

MHY218-induced apoptotic cell death is enhanced by the inhibition of autophagy in AGS human gastric cancer cells

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Abstract. We previously reported the anticancer effects of MHY218, which is a hydroxamic acid derivative, in HCT116 human colon cancer cells. In the present study, the involvement of autophagy in the MHY218-induced apoptotic cell death of AGS human gastric cancer cells was investigated. MHY218 treatment induced growth inhibition and apoptotic cell death in a concentration- and time-dependent manner. The induction of apoptosis was confirmed by observations of decreased viability, DNA fragmentation, and an increase in late apoptosis and sub-G1 DNA, which were detected with a flow cytometric analysis. Western blot analyses showed that MHY218 treatment resulted in decreased protein levels of procaspase-8, -9, and -3; cleavage of poly(ADP-ribose) polymerase (PARP); and alterations in the ratio of Bax/Bcl-2 protein expression. Apoptosis induced by MHY218 was involved in the activation of caspase-8, -9, and -3, and it was blocked by the addition of Z-VAD-FMK, a pan-caspase inhibitor. In addition, autophagy-inducing effects of MHY218 were indicated by cytoplasmic vacuolation, the accumulation of acidic vesicular organelles, the appearance of green fluorescent protein-light-chain 3 (LC3) punctate dots, and increased levels of Beclin-1 and LC3-II protein expression. Pretreatment with the autophagy inhibitors LY294002, 3-methyladenine, chloroquine, and bafilomycin A1 enhanced the induction of apoptosis by MHY218, and this was accompanied by an increase in PARP cleavage. Taken together, these results provide new insights into the role of MHY218 as a potential antitumor agent. The

combination of MHY218 with an autophagy inhibitor might be a useful candidate for the chemoprevention and/or treatment of gastric cancer.

Introduction

According to GLOBOCAN 2012, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012 compared with 12.7 million and 7.6 million, respectively, in 2008. Gastric cancer (GC) is the fifth most common cancer (0.9 million, 6.8%) in the world after lung, breast, colorectal, and prostate cancers and the second leading cause of cancer death worldwide (0.7 million, 8.8%) after lung cancer. Historically, GC is one of the major cancers in Asian countries, such as Korea and Japan. Although the mortality and incidence of GC have decreased worldwide, 31,269 new GC cases were reported in 2012, accounting for 14.2% of all cancer occurrences (219,520 case/year; data available at <http://www.cancerresearchuk.org/cancer-info/cancerstats/world/incidence/>). The most effective treatment for localized GC is surgery, but, even after curative resection, recurrence is noted in more than half of the cases with advanced-stage disease. Moreover, although the effective treatments that are available have increased the survival rates of GC patients during the last three decades, the prognoses of these patients remain relatively poor, with a 5-year survival rate of 63.1% during 2004-2008 (http://www.cancer.go.kr/mbs/cancer/subview.jsp?id=cancer_040101000000). Due to the high recurrence rate after resection surgery, patients with GC are treated with adjuvant chemotherapy (including cisplatin, 5-fluorouracil, capecitabine, oxaliplatin, mitomycin C, and etoposide) and/or radiation therapy. However, the efficacies of these adjuvant therapies are limited because of the side effects, such as nausea, vomiting, the high risk of infection, and diarrhea (1). Thus, the development of novel anticancer agents for patients with GC and effective treatment strategies are urgently required to increase their efficacy.

Autophagy is a process that involves the cellular degradation of unnecessary or dysfunctional cellular components and the maintenance of cellular homeostasis. During autophagy, parts of the cytoplasm and cellular organelles are engulfed within double-membraned autophagosomes, and the autophagosomes fuse with lysosomes to form autolysosomes. This

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results in the degradation of the sequestered cargo by lysosomal hydrolytic enzymes. The degradation is followed by the generation of cellular building blocks for biosynthesis and energy production. Because autophagy reuses cellular components when dealing with cellular stress by clearing damaged proteins, organelles, or pathogens and provides the resources for biosynthesis (e.g., adenosine triphosphate or anabolic building blocks) during starvation, this process was initially recognized as a mechanism that was cytoprotective against environmental stress. Conversely, increasing amounts of recent evidence have revealed that autophagy can also culminate in cell death, which is designated as type-2 programmed cell death or autophagic cell death (2).

The essential role of autophagy in cancer is not clearly understood; however, its role in cell death is conflicting depending on the type of tumor, the stage of tumorigenesis, and the nature and extent of the stimuli (3-5). It is generally believed that these two self-destructive processes, autophagy and apoptosis, often occur in the same cell and that autophagy mostly precedes apoptosis (6,7). Furthermore, some chemotherapeutics that are known to trigger apoptosis also induce autophagy (8). In addition, emerging evidence indicates that the inhibition of autophagy appears to enhance the sensitivity of cancer cells towards anticancer drugs (9). Based on the aforementioned findings, it would be helpful to develop a new anticancer agent that simultaneously induces both autophagy and apoptosis. Thus, the elucidation of the role of autophagy and the determination of whether it is induced by agents in the response of tumor cells to that agent will provide therapeutic advantages by manipulating the autophagic process.

The anticancer effects of the synthetic hydroxamic acid derivative MHY218 [N^1 -hydroxy- N^8 -(4-phenoxyphenol)octanedianide], was first reported by Jeon and colleagues (10). It was found to cause apoptosis in SKOV-3 ovarian cancer cells. This histone deacetylase (HDAC) inhibitor exhibits growth inhibitory effects in several tumor cell lines, including ovarian, breast, and colon cancer cells (10-12). Two studies in animals have shown that MHY218 has antineoplastic activities against tamoxifen-resistant breast cancer and ovarian cancer (10,11). The anticancer mechanisms of MHY218 are still under investigation, and few studies have reported its effects on cell death. It has been shown to induce the pro-apoptotic Bax protein, inhibit the anti-apoptotic Bcl-2 protein (10-12), and suppress the nuclear translocation of nuclear factor- κ B (NF- κ B) (12). Thus, modulations of the NF- κ B-regulated genes such as cyclooxygenase-2, matrix metalloproteinase-9, 5-lipoxygenase (12), and HDACs (10,11) have been implicated in MHY218-induced apoptotic cell death. In addition, this synthetic hydroxamic acid derivative induces G2/M phase arrest in cells, and this is most likely mediated through the regulation of cyclin B1 and its activating partners Cdc25C and Cdc2, and a G2/M phase inhibitor p21^{WAF1/CIP1} (10-12). MHY218 has been shown to significantly inhibit the growth of tumors in mice bearing human ovarian cancer (10). A recent study has indicated that MHY218 can suppress tamoxifen-resistant breast cancer growth *in vivo* through the downregulation of the proliferating cell nuclear antigen (PCNA), the induction of apoptotic cell death, and the upregulation of light-chain 3 (LC3), which is a biomarker for autophagy (10,11). Notably, MHY218 possesses more potent anticancer effects compared

to those of suberoylanilide hydroxamic acid (SAHA, which is also known as vorinostat) against both ovarian and breast cancer *in vivo* animal models (10,11).

The aim of the present study was to determine whether MHY218 can induce apoptotic cell death and autophagy in AGS cells, and finally to access the inhibition of autophagy can potentiate the proapoptotic effect of MHY218 in AGS cells.

Materials and methods

Chemicals. The structure of MHY218 [N^1 -hydroxy- N^8 -(4-phenoxyphenol)octanedianide] that was used in this study is shown in Fig. 1. This compound was kindly provided by Professor Hyung Ryong Moon (Pusan National University, Busan, Korea), dissolved in dimethyl sulfoxide (DMSO), and stored at -20°C before the experiments, and dilutions were made in culture medium. The maximum concentration of DMSO did not exceed 0.1% (v/v) in the treatment range in which there was no influence on cell growth. The DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from AMRESCO LLC (Solon, OH, USA). Antibodies that were specific for procaspase-3, -8, and -9, poly(ADP-ribose) polymerase (PARP), Bax, Bcl-2, Beclin-1, and Z-VAD-FMK were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The polyclonal antibody against LC3B was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Propidium iodide (PI), acridine orange, 3-methyladenine, chloroquine (CQ), bafilomycin A1 (Baf-A1), cycloheximide (CHX), and a monoclonal antibody against β -actin were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

Cell culture and cell viability assay. The human GC AGS cell line was cultured in RPMI-1640 (GE Healthcare Life Sciences, Logan, UT, USA) that was supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences), 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin (GE Healthcare Life Sciences) at 37°C in a humidified 5% CO_2 . Cell viability was determined with an MTT assay. For the MTT assay, AGS cells were seeded in a 24-well culture plate, cultured for 24 or 48 h in the growth media, and then treated with or without various reagents for the indicated concentrations. The cells were incubated in the dark with 0.5 mg/ml MTT at 37°C for 2 h. The formazan granules that were generated by the live cells were dissolved in DMSO, and the absorbance at 540 nm was monitored with a multiwell reader (Thermo Fisher Scientific Inc., Vantaa, Finland).

Nuclear staining with Hoechst 33342. Cells were stained with 4 $\mu\text{g}/\text{ml}$ of Hoechst 33342 (Life Technologies Corp., Grand Island, NY, USA) at 37°C for 10 min. The cells were then examined under a fluorescence microscope.

Measurement of apoptotic cell death by flow cytometry. Apoptotic cell death was determined with Annexin V/PI staining and sub-G1 phase analyses. For the Annexin V/PI staining, the cells were treated with the appropriate conditions for 24 h, subsequently harvested, trypsinized, washed once with cold phosphate-buffered saline (PBS), and then suspended in 1X binding buffer (BD Biosciences, San Jose, CA, USA). The cells were stained in PI and Annexin V-fluorescein isothio-

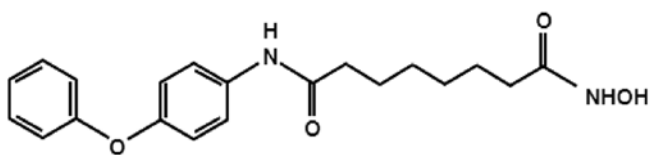


Figure 1. Chemical structure of MHY218 [N1-hydroxy-N8-(4-phenoxyphenol) octanedianide].

cyanate (FITC) solution (BD Pharmingen FITC Annexin V Apoptosis Detection kit) at room temperature for 15 min in the dark. The stained cells were analyzed by flow cytometry within 1 h. For the sub-G1 phase analysis, the cells were trypsinized, washed once with cold PBS, and then fixed in 70% ethanol at -20°C overnight. The fixed cells were stained with cold PI solution (50 µg/ml in PBS) at 37°C for 30 min in the dark. The flow cytometry analysis was performed on Accuri C6 (BD Biosciences).

DNA fragmentation assay. The cells were lysed in buffer containing 5 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. The lysates were vortexed and cleared by centrifugation at 27,000 x g for 20 min. Fragmented DNA in the supernatant was treated with RNase, which was followed by proteinase K digestion, phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v/v) extraction, and isopropanol precipitation. The DNA was separated in a 1.6% agarose gel, stained with 0.1 µg/ml ethidium bromide, and visualized with an ultraviolet source.

Western blot analysis. The total cells were lysed in lysis buffer [25 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 100 µg/ml phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich Co. LLC)]. Equal amounts of the protein extracts were denatured by boiling at 100°C for 5 min in sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The total proteins were subjected to 6-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were probed with the desired primary antibodies overnight, incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), and then visualized with the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Caspase activity. The cells were harvested and washed with cold PBS. The total cells were incubated with the lysis buffer (R&D Systems, Inc., Minneapolis, MN, USA) on ice for 10 min. The lysed cells were centrifuged at 10,000 x g for 1 min, and 100 µg of protein was incubated with 2X reaction buffer and substrates of colorimetric tetrapeptides, including Z-DEVD for caspase-3, Z-IETD for caspase-8, and Ac-LEHD for caspase-9, respectively. The reaction mixture was incubated at 37°C for 2 h, and the enzyme-catalyzed release of p-nitroaniline was quantified at 405 nm with a multiwell reader (Thermo Fisher Scientific Inc.).

Detection of acidic vesicular organelles (AVOs). The formation of AVOs is a well-known feature of autophagy. Cells were

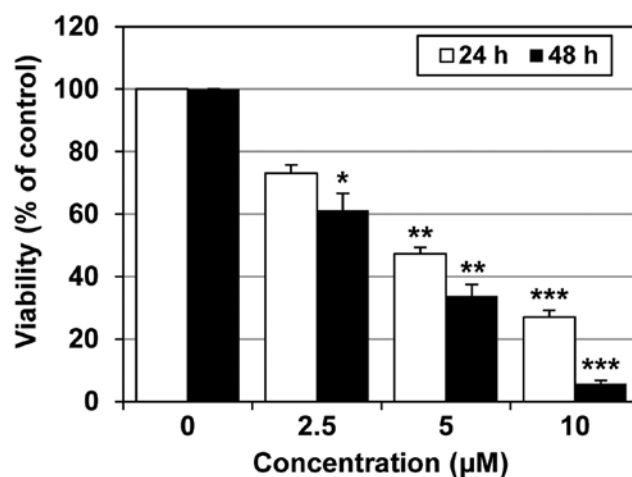


Figure 2. Effects of MHY218 on the viability of AGS cells. The cells were treated with the indicated concentrations of MHY218 for 24 h. Proliferation was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results are expressed as mean \pm standard deviation (SD; n=3) and as a percentage of the vehicle-treated control. The significance was determined by Student's t-tests and ANOVA. *p<0.05, **p<0.01 and ***p<0.001 compared with the vehicle-treated control cells.

treated under the appropriate conditions for 24 h, stained with acridine orange (1 µg/ml) for 15 min, trypsinized, and then washed with PBS. The stained cells were then analyzed with an Accuri C6 flow cytometer (BD Biosciences).

GFP-LC3 assay. Cells were transfected with the LC3-GFP plasmid with the Lipofectamine 2000 reagent (Life Technologies Corp.) according to the manufacturer's protocol. The cells were seeded in Lab-Tek II chamber slides (Thermo Fisher Scientific Inc.). After transfection for 24 h, the cells were treated with 5 µM of MHY218 for 24 h, washed with PBS twice, and then fixed with 4% paraformaldehyde for 20 min. The formation of punctate LC3-positive structures was examined with confocal microscopy. Confocal images were obtained with a FV10i FluoView Confocal Microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis. The results are expressed as the mean \pm standard deviation of three separate experiments, and they were analyzed by Student's t-tests and ANOVA. The means were considered significantly different at *p<0.05, **p<0.01 or ***p<0.001.

Results

MHY218 exhibited a potent cytotoxic effect on AGS cells. In order to determine whether MHY218 inhibited the growth and proliferation of AGS cells *in vitro*, we treated the cells for 24 or 48 h with increasing concentrations of MHY218 that ranged from 0 to 10 µM. An MTT assay was conducted to measure the MHY218-mediated growth inhibition and estimate the concentration of the compound at which cell growth was cut in half (IC₅₀). As shown in Fig. 2, MHY218 effectively suppressed the cell growth of AGS cells in a concentration-dependent manner. Moreover, the IC₅₀ value of MHY218 was ~5 and 3 µM at 24 and 48 h, respectively. The results presented

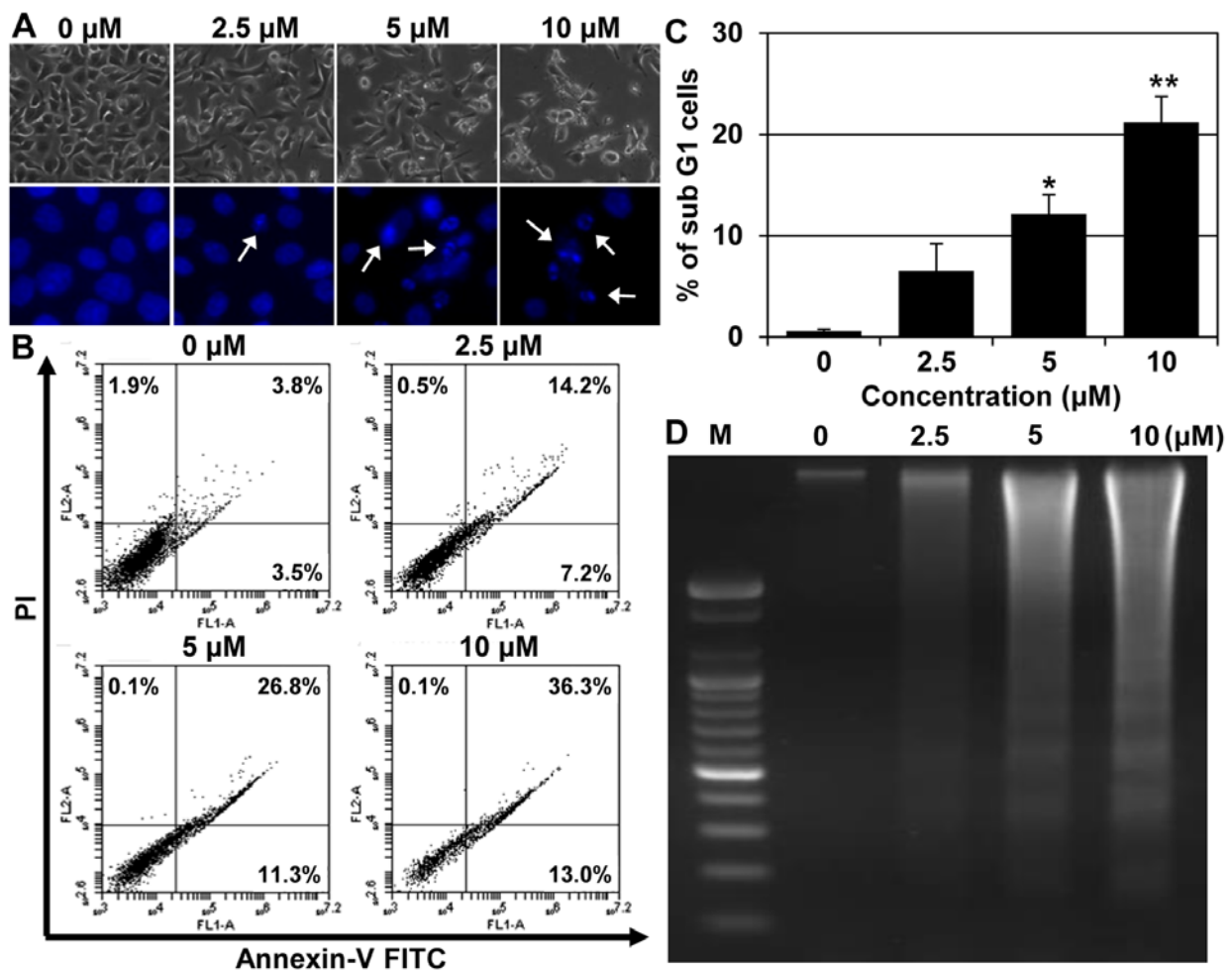


Figure 3. Induction of apoptosis in AGS cells by MHY218. (A) Morphological changes were observed with phase contrast microscopy. The nuclei of AGS cells were stained with the fluorescent DNA-binding dye, Hoechst 33342, and they were then photographed with a fluorescent microscope with a blue filter at a magnification of $\times 400$. Arrows, apoptotic cells. (B) Annexin V-fluorescein isothiocyanate (FITC) binding and propidium iodide (PI) uptake in non-permeabilized cells were analyzed by flow cytometry. (C) The presence of cells with sub-G1 DNA content following treatment with MHY218 was evaluated with flow cytometry in order to quantify the onset of apoptosis. (D) DNA was extracted and analyzed by 1.6% agarose gel electrophoresis in the presence of ethidium bromide (EtBr). A representative result is shown from three independent experiments. M, marker.

in Fig. 2 indicate that MHY218 suppressed the growth of AGS cells in a concentration- and time-dependent manner.

MHY218 triggers apoptotic cell death in AGS cells. In order to address the question of whether the growth-suppressive effects of MHY218 were associated with apoptotic cell death, the morphological changes of the cellular structures were assessed with Hoechst 33342 staining. Fig. 3A shows the morphological and nuclear changes in AGS cells after 24 h of MHY218 treatment. MHY218 treatment caused nuclear condensation and cell death, whereas untreated control cells displayed intact nuclear structures (Fig. 3A). The number of cells with highly condensed nuclei (representing programmed cell death) significantly increased in cells treated with 10 μ M of MHY218 (Fig. 3A). MHY218-induced apoptotic cell death in AGS cells was further verified with Annexin V and PI double staining. As shown in Fig. 3B, there was a prominent increase in the percentage of late apoptotic cells (36.3%, with higher Annexin V and PI-positive signals) in the cells after they were treated with 10 μ M of MHY218 for 24 h compared to the untreated controls (0 μ M, 3.8%). Cells with higher

Annexin V but lower PI signals were indicative of early apoptotic cell death. The percentages of total apoptotic cells (early and late apoptotic) significantly increased in AGS cells in a concentration-dependent manner (Fig. 3B). In addition, we examined MHY218-induced cell death in AGS cells with a cell cycle analysis. As shown in Fig. 3C, a cell cycle assay showed that MHY218 significantly increased AGS cells in the sub-G1 population in a concentration-dependent manner (Fig. 3C).

Finally, we examined the effects of MHY218 on DNA fragmentation. AGS cells were treated with various concentrations of MHY218, and inter-nucleosomal DNA fragmentation was evaluated with a DNA ladder on agarose gel electrophoresis. The genomic DNA of the cells treated with MHY218 for 24 h displayed the characteristic ladder pattern of discontinuous DNA fragments, while untreated cells did not show any signs of fragmentation, as shown in Fig. 3D.

MHY218 modulates the expression of caspases, Bax, and Bcl-2 in AGS cells. The molecular events underlying the MHY218-induced apoptosis in AGS cells were investigated.

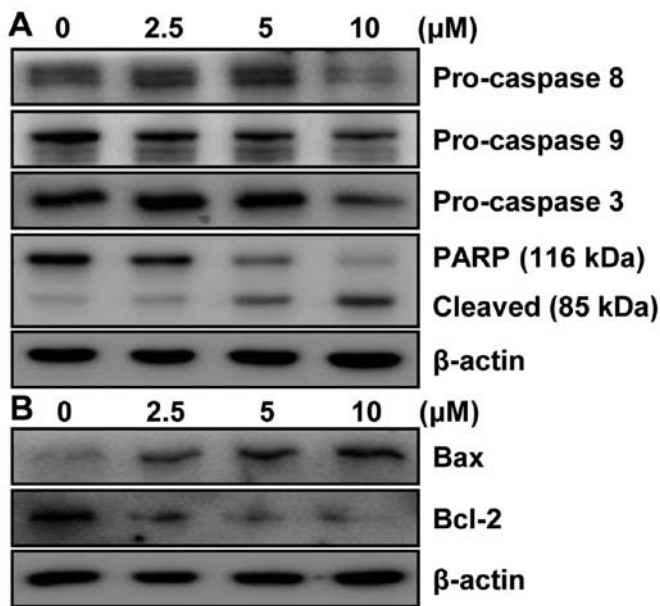


Figure 4. Effects of MHY218 on apoptosis-related protein expression in AGS cells. The cells were treated with variable concentrations of MHY218 for 24 h. (A) Membranes were probed with pro-caspase-8, -9, and -3 and poly(ADP-ribose) polymerase (PARP). (B) The membranes were detected with antibodies against Bax and Bcl-2, and the proteins were visualized with the ECL detection system. Representative results from three independent experiments are shown. Actin was used as a loading control.

The activations of several caspases are important in apoptosis that is induced by various apoptotic stimuli (13). In order to elucidate the mechanisms of MHY218-induced apoptotic cell death in AGS cells, caspase involvement was investigated with a western blot analysis. As shown in Fig. 4A, the exposure of AGS cells to MHY218 markedly decreased the protein levels of pro-caspase-8, -9 and -3 in a concentration-dependent manner. Thus, after treatment with MHY218, the full-length form of the PARP protein (116 kDa), which is a selective substrate for caspase-3, was degraded to the cleaved form (85 kDa) (Fig. 4A).

We next examined the expression of apoptosis-associated proteins after treatment with MHY218. With the balance of anti- and pro-apoptotic proteins arbitrating life-or-death decisions, the Bcl-2 family proteins may regulate mitochondria-dependent apoptosis (14). The expression of pro-apoptotic Bax was increased, while anti-apoptotic Bcl-2 expression was reduced in MHY218-treated cells in a concentration-dependent manner (Fig. 4B). These results suggested that MHY218 promoted cell death in AGS cells through apoptosis with both the intrinsic and extrinsic pathways.

MHY218 induces apoptosis through caspase activation. Because the activation of caspases plays a critical role in apoptosis (15), we examined the activation of caspases by MHY218 in AGS cells. Therefore, the activities of caspases in AGS cells that were treated with various concentration of MHY218 were investigated. After 24 h of treatment with MHY218, the activities of caspase-8, -9, and -3 were increased in a concentration-dependent manner (Fig. 5A). MHY218 markedly stimulated caspase-3 to a >2-fold increase in activity

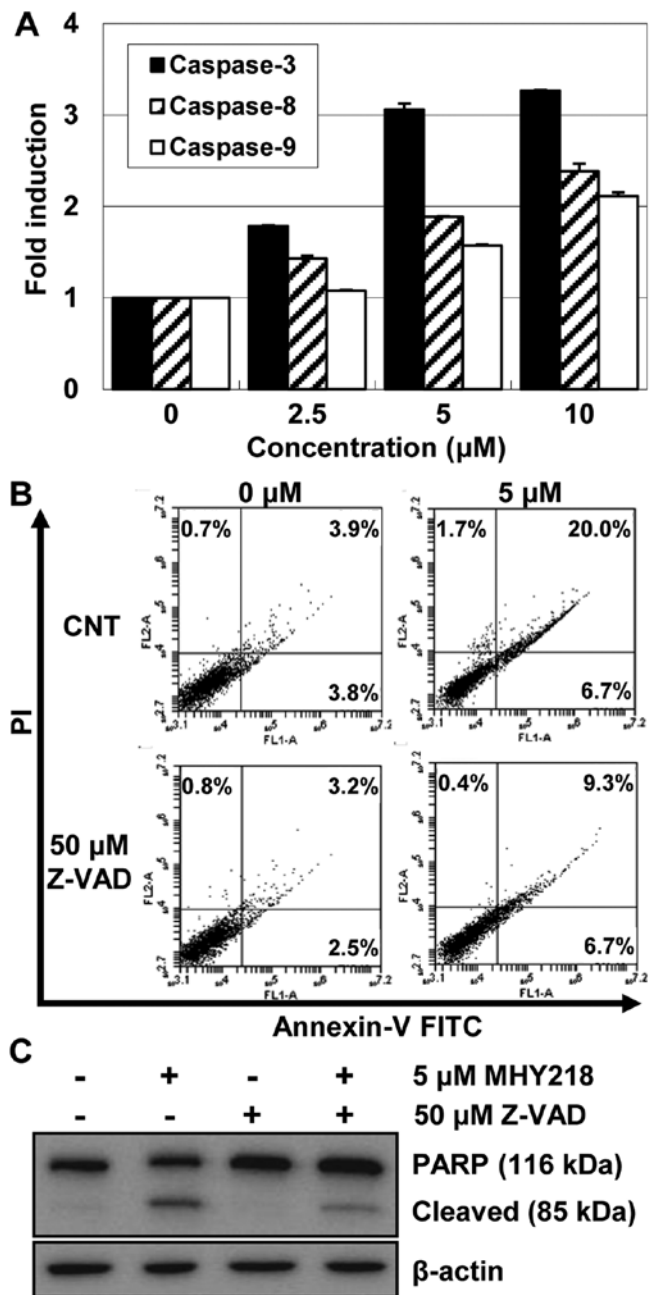


Figure 5. Effects of MHY218 on caspase activation in AGS cells. (A) Cell lysates from cells that were treated with the indicated concentrations of MHY218 for 24 h were assayed for *in vitro* caspase-3, -8, and -9 activity with the substrates of Z-DEVD-pNA, Z-IETD-pNA, and Ac-LEHD-pNA, respectively, at 37°C for 2 h. The fluorescent products that were released were measured. The data represent the mean \pm SD values of duplicate samples. The results are representative of one of three independent experiments. (B) The cells were pretreated with 50 μM of Z-VAD-FMK for 1 h, and they were then treated with 5 μM of MHY218 for 24 h. The cells were stained with Annexin V-FITC/PI and analyzed with flow cytometry. (C) Total cell lysates were prepared and immunoblotted for PARP. The representative results from three independent experiments are shown. Actin was used as a loading control.

in a concentration-dependent manner, while it produced less of an effect on the caspase-8 and -9 activities.

In order to demonstrate the contributions of caspases to MHY218-induced apoptosis, we next examined the effects of the caspase inhibitor Z-VAD-FMK on apoptosis. As shown in Fig. 5B, Z-VAD-FMK, which is a broad-spectrum caspase

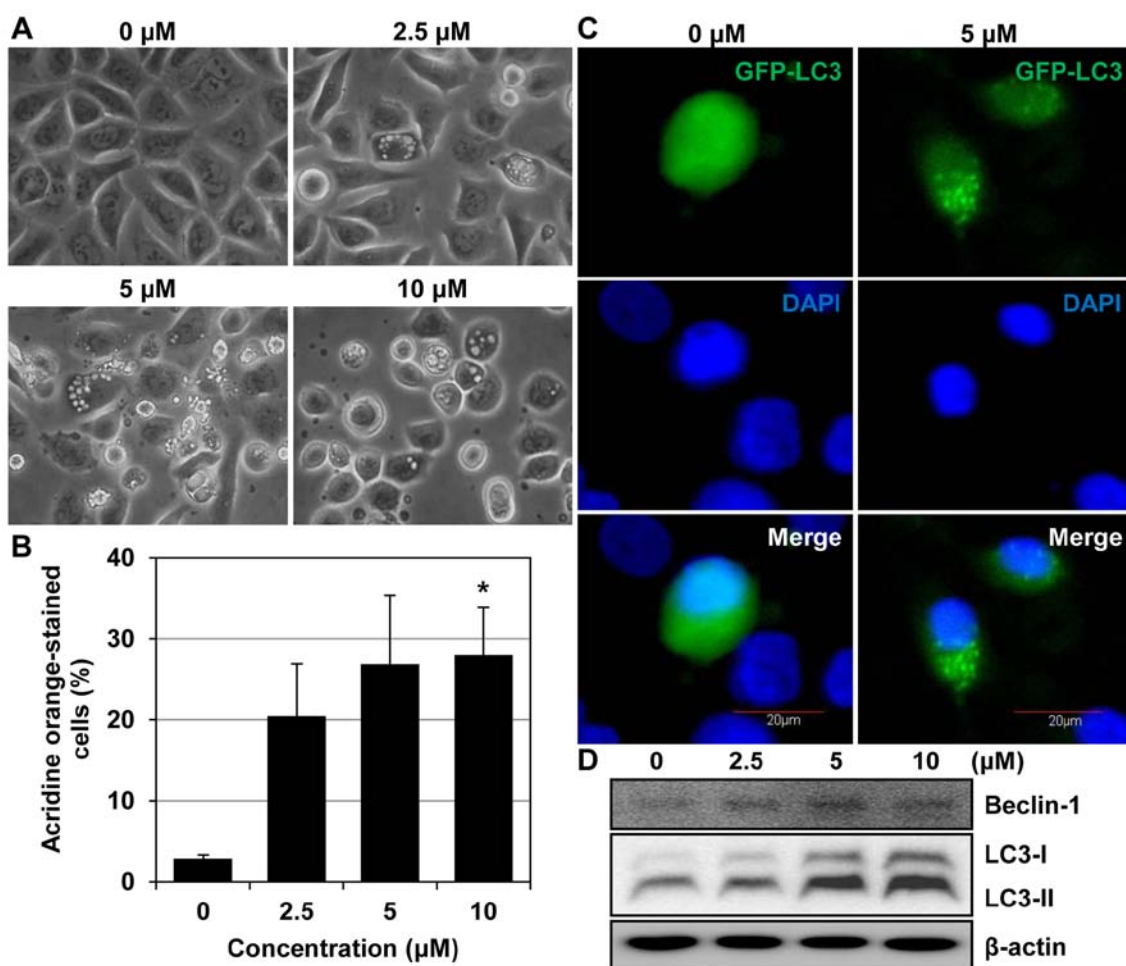


Figure 6. Induction of autophagy in AGS cells by MHY218. (A) AGS cells were treated with different concentration of MHY218 for 24 h. Representative images were obtained by phase contrast microscopy at a magnification of $\times 400$. (B) Cells were treated with MHY218 for 24 h, stained with acridine orange, and then analyzed by flow cytometry to quantify acidic vesicular organelle (AVO) formation. The results are expressed as mean \pm SD ($n=3$). Significance was determined by Student's *t*-tests ($p<0.05$ vs. vehicle-treated control cells). (C) AGS cells were transfected with the light-chain 3 (LC3)-green fluorescent protein (GFP) plasmid for 24 h, and the medium was then replaced with media containing $5 \mu\text{M}$ of MHY218 and incubated for another 24 h. The GFP-LC3 puncta formation was observed with confocal microscopy. The representative results from three independent experiments are shown. Bar, $20 \mu\text{m}$. (D) The cells were lysed, and the levels of Beclin-1 and LC3 proteins were analyzed by western blot analysis.

inhibitor, markedly decreased the proportion of apoptotic cells from 26.7 to 16%, which was the percentage observed in untreated cells. In addition, the effect of the caspase inhibitor on the MHY218-induced cleavage of PARP was examined with a western blot analysis. As shown in Fig. 5C, pretreatment with Z-VAD-FMK inhibited the cleavage of PARP compared to the cells that were treated with MHY218 alone, but not completely. It is interesting to note that MHY218-induced apoptosis was only partially suppressed by Z-VAD-FMK. Taken together, these results suggested that the apoptogenic effect of MHY218 in AGC cells, at least in part, was mediated by activating the caspase cascade.

MHY218 induces autophagy in AGS cells. In recent years, many modes of cell death other than apoptosis have been found to exist, and these include autophagy, necroptosis, and PARP1-mediated cell death (16). In the present study, we examined whether MHY218 also induced autophagy in AGS cells. Cells were treated with the indicated concentrations of MHY218 for 24 h, and the formation of autophagic vacuoles was examined with phase contrast microscopy. As shown

in Fig. 6A, MHY218 induced the formation of autophagic vacuoles in AGS cells in a concentration-dependent manner.

We further conducted a series of experiments to confirm the effects of MHY218 on the autophagy process. Because the formation of cytosolic AVOs is one of the typical features of autophagic cell death, a flow cytometry analysis was performed after staining the cells with acridine orange for the quantification of the AVOs. The number of AVOs in MHY218-treated cells clearly increased in a concentration-dependent manner (Fig. 6B).

Beclin-1 levels and the conversion of microtubule-associated protein 1 LC3 are selective autophagic markers. Next, in order to further confirm that autophagy was induced by MHY218, we examined the LC3 distribution in MHY218-treated AGS cells with fluorescence microscopy. As shown in Fig. 6C, green fluorescent protein (GFP)-tagged-LC3 (GFP-LC3) formed cytoplasmic puncta in cells that were treated with MHY218 but not in untreated control cells. In addition, a western blot analysis showed that LC3 underwent a conversion from LC3-I (the soluble form) to LC3-II (the lipidized form) in MHY218-treated cells, thus indicating the

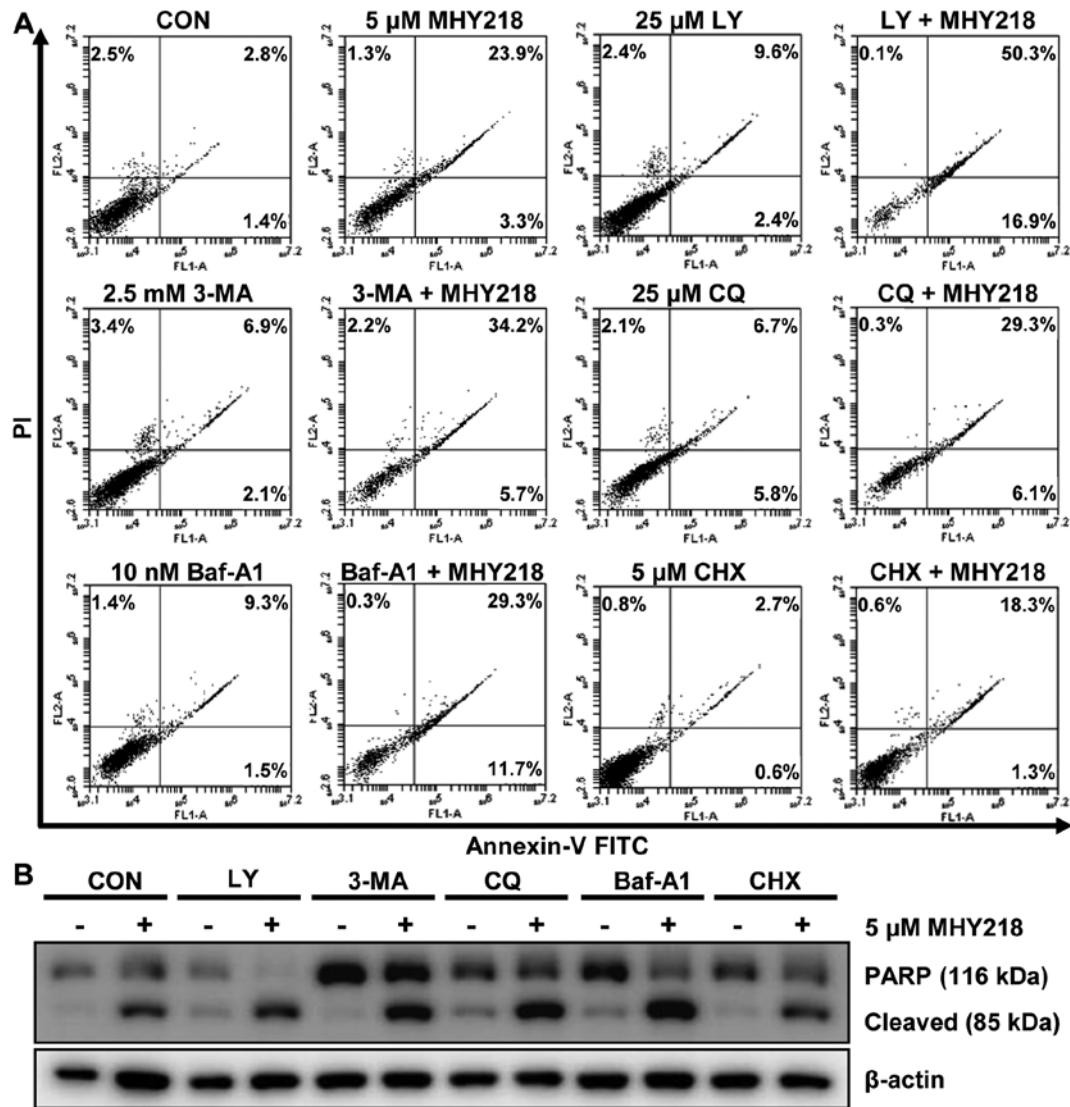


Figure 7. Role of autophagy inhibitors on MHY218-induced apoptosis in AGS cells. (A) AGS cells were pretreated with vehicle, 25 μ M of LY294002, 2.5 mM of 3-methyladenine (3-MA), 25 μ M of chloroquine (CQ), 10 nM of bafilomycin A1 (Baf-A1), and 5 μ M of cycloheximide (CHX) for 1 h, and they were then treated with 5 μ M of MHY218 for 24 h. Annexin V-FITC binding and PI uptake in non-permeabilized cells were analyzed by flow cytometry. (B) Total proteins were prepared and immunoblotted for PARP. The representative results from three independent experiments are shown. Actin was used as a loading control.

induction of autophagy (Fig. 6D). Consistent with the findings of LC3 conversion, the upregulation of Beclin-1 by MHY218 was observed in AGS cells (Fig. 6D).

The suppression of autophagy modulates MHY218-induced apoptotic cell death in AGS cells. Accumulating data have suggested that the inhibition of autophagy may enhance chemosensitization in human cancer cells (17). Thus, we investigated the role of autophagy in MHY218-induced cell death by examining the effects of pharmacological autophagy inhibitors. Thus, we first treated cells with various autophagy inhibitors for 1 h, incubated them with MHY218 for another 24 h, and then performed Annexin V/PI staining to evaluate cell death. Apoptotic cell death was induced at a level of 27.2% by MHY218, at a level of 12% by LY294002, which is an autophagy inhibitor that acts by blocking the class-III phosphoinositide 3-kinases (PI3Ks) that are critical during the late stage of vesicle expansion (autophagosome formation), and at a level of 67.2% by the combination of the two agents

(Fig. 7A). However, cells that were treated with 3-methyladenine (3-MA), which is an autophagy inhibitor that acts by blocking the class-III PI3Ks, which was followed by the addition of MHY218, exhibited an additive effect on the induction of apoptosis. Similar results were obtained with the use of two autophagy flux blockers, CQ and Baf-A1, on MHY218-induced apoptosis. CQ, which is a lysosomotropic agent, prevents lysosome acidification and thereby the degradation of the products of autophagy, which results in autophagolysosome accumulation, whereas Baf-A1, which is a vacuolar-type H⁺-ATP inhibitor, prevents the maturation of autophagic vacuoles by inhibiting the fusion of autophagosomes and lysosomes. We also employed CHX, which is an inhibitor of protein biosynthesis and which may inhibit autophagosome formation, to evaluate the effects of MHY218-induced cell death. As shown in Fig. 7A, CHX failed to potentiate the apoptosis-inducing effects of MHY218 in AGS cells, indicating that the effects of the autophagy inhibitor CHX with MHY218 on cell death seemed to be additive.

To verify the observation that the inhibition of autophagy affected the apoptotic cell death that was induced by MHY218, we performed a western blot analysis to measure PARP cleavage, which is an executioner of apoptosis. MHY218 induced PARP cleavage compared to untreated cells (Fig. 7B). In accordance with the apoptotic cell data (Fig. 7A), cells cotreated with MHY218 and 3-MA, CQ, Baf-A1, or CHX exhibited a measurable increase in PARP cleavage; whereas the combination of the autophagy inhibitor LY294002 and MHY218 resulted in the most profound amounts of PARP cleavage of all of the tested autophagy inhibitors (Fig. 7B). Altogether, these results indicated that the inhibition of autophagy by autophagy inhibitors, such as LY294002, enhanced the MHY218-induced cell death in AGS cells. However, the potency of autophagy inhibition on apoptotic cell death may vary depending on the selectivity of the autophagy inhibitors.

Discussion

With the development of new therapeutics and the advances in diagnostic technology, the incidence of GC has decreased during the past three decades. However, GC is still the fifth most lethal neoplasm on a worldwide basis, and, thus, Korea continues to have one of the highest rates of GC (18). While surgery is the most common therapy used for patients with stomach cancer, advanced GC (AGC) needs to be treated with multimodal treatments, including chemotherapy and radiotherapy. Thus, the modest efficacies in GC and considerable toxicities that are associated with the current chemotherapy modalities have prompted the pursuit of novel treatment strategies. In this study, we investigated the mechanisms of MHY218, which is a hydroxamic acid derivative, in the induction of autophagy and the role of autophagy in tumor cell survival in the presence of MHY218. Our results indicated that MHY218 triggered both apoptosis and autophagy in AGS cells. Furthermore, we found that autophagy inhibition resulted in higher levels of apoptotic cell death in response to MHY218 treatment. Collectively, our findings suggest that targeting autophagy during cancer treatment with MHY218 may augment the therapeutic effects.

One of the common features of human tumors is the deregulation of HDAC isoenzymes, but no conclusive data about the patterns of HDAC expression in human cancers are available (19-21). Because studies of GC tissues have shown an increase in class-I HDAC expression, HDAC is becoming a prominent therapeutic target for GC treatment (22-24). HDAC inhibitors (HDACi) have been recognized as a new class of therapeutic agents with promising outcomes during the treatment of a broad range of cancer types (25,26). Therefore, HDACi have so far been approved by the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma, and preclinical and clinical trials have provided evidence that HDACi can be used in the treatment of solid tumors in combination with other anticancer drugs or radiation (26-28). MHY218, which is a synthetic hydroxamic acid, was primarily designed to target HDAC, and it has been shown to suppress HDAC activity and HDAC expression in cancer cells from two solid tumors (10,11). These HDAC expression profiles and previous reports on MHY218 provide the rationale for examining the anticancer potential of MHY218 in GC cells.

Mechanistically, HDACi have been reported to promote apoptosis through alterations in the expression of genes that are involved in apoptotic cell death and activation of the caspase cascade (29,30). We found that MHY218 inhibited cell growth and induced apoptosis in AGS cells. The observed growth-inhibitory and apoptosis-inducing properties of MHY218 were in agreement with those observed by others in ovarian, breast, and colon cancer cells (10-12). When we examined the mechanism by which MHY218 mediated apoptotic cell death in AGS cells, we found that MHY218 activated caspases in AGS cells. In agreement with these observations, previous studies have shown that MHY218 induces caspase activation (10,12). Our observations further revealed that caspase activation in response to MHY218 may be involved in part of the MHY218-induced apoptosis. It is estimated that about half of the induced apoptosis can be inhibited by the pan-caspase inhibitor Z-VAD-FMK, and this is therefore mediated by caspases, whereas the other half is executed in a caspase-independent manner. Likewise, caspase-dependent and -independent apoptosis that is induced by SAHA, which is a widely used HDACi, has also been reported in several cell lines (31-34). Overall, these observations suggest that some other mechanism is likely to be involved in MHY218-induced apoptosis.

We found that MHY218 mediated anticancer activity by modulating the expression of genes that are involved in apoptosis. This hydroxamic acid-derivative treatment resulted in the upregulation of Bax and the downregulation of Bcl-2 expression levels, which was in agreement with the findings of previous reports (10-12). Other HDACi, such as SAHA (35,36), trichostatin A (37), and valproic acid (38), have also been reported to exert apoptosis-inducing effects through the induction of Bax as well as the inhibition of Bcl-2 expression in cancer cells.

As with many other anticancer therapies, HDACi induces autophagy, and this HDACi-mediated autophagy appears to act as a cytoprotective mechanism to counteract the cytotoxic activities of HDACi (33,39-41). We observed the upregulation of Beclin-1 and an increase in LC3-II conversion in MHY218-treated cells. These observations were supported by a previous report that showed that MHY218 induced autophagy in tamoxifen-resistant MCF-7 cells (11). However, Park *et al* (11) did not rule out the relationship between apoptosis and autophagy that were induced by MHY218 and that cooperate or counteract each other.

Although MHY218 enhanced autophagy, we found that the inhibition of autophagolysosome formation was ineffective on the synergistic induction of apoptosis by MHY218. The inhibition of MHY218-stimulated autophagy by CQ turned out to be ineffective on the synergistic induction of apoptosis by MHY218-CQ cotreatment. This conclusion was further supported by our data that showed that the lysosomal H⁺-ATPase inhibitor Baf-A1, which similarly interrupted autophagolysosome formation, failed to produce a synergistic effect on MHY218-inducing apoptosis (Fig. 7A). These results indicated that the disruption of autophagolysosome formation *per se* was not effective on the sensitization of cells for MHY218-mediated apoptosis.

A group of PI3K inhibitors, including 3-MA, wortmannin, and LY294002, have been well established and extensively used

as autophagy inhibitors, while the inhibition of the PI3K-Akt pathway has previously been reported in certain cancers to trigger autophagy as a survival mechanism (42-44). The effects of PI3K inhibitors as autophagy inhibitors on MHY218-induced apoptosis are intriguing. The combination of MHY218 and the autophagy inhibitor 3-MA enhanced cell death in an additive manner. LY294002, in comparison, dramatically promoted MHY218-induced apoptosis. The cells that were treated with MHY218 exhibited different apoptotic percentages in response to PI3K-targeting autophagy inhibitors. This discrepancy may be explained by the different properties of 3-MA compared to other PI3K inhibitors. A recent report by Wu and colleagues showed a dual role of 3-MA in modulating autophagy (45). They found that, surprisingly, 3-MA promoted autophagy under full (nutrient rich) medium for a prolonged period (≤ 9 h), whereas it was still capable of inhibiting starvation-induced autophagy. However, the PI3K inhibitor wortmannin was able to suppress autophagy regardless of the nutrient status. An increase in LC3-II levels was observed after 24 h of treatment with 3-MA (data not shown), even though Wu *et al* (45) did not determine the LC3 levels with the same experimental period, the possibility that 3-MA activated autophagy as a survival mechanism rather than suppressed MHY218-induced cytoprotective autophagy was proposed. In addition, the effectiveness of the PI3K inhibitors (i.e., 3-MA, wortmannin, and LY294002) was varied in order to induce the autophagic marker LC3-II in different cell types (46), thus further supporting the discrepancy in the results with 3-MA and LY294002 in our present study. Future studies are required to investigate the molecular basis and therapeutic significance of autophagy in the MHY218-derived anticancer effects.

In patients with advanced GC, the efficacy of systemic chemotherapy is still limited, and the toxicity of combination chemotherapy is substantial. Hydroxamate-based HDACi, such as vorinostat (SAHA), belinostat (PXD101), and panobinostat (LBH-589), have been shown to exhibit a good toxicity profile, and they are now under phase-I and -II clinical trials in solid tumors with promising results in selected neoplasms, such as hepatocarcinoma (27). A recent phase-I trial of the safety, tolerability, pharmacokinetic, and pharmacodynamic characteristics of hydroxychloroquine (HCQ) in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors established that 600 mg of HCQ and 400 mg of vorinostat was the maximum tolerated dose and recommended phase-II regimen (47). It is worth noting that the anticancer potency of MHY218 (10 mg/kg, twice a week for 21 days) is greater than that of SAHA (25 mg/kg, twice a week for 21 days) in tumor-bearing nude mice (10,11). These findings and the promising results from clinical trials encourage further consideration of MHY's ability to be used as a therapeutic target in gastric cancer. However, evaluations in non-human primates of the pharmacokinetics, pharmacodynamics, safety, and efficacy and studies that use clinically relevant animal models to explore more action mechanisms are needed to fully realize the potential of this fascinating molecule in the treatment of gastric cancer.

Altogether, our data suggested that the novel synthetic hydroxamate MHY218 possessed anticancer activity by triggering and autophagy induction in GC cells. The results from the present study provide evidence of MHY218-induced

autophagy, and autophagy inhibition by LY294002 dramatically augmented apoptotic cell death that was induced by MHY218. Our findings suggest that MHY218-induced autophagy acts as a prosurvival mechanism against MHY218-induced apoptosis.

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