A novel hydroxamic acid derivative, MHY218, induces apoptosis and cell cycle arrest through downregulation of NF-κB in HCT116 human colon cancer cells

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Abstract. Colorectal cancer (CRC) is one of the most common malignant diseases and frequent cause of cancer deaths in the world. In spite of the significant advances in conventional therapeutic approaches to CRC, most patients ultimately die of their disease. There is a need to develop novel preventive approaches for this malignancy. This study was carried out to investigate the anticancer effect of MHY218, a hydroxamic acid derivative, in HCT116 human colon cancer cells. Treatment of cells with MHY218 resulted in growth inhibition and induction of apoptosis in a concentration-dependent manner. MHY218 induced G2/M phase arrest in the cell cycle progression which was observed by flow cytometry analysis, and a decrease in the protein expression of cyclin B1 and its activating partners Cdc25C and Cdc2. MHY218 also caused an increase in the expression levels of p21^{WAF1/CIP1}, a G2/M phase inhibitor, in a p53-independent pathway. The induction of apoptosis was observed by decreased viability, DNA fragmentation, cleavage of poly(ADP-ribose) polymerase, alteration in the ratio of Bax/Bcl-2 protein expression, and activation of caspase-3, -8 and -9. In addition, MHY218 treatment showed downregulation of the expression levels of the transcription factor nuclear factor-kappa B (NF-κB) in the nucleus, which has been reported to be implicated in the apoptotic cell death of several types of cancer cells, suppression of TNF-α-induced NF-κB activation, inhibition of cyclooxygenase-2 expression, repression of matrix metalloproteinase-9 activation and decrease of 5-lipoxygenase in a concentration-dependent manner. These results suggest that MHY218 may be a useful candidate to be used in the chemoprevention and/or treatment of colon cancer.

Introduction

Colorectal cancer (CRC) is a cancer from uncontrolled cell growth in the lining of the large intestine, such as colon and rectum. CRC is the fourth most common cancer in the world, but it is more common in developed countries. CRC is the third most common cancer and the fourth leading cause of cancer-related death in Korea and the incidence of CRC have been increased rapidly over the past few decades (1,2). While surgical resection cures >50% of CRC patients, 40-50% of these subjects eventually experience recurrences, leaving only a minority amenable to re-operation (3). Because of unsatisfactory treatment options for CRC, there is an urgent need to develop novel preventive treatment approaches for this malignancy.

Progressive inhibition or evasion of apoptosis has been found during the transformation of colorectal epithelium to carcinoma (4), indicating that dysfunction of apoptosis has important roles in colorectal carcinogenesis. The cytotoxic action of most chemotherapeutic drugs is often mediated by the activation of apoptotic pathways (5). Recent progress in understanding the molecular mechanisms and role of apoptosis in CRC development has provided novel targets for therapy.

Nuclear factor-κB (NF-κB), a transcription factor, controls the expression of genes involved in tumor cell growth, proliferation, angiogenesis, invasion and survival. NF-κB is composed of two subunits, p65 and p50, and is normally sequestered in the cytosol by an inhibitory protein, IκBα. Exposure of cells to a variety of extracellular stimuli leads to the rapid phosphorylation, ubiquitination, and ultimately

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proteolytic degradation of IκBα, resulting in the release of NF-κB from its inhibitory protein to translocate to the nucleus where it regulates transcription of various genes (6). Increased NF-κB activity has been demonstrated in colon cancer, which is believed to enhance cancer cell survival by inhibiting apoptosis. In addition, the inflammatory colon diseases such as Crohn's disease and ulcerative colitis are associated with the constitutive activation of NF-κB (7). Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth to inflammation-induced tumor regression mediated by TNF-α and TRAIL (8). Therefore, inhibition of NF-κB signaling pathway provides attractive targets for new chemopreventive and chemotherapeutic approaches.

Hydroxamic acids are known as iron chelators and microbial siderophores that show diverse biological activities such as antibacterial, antifungal, antitumor and anti-inflammatory properties (9). Some hydroxamates, such as suberoylanilide hydroxamic acid, has been used clinically for the treatment of cancer (10). For the purpose of creating more effective antitumor drugs many derivatives that possess the hydroxamic acid functional group were synthesized (11). The newly designed hydroxamic acid derivatives have been shown to have anticancer effect in various cancer cells (12). MHY218 [N'-hydroxy-N'-[4-(phenoxyphenol)octanedianide] was synthesized and it was a novel hydroxamic acid derivative. MHY218 has inhibitory activity of histone deacetylase and anticancer effects against human ovarian cancer cells (13) and tamoxifen-resistant MCF-7 breast cancer cells (14). The chemical structures of hydroxamic acids and MHY218 are shown in Fig. 1. However, the effects of MHY218 were not studied in HCT116 human colon cancer cells in previous reports. Therefore, the main purpose of this study was to focus on investigating the effects of MHY218 on cell cycle and apoptosis in HCT116 human colon cancer cells.

Materials and methods

Chemicals. The simplified code name and structure of MHY218 [N'-hydroxy-N'[4-(phenoxyphenol)octanedianide] used in this study is shown in Fig. 1B. Detailed method for the design and synthesis of this compound is described elsewhere (13). This was dissolved in sterile dimethyl sulphoxide (DMSO) to generate 10 mM stock solution. The solutions were stored at -80°C. Subsequent dilutions were made in RPMI-1640 (Hyclone, Logan, UT, USA). The maximal concentration of DMSO did not exceed 0.1% (v/v) in the treatment range, where there was no influence on the cell growth. All other chemicals with the highest purity available were from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cell culture. HCT116 human colon cancer cells (p53 wild-type) were obtained from American Type Culture Collection (Mansssas, VA, USA) and were cultured in RPMI-1640 (Hyclone) supplemented with 10% fetal bovine serum (PBS, Hyclone), 2 mM glutamine (Sigma-Aldrich), 100 U/ml penicillin (Hyclone), and 100 µg/ml streptomycin (Hyclone) at 37°C in a humidified 5% CO2.

Cell viability assay. Cell viability was determined by MTT assay. For the MTT assay, HCT116 cells were seeded in a 24-well culture plate at a density of 4x10^4 cells/well, cultured for 24 h in the growth media, and then treated with or without various reagents for the indicated concentrations. The cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) 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 by using a multi-well reader.

Nuclear staining with Hoechst 33342. Cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4 µg/ml Hoechst 33342 for 20 min at room temperature. The cell were washed two more times with PBS and analyzed via a fluorescent microscope.

DNA fragmentation assay. Cells were lysed in a buffer, containing 5 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.5% Triton X-100, for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 14,000 rpm for 20 min. Fragmented DNA in the supernatant was treated with RNase, followed by proteinase K digestion, phenol:chloroform:isoamyl alcohol mixture (25:24:1) extraction and isopropanol precipitation. DNA was separated through a 1.5% agarose gel, was stained with 0.1 µg/ml ethidium bromide, and was visualized by UV source.

Cell cycle analysis. The DNA content was measured following the staining of the cells with propidium iodide. The cells were treated under the appropriate conditions for 24 h, subsequently trypsinized, washed once in cold PBS, and then fixed in 70% ethanol at 4°C overnight. The fixed cells were pelleted and stained in cold propidium iodide (PI, Sigma-Aldrich) solution (50 µg/ml in PBS) at room temperature for 30 min in the dark. Flow cytometry analysis was performed on a FACScan flow cytometry system (Becton-Dickinson, San Jose, CA, USA).

Caspase activity assay. The cells were harvested and washed with cold PBS. Total cells were lysed with the lysis buffer [40 mM Tris (pH 8.0), 120 mM, NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 µg/ml aprotinin, 2 µg/ml leupeptin and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF)] at 4°C for 30 min. Cell lysate protein (100 µg) was mixed in assay buffer in a final volume of 100 µl, followed by addition of 10 µl of 2 mM of the substrate caspase-8 (Z-IETD-pNA), caspase-9 (Ac-LEHD-pNA), or caspase-3 (Z-DEVD-pNA) for the respective caspase assay. The reaction mixture was incubated at 37°C for 30 min and liberated p-nitroanilide (pNA) was measured at 405 nm using a multi-well reader.

Preparation of cytosolic and nuclear protein extracts. The cells were washed with cold PBS and resuspended in Buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and Protease inhibitor cocktail (Sigma-Aldrich)] and incubated on ice. After 15 min, 0.5% Nonidet P (NP)-40 was added to lyse the cells, which were vortexed for 10 sec. Cytosolic extracts were obtained after centrifuging at 12,000 rpm for 60 sec at 4°C. Nuclear extracts were resuspended in Buffer C [20 mM HEPES (pH 7.9), 1.5 mM MgCl2,
300 mM NaCl, 0.2 mM EDTA, 20% v/v glycerol, 0.5 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) and incubated on ice for 20 min with gentle vortexing every 5 min. Nuclear cell extracts were recovered after centrifugation for 10 min at 12,000 rpm at 4°C. Protein concentration was determined by Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA).

Western blot analysis. The cells were treated with the appropriate conditions, harvested, and washed with cold PBS. Total cells lysates were lysed in lysis buffer [40 mM Tris (pH 8.0), 120 mM, NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 µg/ml aprotinin, 2 µg/ml leupeptin and 100 µg/ml PMSF]. The supernatant was collected and protein concentrations were then measured with protein assay reagents (Bio-Rad, Rockford, IL, USA). Protein extracts were denatured by boiling at 100°C for 5 min in sample buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol). Equal amount of the total proteins were subjected to 6-15% SDS-PAGE and transferred to PVDF. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBS-T) (20 mM Tris, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature. Then, the membranes were incubated overnight at 4°C with primary antibodies. The membranes were washed once for 10 min, 4 times with TBS-T buffer and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Santa Cruz, CA, USA). The membranes were washed again for 10 min, 4 times with TBS-T buffer. Antigen-antibody complexes were detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences Corp., Little Chalfont, Bucks, UK).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. For RT-PCR analysis, total RNA was extracted from cultured cells using a TRIzol reagent as described by the manufacturer (Invitrogen). PCR amplification cDNA was prepared using a Bioneer RT/PCR PreMix containing 1 U Taq DNA polymerase, 250 µM dNTPs, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgCl2 (Bioneer, Korea). The assay was carried out in a 20 µl reaction mixture containing 1.0 µg of total RNA, 30 pmol of each primer, 1 µg Oligo dT (Bioneer), using a PCR Thermal Cycler Dice Takara TP600 (Takara, Otsu, Japan). The mRNAs were amplified with the primers indicated in Table I. GAPDH served as an internal control. The cycling conditions were as follows: cDNA synthesis at 42°C for 60 min, RTase inactivation at 94°C for 5 min, 1 x denaturation (94°C for 30 sec), 30 x annealing (58°C for 30 sec), and 1 x extension (72°C for 1 min) for 30 cycles. PCR products were analyzed by electrophoresis on 1% agarose gel (Bio Basic Inc., Markham, Ontario, Canada) in the presence of ethidium bromide, and were visualized with a UV trans-illuminator (MultiImage™ Light Cabinet, Alpha Innotech Co., San Leandro, CA, USA).

Luciferase reporter assay for NF-κB activity. The activity of NF-κB was examined using a luciferase plasmid DNA, pTL-κB that contains a specific binding sequence for NF-κB (BD Biosciences Clontech, CA, USA). Transfection was carried out using TransIT-LT1 transfection reagent (Promega, Madison, WI, USA). Briefly, HCT116 cells were seeded in 6-well plates. When cultured cells reached ~50% confluence, cells were treated with 2 µg DNA/6 µl transfection complexes in a total volume of media with 2 ml for 24 h. Subsequently, various concentrations of MHY218 were treated and incubated for 1 h, and then 10 ng/ml TNF-α was treated and incubated for 6 h. Cells were washed with PBS and added by Stead-Glo Luciferase Assay System (Promega) to the plate. Luciferase activity was measured by a luminometer (GENious, Tecan, Salzburg, Austria). The obtained raw luciferase activities were normalized by protein concentration in each well.

Zymography. Zymography was used to semiquantitatively determine the gelatinolytic activity of matrix metalloproteinase-9 (MMP-9) secreted into culture media. Equal amount of conditioned culture media from equal number cells were applied to SDS-PAGE containing 0.25% gelatin. The gel was incubated with the renaturing buffer (2.5% Triton X-100) with gentle agitation for 30 min at room temperature. The gel was washed once for 10 min, 2 times with distilled water and incubated overnight at 37°C with the Developing buffer [1 M Tris-HCl (pH 7.5), 1 M CaCl2, 10% NaN3, 1 mM NaCl]. The gel was stained with 0.5% (w/v) Coomassie Blue R-250 for 30 min at room temperature and then destained with the destaining solution (methanol:acetic acid:distilled water = 50:10:40). Areas of protease activity appeared as clear bands against a dark blue background where the protease had digested the substrate. The product of zymography was visualized with a White light transilluminator (BioSpectrum™ Imaging System, Upland, CA, USA).

Statistical analysis. Results were expressed as the mean ± SD of three separate experiments and analyzed by Student’s t-test. Means were considered significantly different at p<0.05 or p<0.01.

Table I. Primer sequences for RT-PCR.

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<tr>
<th>Gene</th>
<th>Sequence of primers (5'–3')</th>
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<tbody>
<tr>
<td>Cox-2</td>
<td>Sense AGA TCA TCT CGT CCT GAG TAT CTT</td>
</tr>
<tr>
<td></td>
<td>Antisense TTC AAA TGA GAT TGG TGG AAA ATT GCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense CGG AGT CAA CGG ATT TGG TCG TAT</td>
</tr>
<tr>
<td></td>
<td>Antisense AGC CTT CTC CAT GGT GGT GAA GAC</td>
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Figure 1. Chemical structures of hydroxamic acid (A) and MHY218 (B). R, an organic residue.
Results

MHY218 inhibits the growth of HCT116 cells. To investigate the effects of MHY218 on the viability of HCT116 cells, the MTT assay was performed. As shown in Fig. 2, MHY218 showed concentration-dependent cytotoxicity on HCT116 cells. The IC\textsubscript{50} value of MHY218 on HCT116 cells was ~3.0 µM.

MHY218 modulates the cell cycle in HCT116 cells. To investigate whether the inhibition of HCT116 cell growth was mediated, at least in part, by regulating the cell cycle, flow cytometry analysis of PI-stained HCT116 nuclei was performed. This flow cytometry analyses data showed that MHY218 treatment induced the accumulation of cells in G2/M phase of the cell cycle, with the occurrence of sub-G1 peak, indicating DNA degradation through either necrosis or apoptosis (Fig. 3A). G2/M phase arrest by MHY218 treatment reached the maximum percentage at 24 h. After 24 h incubation with different concentrations of MHY218, the population of the cells at the G2/M phase increased from 27.89% (vehicle alone) to 57.07% (10 µM MHY218) (Fig. 3A). As shown in Fig. 3A, the increase of cell population in G2/M phase consequently occurred with the decrease in G0/G1 cells as compared to those of control. In addition, we also observed the appearance of the peak corresponding to a population of cells with sub-G1 DNA content. This peak represented MHY218-induced DNA degradation either by necrosis or by apoptosis in HCT116 cells. After 24 h incubation of 10 µM MHY218, as shown in Fig. 3A, the fractions of sub-G1 peak increased from 3.55% (vehicle alone) to 19.72% (10 µM MHY218). These result supported that the MHY218 treatment for 24 h mainly induced the inhibition of cell growth via G2/M phase arrest in the cell cycle.

MHY218 modulates cell cycle regulatory proteins in HCT116 cells. To assess the effect of MHY218 on the intracellular protein expression levels of G2/M phase in the cell cycle, we performed western blot analysis. As shown in Fig. 3B, the expression levels of cyclin B1, Cdc25C and Cdc2 were decreased
by MHY218 treatment as compared to the basal levels in a concentration-dependent manner. The induction of \( p21^{WAF1/CIP1} \) causes subsequent arrest in the G0/G1 or G2/M phase of the cell cycle by binding of the cyclin-cyclin-dependent kinase (CDK) complex. Thus further studies were performed to elucidate whether \( p21^{WAF1/CIP1} \) was induced by the MHY218 treatment either via a p53-dependent or a p53-independent pathway in HCT116 cells. The results show that p53 was not significantly changed and \( p21^{WAF1/CIP1} \) was increased without the change in the level of MDM2 by MHY218 treatment in HCT116 cells (Fig. 3B). Therefore, these results suggest that MHY218 treatment induces G2/M phase arrest in cell cycle by downregulating expressions of cyclin, CDKs, and induction of \( p21^{WAF1/CIP1} \) via p53-independent pathway.

**MHY218 induces morphological changes and apoptosis in HCT116 cells.** To assess whether there are any morphological changes in MHY218-treated HCT116 cells, we examined the cells by phase-contrast light microscopy after 24 h of incubation with or without MHY218. Under the light microscope, untreated HCT116 cells spread regularly in the culture plate and grew to near confluence (Fig. 4Aa). In contrast, MHY218-treated HCT116 cells were shrunken and changed to round form. Additionally, cell numbers were decreased in a concentration-dependent manner (Fig. 4Ab-f). To investigate whether the growth inhibitory effects of MHY218 were due to the induction of apoptosis in HCT116 cells, the morphological changes were assessed with Hoechst 33342 staining. As shown in Fig. 4B, nuclei with chromatin condensation and formation of apoptotic bodies, which are characteristics of apoptosis, were seen in cells cultured with MHY218 in a concentration-dependent manner (Fig. 4Bb-4f), whereas the control cells maintained nuclear structure intact (Fig. 4Ba). We also analyzed whether DNA fragmentation, another hallmark of apoptosis, was induced by MHY218 treatment on HCT116 cells. Following agarose gel electrophoresis of HCT116 cells treated with MHY218 for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed in a concentration-dependent manner (Fig. 4C). Polypeptide degradation, including poly(ADP-ribose) polymerase (PARP), was examined to see the possible involvement of apoptosis-associated protease activity during the growth inhibition of the colon cancer cells. PARP cleavage was evident by the appearance of the p85 PARP cleavage fragment (Fig. 4D) and clearly observed in the 5 µM and 10 µM of MHY218 treatment.

**MHY218 modulates the expression levels of apoptosis-related proteins in HCT116 cells.** To determine whether the expression levels of apoptosis-related proteins were modulated by MHY218, western blot analysis was performed. The expression level of Bax protein was markedly upregulated, but Bcl-2 was downregulated in a concentration-dependent manner (Fig. 5A). The Bax/Bcl-2 ratio was also significantly increased with increase of MHY218 concentration (Fig. 5B). These data suggest that MHY218 induces apoptosis by the
alterations in expression levels of Bax/Bcl-2 protein. The expression levels of pro-caspase-3, -8 and -9 were decreased, indicating the activation of these caspases (Fig. 5C). In an attempt to further characterize the mechanisms of apoptosis, the activity of caspase-3, -8 and -9 was determined with the treatment of MHY218 in a concentration-dependent manner (Fig. 5D). These results, taken all together, imply that MHY218 seems to induce apoptosis through the internal and external pathway in HCT116 cells.

MHY218 modulates the expression levels of NF-κB, COX-2, MMP-9 and 5-LOX protein in HCT116 cells. NF-κB regulates the expression of a wide variety of genes involved in tumor cell growth and survival. We examined whether MHY218 has the potential to inhibit NF-κB activation in MHY218-treated HCT116 cells. After 24 h exposure with the indicated concentration of MHY218, the levels of nuclear NF-κB p65 and NF-κB p50 were examined, with western blot analysis, because the nuclear translocation of the NF-κB subunits p65 and p50 is essential for NF-κB activation. As shown in Fig. 6A, treatment with MHY218 decreased nuclear translocations of p65 and p50, the functionally active subunits of NF-κB, to the nucleus, in a concentration-dependent manner. Next to determine the effect of MHY218 on the TNF-α-induced NF-κB transcriptional activity, HCT116 cells were transiently transfected with the NF-κB-regulated luciferase
reporter construct, and the transfected cells were then stimulated with TNF-α alone or with a combination of TNF-α and MHY218. As shown in Fig. 6B, MHY218 significantly suppressed TNF-α-induced activation of NF-κB as compared to untreated cells. These data indicated that MHY218 treatment resulted in a significant inhibition of NF-κB activation.

To further investigate the effect of MHY218 on expression level of the COX-2 protein, an NF-κB downstream target gene, in HCT116 cells, the RT-PCR and western blot analyses showed no change in COX-2 mRNA (Fig. 7A) but a significant decrease in COX-2 protein expression after MHY218 treatment in a concentration-dependent manner (Fig. 7B). These data suggested that the inhibition of the COX-2 expression is consistent with the results that MHY218 inhibited the NF-κB activation. We next examined the effect of MHY218 on MMP-9 activity in HCT116 cells. Gelatin zymography showed that MMP-9 activity was significantly inhibited by MHY218 in a concentration-dependent manner (Fig. 7C). Another downstream target gene of NF-κB is 5-lipoxygenase (5-LOX). We next examined the effect of MHY218 on 5-LOX protein level in HCT116 cells. 5-LOX protein level was also significantly reduced in HCT116 cells treated with MHY218 in a concentration-dependent manner (Fig. 7D). These data suggested that MHY218 inhibited TNF-α-induced NF-κB activation through the suppression of nuclear translocation of NF-κB, leading to reduced expression of NF-κB-regulated gene products.

Discussion

This study was conducted to investigate the effects of MHY218 on HCT116 human colon cancer cells. MHY218 induced cell cycle arrest and apoptosis in HCT116 human colon cancer cells. MHY218 also modulated the activity of NF-κB and downregulated the expression levels of COX-2, MMP-9 and 5-LOX protein in HCT116 cells.

The treatment of HCT116 cells with MHY218 resulted in growth inhibition concentration-dependently. Flow cytometric analysis revealed that MHY218 induced G2/M phase arrest. Different classes of cyclins and their CDK control cell cycle progression. G2/M transition provides an effective checkpoint in the cell cycle progression that is regulated by cyclin B1, Cdc2 and Cdc25C (15). In this study, treatment of HCT116 cells with MHY218 resulted in arrest of cells in G2/M phase and is associated with a decrease in the protein levels of cyclin B1, Cdc2 and Cdc25C.

p21WAF1/CIP1, a cyclin-dependent kinase inhibitor, is commonly associated with the G1 checkpoint and G2/M phase, its association with inhibiting the expression of the Cdc2/cyclin B1 complex has also been demonstrated (16,17). p21WAF1/CIP1 transcription can be regulated through p53-dependent (18) and p53-independent pathways (19). In the present study, the protein level of p53 was not significantly increased, whereas p21WAF1/CIP1 was increased without a change on the expression of MDM2 by MHY218 in HCT116 cells. These results suggested that MHY218 activated p21WAF1/CIP1 expression and that this induced G2/M phase arrest of the p53-independent pathway. Therefore, the upregulation of p21WAF1/CIP1, and the downregulation of cyclin B1, Cdc2 and Cdc25C may be one of the molecular mechanisms by which MHY218 inhibited HCT116 cells growth and induced cell cycle arrest.

The treatment of MHY218 also induced apoptosis as demonstrated by the formation of apoptotic bodies and DNA fragmentation. Apoptosis (programmed cell death), is an important process required for homeostasis (20). Apoptosis occurs through two broad pathways: the intrinsic pathway (the mitochondrial pathway) and extrinsic pathway (the death receptor pathway) (21). Caspases are key players in the extrinsic pathway. Another group of key players in the intrinsic pathway of apoptosis is the Bcl-2 family, which consists of ~20 members of pro-apoptotic proteins (including Bax, Bak, Bok, Bad and Bid), and anti-apoptotic proteins (including Bcl-2, Bcl-XL, Mcl-1 and Bfl-1/A1) (22).

The activation of effector caspase-3, in response to MHY218 treatment also resulted in cleavage of PARP in HCT116 cells. The ratio between Bcl-2 and Bax has been suggested as a primary event in determining the susceptibility to apoptosis through maintaining the integrity of the mitochondria and inhibiting the activation of caspase cascade.
A new synthetic HDAC: Theaflavins target anticancer effects of the research information. We thank Aging Tissue Bank for providing material. This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP). The present study investigated the effect of MHY218 on the NF-κB pathway. Treatment of MHY218 to HCT116 cells resulted in inhibition of nuclear translocation of NF-κB concentration-dependently. NF-κB transcription factors can block apoptosis induced by TNF-α (29). TNF-α normally has less or no cytotoxic effect unless NF-κB activation or protein synthesis is blocked (30). In line with this, luciferase reporter assay revealed significant suppression of TNF-α-induced NF-κB transcriptional activity in MHY218-treated HCT116 cells in a concentration-dependent manner. COX-2 is the inducible form of cyclooxygenase that catalyzes the rate limiting step in prostaglandin synthesis from arachidonic acid and plays an important role in cancer and tumor promotion (31). It has been suggested that COX-2 induction mediated by NF-κB pathway could lead to malignant cell proliferation and invasion (32). These carcinogenic effect of COX-2 can be reversed by NSAIDs, elucidating the importance of COX-2 inhibition in cancer therapy (33). It has been shown to inhibit COX-2 expression by repressing degradation of the inhibitory protein IkBα and hindering the nuclear translocation of the functionally active subunit of NF-κB, thereby blocking improper NF-κB activation (34). The western blot analysis data also revealed that treatment of cells with MHY218 markedly inhibited COX-2, MMP-9 and 5-LOX protein expression in a concentration-dependent manner. However, RT-PCR analysis data showed no change in COX-2 mRNA. These results suggest that MHY218 may also be effective against colon cancer cells through suppression of NF-κB activity and NF-κB gene products. In conclusion, MHY218 suppressed growth of HCT116 cells by causing G2/M cell cycle arrest and apoptosis. These results suggest that MHY218-induced cell cycle arrest and apoptosis are associated with inhibition of NF-κB pathway. Taken together, these results suggested that the novel compound MHY218 may be useful in the chemoprevention and/or treatment of colon cancer.

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