EN.aspx). Leukemia involves progressive disruption of cell differentiation, proliferation and apoptosis (1). Bone marrow transplant, radiotherapy and chemotherapy are applied for the treatment of leukemia patients (2).

The novel synthesized compounds, designed for providing chemotherapeutic effectiveness, are not detrimental to normal cells, which exert specific cytotoxic effects on leukemia cells through cell cycle arrest and apoptosis (3). Cyclin-dependent protein kinases (CDKs) and cyclins play important regulatory roles in cell cycle transition (4). Particularly, G2-M transition is regulated by cyclin-dependent protein kinase 1 (p34cdc2; CDK1) and cyclins A, B (5). Apoptosis is characterized by a series of morphological changes involving cell shrinkage and chromatin condensation (6). At least, two distinct pathways are involved in apoptotic cell death. The intrinsic pathway involves disrupting the mitochondrial membrane and releasing...
cytotoxic effects of Smh-3 on leukemia cells and normal cells, nor the molecular mechanisms underlying its anticancer activity have been investigated. Therefore, this study investigated the molecular mechanisms of Smh-3 in anti-leukemia effects on HL-60 cells in vitro.

Materials and methods

Chemicals and reagents. Smh-3 (2-(3-(methylamino)phenyl)-6-(pyrrolidin-1-yl)quinolin-4-one; Smh-3, as shown in Fig. 1, is a most potential candidate for anti-leukemia activities. However, neither the cytotoxic effects of Smh-3 on leukemia cells and normal cells, nor the molecular mechanisms underlying its anticancer activity have been investigated. Therefore, this study investigated the molecular mechanisms of Smh-3 in anti-leukemia effects on HL-60 cells in vitro.

Cell culture. The leukemia cell lines HL-60 (human promyelocytic leukemia), U937 (human lymphoma cancer cell), K562 (human chronic myelogenous leukemia), and HUVEC (human umbilical vein endothelial cells) were purchased from Culture Collection and Research Center (CCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37°C in a 5% CO2 humidified incubator. The HUVEC were passaged at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen) (13).

Determinations of cell viability. Cells (2.5x10^5/well) were seeded in each well of 24-well plates and incubated with 0, 50, 100, 200 and 400 nM Smh-3 for 0, 24 and 48 h. Cells were harvested, washed and re-suspended with phosphate-buffered saline (PBS) containing 4 µg/ml PI, and then analyzed by using a PI exclusion method and flow cytometry (FACSCalibur™, Becton-Dickinson, NJ, USA) equipped with a laser at 488 nm wavelength. The percentage of cell viability was calculated as a ratio of the number of Smh-3-treated cells to that of 0.1% DMSO as a vehicle-control group (14,15). Viability assays were performed in triplicate from three independent experiments. The 50% inhibitory concentration (IC50) of Smh-3 was calculated using the software of dose-effect analysis with microcomputers as described previously (13).

Cell morphology. HL-60 cells (2x10^5 cells/well) were maintained in 24-well plates with RPMI-1640 medium and then were treated with 0, 50, 100 and 200 nM of Smh-3. Cell morphology was examined and photographed under a phase contrast microscope. Chromatin condensation was detected using the DAPI (4,6-diamidino-2-phenylindole dihydrochloride) staining method. HL-60 cells were treated with 100 nM Smh-3 for 0, 12, 24 and 48 h. After incubation for the indicated time, HL-60 cells were fixed gently by putting 70% ethanol, stained with DAPI, and then photographed using a fluorescence microscope as described previously (14,16).

DNA content and cell cycle distribution analysis. HL-60 cells were seeded in 24-well cell culture plates at a density of 2.5x10^5 cells/well, and incubated with 100 nM Smh-3 for 0, 12, 24 and 48 h. For determination of cell cycle phase and apoptosis, cells were fixed gently by putting 70% ethanol and 70% acetone overnight, and then re-suspended in PBS containing 40 µg/ml PI, 0.1 mg/ml RNase and 0.1% Triton X-100 in dark room for 30 min. Cell cycle distribution and apoptotic nuclei were determined by flow cytometry as described previously (13,17).

CDK1 kinase assay. CDK1 kinase activity was analyzed according to the protocol of Medical & Biological Laboratories CDK1 kinase assay kit (MBL International, Nagoya, Japan). In brief, the ability of cell extract prepared from each treatment was measured to phosphorylate its specific substrate, MV peptide as described previously (13,18).

TUNEL assay. HL-60 cells were seeded in 24-well cell culture plates at a density of 2.5x10^5 cells/well, and then incubated with 100 nM Smh-3 for 0, 24, 48 and 72 h. For the specific inhibitor assay, cells were seeded in 24-well plates, and then pretreated with a pan-caspase inhibitor (Z-VAD-fmk) for 1 h, followed by treatment with or without 100 nM Smh-3. After incubation for the indicated time, in situ apoptosis detection of DNA fragmentation was determined by using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche Diagnostics, Hillsdale, MI, USA). Following TUNEL staining, all samples were washed once and re-suspended in 0.5 ml of PBS containing PI (10 µg/ml) and DNase free-RNase A (200 µg/ml). TUNEL positive cells were analyzed by flow cytometry. The median fluorescence intensity was quantified by BD ProCellQuest software. TUNEL assays were performed in triplicate from three independent experiments as described previously (13).
Caspase activity assay. HL-60 cells (2.5x10^6 cells/well) were seeded in 6-well cell culture plates and incubated with 100 nM Smh-3 for 48 h. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT). About 50 µg of cytosol proteins were incubated with caspase-9, -8, -7 and -3 specific substrates (R&D System) for 1 h at 37˚C. The caspase activity was determined by measuring OD 405 as described previously (19).

Western blotting. HL-60 cells were seeded in 10 cm-dishes at an initial concentration of 1x10^7 cells and incubated with 100 nM Smh-3 for 0, 12, 24 and 48 h. Cytosolic fraction and total protein were prepared and determined. Equal amounts of protein (30 µg) were separated by 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electro-transferred to a nitrocellulose membrane by using iBot™ Dry Blotting System (Invitrogen). Blots were blocked in PBST buffer (0.05% Triton X-100 in PBS) containing 5% non-fat milk for 1 h, and incubated with specific primary antibodies at 4˚C overnight. The membrane was washed with PBST buffer and incubated with secondary antibodies conjugated horseradish peroxidase (HRP). The specific protein was detected by using enhanced chemiluminescence kits (Amersham, ECL Kits) as described previously (13,19).

Determination of reactive oxygen species (ROS). Approximately 2x10^5 cells/well of HL-60 cells in 24-well plates were treated with 100 nM Smh-3 and incubated for 0, 3, 6, 12 and 24 h. At the end of incubation, cells from each treatment were harvested by centrifugation, washed twice with PBS, and then re-suspended in 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; 10 µM) for ROS determination. Cells were incubated for 30 min at 37˚C in the dark room and analyzed immediately by flow cytometry as described previously (15,19).

In vitro cell AKT kinase assay. This assay was performed followed as the protocol of the manufacturer's instructions from an AKT kinase assay Kit (Cell Signaling Technology, Beverly, MA, USA). In brief, about 1x10^6 cells/well of HL-60 cells in a 75-T flask were treated with 0, 50, 100, 200 and 400 nM of Smh-3 for 2 h. Cells were then harvested, washed twice with PBS, and lysed in ice-cold lysis buffer provided in this kit. The 200 µg of protein from each time-point treatment were immunoprecipitated with 2 µg of anti-AKT antibody overnight. All samples were extensively washed, and then the immunoprecipitates were incubated with 1 µg of glycogen synthase kinase-3 α/β (GSK-3 α/β) fusion protein substrate in 50 µl of kinase buffer for 30 min at 30˚C. Reactions were stopped with SDS loading buffer. The samples were separated on 12% SDS-PAGE, and the phospho-GSK-3 α/β (Ser219) was detected by immunoblotting (20).

Statistical analysis. Data are presented as the mean ± SEM for the indicated number of separate experiment. Statistical analyses of data were performed by Student's t-test, and *P<0.05, ***P<0.001 were considered significant.

Results
Smh-3 inhibited cell growth of human leukemia cells. Initially, cells were determined the growth inhibition effects of Smh-3 on cell viability by using a PI exclusion assay and flow cytometric analysis. As shown in Fig. 2A, Smh-3 inhibited cell growth of
HL-60, U937 and K562 cells in a dose-dependent manner. Smh-3 shows much less cytotoxic effect on the normal HUVE than that on HL-60 cells as compared this effect of Smh-3 on HL-60 to normal HUVEC cells (Fig. 2A). Smh-3 inhibited cell growth of HL-60 cells in a dose- and time-dependent manner (Fig. 2B). The half maximal inhibitory concentration IC_{50} for 48 h treatment of Smh-3 in HL-60 cells was 103.26±4.59 nM. It can be seen in Fig. 2C and indicated that Smh-3 induced cell morphological changes, and decreased the cells number in HL-60 cells. Apoptotic HL-60 cells showed smaller, round and blunt in size after Smh-3 exposure, and these effects are dose-dependent (Fig. 2C).

Smh-3 induced G2/M phase arrest by decrease CDK1 activity in HL-60 cells. We investigated the possible mechanisms and whether Smh-3 is able to promote cell cycle arrest in HL-60 cells. As shown in Fig. 3, 100 nM Smh-3 induced cell cycle arrest in G2/M phase in HL-60 cells and this effect was time-dependent. We also examined the CDK1 activity in Smh-3-treated HL-60 cells. Results shown in Fig. 4A indicate that Smh-3 caused a significant decrease in CDK1 activity for 12, 24, and 48 h treatment. We characterized the cell cycle-regulated protein levels in G2/M phase. As shown in Fig. 4B, Smh-3 caused a decrease the protein levels of cyclins A, B and CDK1 in HL-60 cells. Our results suggest that cyclins A, B and CDK1 activities play important roles in G2/M phase arrest in Smh-3-treated HL-60 cells.

Smh-3 induced chromatin condensation and DNA fragmentation in HL-60 cells. To investigate the incidence of chromatin condensation in Smh-3-treated HL-60 cells, we assessed the nuclear chromatin changes by DAPI staining. As shown in Fig. 5A, cells exhibited nuclear shrinkage and chromatin condensation after incubation with 100 nM Smh-3 for 12, 24 and 48 h. We also demonstrated that Smh-3 induced DNA fragmentation which was examined by TUNEL staining and flow cytometric analysis as shown in Fig. 5B. Smh-3 induced DNA fragmentation (TUNEL positive cells) in HL-60 cells and this response was increased in a time-dependent manner. Based on our results, it is suggested that Smh-3-induced chromatin condensation and DNA fragmentation for cell apoptosis in HL-60 cells.

Smh-3 stimulates the activities of caspase-3, -7 and -9 in HL-60 cells. To verify if caspase activity is involved in Smh-3-induced apoptosis in HL-60 cells, the cells were pretreated with 10 µM Z-VAD-fmk (a pan-caspases inhibitor) and exposed to 100 nM Smh-3. The cells were harvested for measuring the TUNEL positive cells by flow cytometric assay. The results are shown in Fig. 6A and revealed that Z-VAD-fmk decreased the percentage of TUNEL positive cells in Smh-3-treated HL-60 cells. Our finding indicated that Smh-3-induced apoptosis was through an increase of caspase activity. To examine whether Smh-3-induced apoptosis is involved in the activations of caspase cascades, cells were harvested after exposure to 100 nM Smh-3 and then the...
activities of caspase-3, -7, -8 and -9 were determined by colorimetric assays or examined the protein levels of caspase-3, -7, -8 and -9 by Western blotting. As shown in Fig. 6B, Smh-3 promoted the activation of caspase-3, -7 and -9, but not caspase-8 after 48 h treatment. Therefore, Smh-3 increased the active form levels of caspase-3, -7 and -9 in a time-dependent manner (Fig. 6C). Our results suggest that Smh-3-triggered apoptosis may be mediated through mitochondria-dependent signaling pathway in HL-60 cells.

**Smh-3 induced production of reactive oxygen species (ROS) and release of the apoptosis-associated proteins in HL-60 cells.** To verify if ROS are involved in Smh-3-induced apoptosis in HL-60 cells, the cells were exposed to 100 nM Smh-3 for 0, 3, 6, 12 and 24 h, and then harvested for measurement in the level of ROS production by flow cytometric assay. The results in Fig. 7A indicated that Smh-3 promoted the ROS production from 3 to 24 h time-dependently. Smh-3 (100 nM) increased the protein levels of cytosolic cytochrome c, AIF and Apaf-1 (Fig. 7B). Our results indicated that Smh-3-induced cell death may be mediated through the mitochondria-dependent apoptotic signaling pathways in HL-60 cells.

**Smh-3 inhibited the activity of AKT and down-regulated the expression of phospho-BAD in HL-60 cells.** To examine the involvement of AKT pathway in Smh-3-regulated apoptosis in HL-60 cells, we assessed the effects of Smh-3 on AKT activity at 0, 50, 100, 200 and 400 nM of Smh-3 for 2 h treatment. The protein levels of phospho-AKT and phospho-mTOR after treatment with 100 nM of Smh-3 for 0, 1, 2 and 4 h were investigated. Our results in Fig. 8A show that Smh-3 inhibited the activity of AKT and down-regulated the expression of phospho-BAD in HL-60 cells.
decreased the AKT activity after treatment with 50 to 400 nM of Smh-3 and this effect is dose-dependent. In Fig. 8B, results from Western blot analysis show that Smh-3 caused a decrease in protein levels of phospho-AKT, AKT, phospho-mTOR and mTOR in HL-60 cells. It is reported that BAD is a major regulator of AKT signaling (10), we next determined the effect of Smh-3 on the protein levels of BAD and phospho-BAD in HL-60 cells. As seen in Fig. 8C, we found an increase in the expression of BAD, but a decrease in the expression of phospho-BAD in a time-dependent manner. Our data suggest that Smh-3-affected apoptotic cell death in HL-60 cells is through inhibition of the AKT activity, leading to the decrease in the level of phospho-BAD protein.

Discussion

Several reports have been demonstrated that 2-phenyl-4-quinolones series compounds exhibited growth inhibitory effects on several human cancer cell lines (18,21). 2-phenyl-4-quinolone series compounds might inhibit CDK1 activity and act as anti-mitotic agents (18). In recent years, we have designed and synthesized a new series of 2-phenyl 6-pyrrolidinyl-4-quinazolinone derivatives as new anti-leukemia agents and Smh-3 (Fig. 1) (2-(3-(methylamino)phenyl)-6-(pyrrolidin-1-yl)quinolin-4-one) is the most potential compound against cancer cells in vitro. However, the growth inhibition effects of Smh-3 on leukemia cells and normal cells or the molecular mechanisms underlying its anti-leukemia activity have not been clarified. In this study, we first demonstrated that Smh-3 induced growth
our results demonstrated an increase of ROS production after Smh-3 treatment for 3 h (Fig. 7), and then promoted the release of cytochrome c, Apar-1 and AIF protein levels from mitochondria into the cytosol (Fig. 7). In addition, caspase-8 activity in Smh-3-treated HL-60 cells had no significant increase (Fig. 6). Based on the above evidence (Fig. 6), we might rule out that Smh-3-stimulated apoptotic cell death is involved in the extrinsic apoptotic pathway.

BCL-2 family proteins are classified into the following three categories according to their function: i) proteins were involved in promoting apoptosis: such as BCL-2, BCL-xL; ii) proteins were involved in promoting apoptosis: such as Bax, Bid; iii) BH3-only protein: such as BAD (23-25). BAD is activated by dephosphorylation, and has a conserved BH3 domain that binds and regulates the anti-apoptotic protein to promote cell apoptosis (10). Considerable evidence indicates that BAD can affect Bax by directly binding and inhibiting BCL-2 (26,27). Our study suggests that BAD was dephosphorylated and activated after HL-60 cells were treated with Smh-3 (Fig. 8C), which may lead to activation of Bax and inactivation BCL-2, then triggering apoptosis. It has been reported that AKT is involved in survival signaling pathway by phosphorylating BAD (11). The phospho-BAD bound to 14-3-3 proteins, but not BCL-2. In the present study, we demonstrated that Smh-3 inhibited the activity of AKT in HL-60 cells (Fig. 2A), leading to that BAD was reported to be dephosphorylated and then induced apoptosis in Smh-3-treated HL-60 cells (11,28).

Overall, the molecular signaling pathways are summarized in Fig. 9. Our results demonstrated that Smh-3 exposure causes G2/M phase arrest and inhibits AKT activity in HL-60 cells. Dephosphorylation of BAD promotes the release of caspase-9, Apar-1, AIF from mitochondria to cytosol which activates apoptosis. Hence, Smh-3 could possibly be used as a novel therapeutic agent for the treatment of leukemia in the future.

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