A New Synthetic Histone Deacetylase Inhibitor, MHY2256, Induces Apoptosis and Autophagy Cell Death in Endometrial Cancer Cells via p53 Acetylation

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Abstract: We previously found a novel sirtuin (SIRT) inhibitor MHY2256 that exerts anticancer activity through p53 acetylation in MCF-7 human breast cancer cells. Here, we investigated the anticancer activity of MHY2256 against hormone-related cancer, which is an endometrial cancer with poor prognosis. We found that MHY2256 markedly reduced cellular proliferation at low concentrations against Ishikawa endometrial cancer cells. The IC$_{50}$ values of MHY2256 were much lower than that of salermide. Furthermore, MHY2256 significantly reduced the protein expression and activities of SIRT1, 2, and 3 with similar effects as salermide, a well-known SIRT inhibitor. Particularly, MHY2256 markedly inhibited tumor growth in a tumor xenograft mouse model of Ishikawa cancer cells. During the experimental period, there was no significant change in the body weight of mice treated with MHY2256. Detailed analysis of the sensitization mechanisms of Ishikawa cells revealed that late apoptosis was largely increased by MHY2256. Additionally, MHY2256 increased G1 arrest and reduced cell cyclic-related proteins, suggesting that apoptosis by MHY2256 was achieved by cellular arrest. Particularly, p21 was greatly increased by MHY2256, suggesting that cell cycle arrest by p21 is a major factor in MHY2256 sensitization in Ishikawa cells. We also detected a significant increase in acetylated p53, a target protein of SIRT1, in Ishikawa cells after MHY2256 treatment. In a mouse xenograft model, MHY2256 significantly reduced tumor growth and weight without apparent side effects. These results suggest that MHY2256 exerts its anticancer activity through p53 acetylation in endometrial cancer and can be used for targeting hormone-related cancers.

Keywords: histone deacetylase inhibitor, MHY2256, p53, apoptosis, autophagy, Ishikawa, endometrial cancer
1. Introduction

Endometrial cancer is one of the most common gynecological malignancies. Endometrial cancer affects approximately 142,000 women and causes an estimated 42,000 deaths worldwide [1,2]. Although the standard treatment for early-stage endometrial cancer is surgery, more advanced stages may require multimodality treatment [3,4]. Therefore, new targets therapy or therapeutic options are needed to decrease the morbidity and mortality observed for advanced stage endometrial cancer [5,6]. Recently, endometrial cancer has been extensively studied at the molecular level to develop effective therapies using histone deacetylase (HDAC) inhibitors, which have shown potential as therapeutic agents for endometrial cancer [7,8]. HDACs play an important role in regulating epigenetic processes that lead to expression of target genes in the development of multiple cancers [9,10].

Numerous HDAC inhibitors have been evaluated in clinical studies for the treatment of breast, cervical, or ovarian cancers, which are commonly correlated with hormone-dependent cancers in women. The differential activities of HDAC are associated with certain types of human cancer affecting signal transduction pathways and expression of tumor suppressor genes such as p53 and p21WAF [11-13]. Particularly, more specific targeting inhibitors for HDAC focused personalized medicine for treating cancers affecting women [14,15]. A previous study demonstrated that HDAC1, 2, 4, 6, or 7 series showed strong immunoreactivity in undifferentiated endometrial sarcoma and can be considered as potential therapeutic targets [7]. Li et al. [11] found that a new HDAC inhibitor, FK228, significantly inhibited endometrial cancer cell proliferation and significantly induced apoptosis and cell cycle arrest at G0/G1 phase in endometrial cancer cells. Moreover, FK228 treatment significantly increased the mRNA and protein expression of p53, p21, cleaved caspases such as 3, 7, and 8, and PARP. In our previous study, a novel synthesized class III HDAC inhibitor, MHY2256 (Fig. 1A), reduced
breast and ovarian cancer cell proliferation and induced apoptosis [16]. However, the exact role of class III HDAC (SIRT) in p53 activation in endometrial cancer remains unclear.

In the present study, we synthesized the novel SIRT inhibitor MHY2256 and investigated its anticancer activity against human endometrial cancer cells. Additionally, the anticancer potency of MHY2256 was compared to that of salermide, a selective SIRT inhibitor. To determine the anticancer activity of MHY2256 by SIRT inhibition, cell viability, cell cycle regulation, and apoptosis- and autophagy-related molecule levels were measured.

2. Results

2.1. MHY2256 highly sensitizes proliferation of Ishikawa endometrial cancer cells

The chemical structure of MHY2256 is shown in Fig. 1A. Previously, we found that MHY2256 inhibited breast and ovarian cancer cell proliferation [16]. In the study, we tested whether MHY2256 also sensitizes endometrial cancer cells, another type of hormone-related cancer. We used the Ishikawa cancer cell line, which are well-established endometrial cancer cells. As shown in Fig. 1B, MHY2256 significantly reduced the viability of Ishikawa cells in a concentration-dependent manner. We compared cytotoxicity using salermide, a well-known SIRT inhibitor. Our data indicated that the IC$_{50}$ value of MHY2256 against Ishikawa cells was 5.6 μM, which is much lower than that of salermide by approximately 10-fold. The result suggests that the novel SIRT inhibitor MHY2256 is highly cytotoxic towards endometrial cancer cells.

2.2. MHY2256 reduces both SIRT1 enzyme activity and SIRTs protein levels in Ishikawa cells

The effect of MHY2256 on SIRT1 enzyme activity was measured using the SensoLyte® 520 SIRT1 assay kit. Salermide was used as a positive compound for the SIRT1 inhibitor. As shown
in Fig. 1C, MHY2256 significantly inhibited SIRT1 enzyme activity in a concentration-dependent manner. The IC₅₀ of MHY2256 against SIRT1 enzyme activity was 1.89 mM, which was lower than that of salermide (IC₅₀, 4.8 mM). Next, the effect of MHY2256 on SIRT protein expression was examined by western blot analysis. We found that SIRT1, 2, and 3 levels were down-regulated in Ishikawa cancer cells following high dose MHY2256 treatment (Fig. 1D), suggesting that MHY2256 might be targeted various SIRT proteins. Thus, MHY2256 showed cytotoxicity against endometric cancer cells by targeting SIRT proteins.

2.2. MHY2256 inhibits cell cycle distribution

Previous studies indicated that SIRT1 inhibitors block the proliferation of cancer cells via cell cycle arrest at a specific phase depending on the exposure conditions [17,18]. We examined the effect of MHY2256 on the cell cycle distribution by flow cytometry. The cells were treated with the indicated concentrations of MHY2256 (0.2, 1, or 5 μM) and salermide (50 μM) for 48 h. MHY2256 markedly increased the number of Ishikawa cells at the G1 phase (Fig. 2A). The MHY2256-mediated cell cycle distribution was similar to that of salermide, suggesting that SIRT1 inhibitor arrest G1 phase of Ishikawa cells. The effect of MHY2256 on the expression levels of cell cycle-related proteins was confirmed by western blot analysis. MHY2256 markedly reduced cyclins and CDKs protein levels, indicating that these molecules are closely associated with the G1 phase cell cycle checkpoints (Fig. 2B). Additionally, MHY2256 significantly increased the expression of p21, suggesting that MHY2256 arrests the cell cycle mainly through p21 upregulation.

2.3. MHY2256 increases p53 proteins by reducing MDM2

It has been reported that SIRT inhibitors up-regulate p53 in various cancer cells [16,19].
Therefore, we investigated whether MHY2256 regulate p53 levels to sensitize Ishikawa cells. As shown in Fig. 2C, the basal level of p53 levels was highly expressed in Ishikawa cells. MHY2256 markedly increased the levels of acetylated p53 and total p53. Moreover, MHY2256 markedly reduced the expression of MDM2, an important negative regulator of p53 (Fig. 2D). The changes in the MHY2256-mediated p53 signaling pathway were similar to those caused by salermide, suggesting that SIRT1 inhibitors target p53 activation by degrading MDM2. This suggests that blocking MDM2-p53 binding by inhibiting p53 deacetylation via MHY2256 can serve as mechanism of sensitization in Ishikawa endometrial cancer cells. This led us to investigate whether Ishikawa cells subsequently induce apoptosis because of p53 activation.

2.4. MHY2256 induces apoptotic cellular death in Ishikawa cells

To assess the extent of apoptosis induced in Ishikawa cancer cells after MHY2256 treatment, Annexin V/FITC double staining was performed. The percentage of late-stage apoptotic cells was increased in a concentration-dependent manner after treatment with MHY2256 (Fig. 3A). To identify the apoptotic pathway underlying the cytotoxic effect of MHY2256, the expression levels of apoptosis-relative proteins were measured by western blot analysis. MHY2256 significantly increased the levels of cleaved PARP and Bax (Fig. 3B). Increased cytochrome c release was also detected following MHY2256 treatment (Fig. 3B). These results confirm that MHY2256 sensitizes Ishikawa cancer cells to apoptosis. However, Bcl-2 expression was not increased by either MHY2256 or salermide treatment (Fig. 3B), suggesting that Bcl-2 protein in Ishikawa cancer cells does not contribute to apoptosis. Considering that MHY2256 has large effects on late apoptosis, apoptotic cellular death by MHY2256 is highly cytotoxic even at low doses.
2.5. MHY2256 induces autophagic cell death

We previously showed that MHY2256 significantly increases autophagic cell death of breast cancer cells [16]. To assess whether MHY2256 induces autophagic cell death of Ishikawa cells, western blot analysis and acridine orange staining were performed. As shown in Fig. 3C, low concentrations of MHY2256 (0.2 and 1 µM) markedly increased the level of LC3-II and autophagy-related gene 5 (ATG5). In contrast, salermide did not greatly increase LC3-II (Fig. 3C), suggesting that MHY2256 is a novel SIRT inhibitor with different mechanisms from the well-known SIRT inhibitor salermide. Autophagy induction was confirmed by acridine orange staining. MHY2256-treated cells showed a significant increase in red fluorescence AVOs at 48 h (Fig. 3D). Flow cytometric analysis after acridine orange staining also showed increased red fluorescence intensity following drug treatment. Histogram profiles were drawn to show the mean fluorescence intensity of control and drug-treated cells (Fig. 3E). Increased autophagy in Ishikawa cells contributes to highly apoptotic cytotoxicity by MHY2256.

2.6. MHY2256 inhibits Ishikawa endometrial cell tumors in a xenograft model

To evaluate the anticancer effects of MHY2256 in an in vivo xenograft model, nude mice were inoculated with Ishikawa cells and treated with MHY2256 (5 mg/kg) or salermide (30 mg/kg) for 4 weeks. The MHY2256 treatment group showed significantly reduced tumor volume and tumor weight by 60% relative to the control group (Figs. 4A and 4B). Similarly, salermide time-dependently inhibited tumor growth compared to in the control group (Figs. 4A and B). No significant adverse effects or body weight changes were observed following MHY2256 treatment (data not shown). Taken together, these data demonstrate that the antitumor activity of MHY2256 towards endometrial tumor cell proliferation was related to reduce proliferating cell nuclear antigen expression in tumor tissues (Fig. 4C).
3. Discussion

SIRT-targeted show promising anticancer effects but have not been widely examined in clinical trials. We investigated the mechanisms of SIRT inhibitors; sirtinol significantly increased autophagic cell death in MCF-7 cells [20]. Moreover, we screened and identified a novel synthesized SIRT inhibitor, MHY2256, which is highly cytotoxic towards MCF-7 and SKOV-3 cancer cells [16]. In the current study, we evaluated the anti-cancer ability of MHY2256 in endometrial cancer.

First, we demonstrated that MHY2256 inhibited the proliferation of Ishikawa endometrial cancer cells by directly inhibiting SIRT protein levels and activity. Thus, MHY2256 can greatly inhibit female-related cancers by inhibiting SIRT protein levels. Importantly, the anti-tumor effects of MHY2256 were demonstrated in an in vivo xenograft model, in which Ishikawa tumor growth was significantly retarded by MHY2256 injection. Our in vivo results revealed no side effects in animals injected with MHY2256, suggesting that MHY2256 can be applied in endometrial cancer in combination or single treatment. To determine the molecular mechanisms of MHY2256 against endometrial cancer cell proliferation, we first measured the apoptotic cell death pathway underlying the cytotoxic effect of MHY2256. We found that the expression levels of apoptosis-related proteins were altered by MHY2256 treatments. Interestingly, the cytotoxic effects of low-dose MHY2256 were observed in late-stage apoptosis of Ishikawa cancer cells, suggesting that MHY2256 is selectively cytotoxic towards endometrial cancer cells. Therefore, our data indicate that this inhibitor can be used for targeted cancer therapeutics for treating endometrial carcinoma.

Previous studies showed that SIRT inhibitors induce apoptosis through several mechanisms in a variety of cancer cells [21,22]. Furthermore, SIRT inhibitor administration
activates the proapoptotic protein, an upstream mediator of mitochondrial membrane disruption, through overexpression of antiapoptotic Bcl-2, which is known to be down-regulated by SIRT expression [23,24]. Previous studies indicated that modifications in histone proteins are associated with the tumorigenesis of endometrial cancer development [25-27]. However, a dual function of SIRT1 in tumor promotion and suppression has been described in different cancer types [28,29]. SIRT7 functions as an oncogene and is upregulated in many cancer types [30]. In contrast, no significant associations were found between SIRT1/7 immunoexpression and histological subtype, grade, lymphovascular invasion, or stage [31]. Thus, the diverse expression patterns of SIRTs may have distinctive roles in endometrial cancer, as has been described for other cancer models.

Our previous study showed that MHY2256 significantly increased autophagic cell death in female-related cancer cells [16]. We also tested whether autophagic apoptosis was increased by MHY2256 treatment of Ishikawa cancer cells. We found that increased autophagy in Ishikawa cells contribute to highly apoptotic cytotoxicity by MHY2256. Thus, MHY2256 may use conserved autophagic death mechanisms to induce highly cytotoxic apoptosis in female-related cancers. We found that p21 was greatly increased by MHY2256. Considering that G1 arrest was increased by MHY2256, p21 may be a key target for arresting G1 phase by MHY2256 and may also contribute to increased late apoptosis caused by MHY2256. It has been reported that SIRT inhibitors up-regulate p53 in some cancers [15, 17-18]. We also found that MHY2256 regulated p53 levels to sensitize Ishikawa cells, suggesting that blocking MDM2-p53 binding by inhibiting p53 deacetylation via MHY2256 can serve as mechanism of sensitization of Ishikawa endometrial cancer cells. MHY2256 shows potential the development of drugs targeting p53.

Lara et al. [22] reported that the SIRT1-dependent proapoptotic effect of salermide was
The apoptotic effect of salermide was suggested to involve reactivation of proapoptotic genes, which are epigenetically repressed in cancer cells by SIRT1. Consistently, sirtinol and salermide treatment resulted in in vivo acetylation of the SIRT1/2 target p53 and SIRT2 target tubulin in MCF-7 cells [18,32]. In this study, we found that MHY2256 profoundly inhibited the growth of endometrial cancer cells and caused morphological changes consistent with apoptosis. Together, our results reveal the specificity and cellular targets of these novel inhibitors and suggest that SIRT inhibitors require combined targeting of both SIRT1 and SIRT2 to induce p53 acetylation and cell death. Furthermore, acetylation of p53 following SIRT inhibition may increase its activity and reduce targeting of p53 for degradation [22,33]. Additional experiments are required to define the expression and role of p53 in SIRT inhibitor-mediated apoptosis in endometrial cancer cells. Based on the in vitro anticancer effect of MHY2256, its anti-tumor effect on Ishikawa endometrial cancer cells was evaluated in a tumor xenograft model using nude mice. MHY2256 inhibited tumor growth by 53% in the Ishikawa cell xenografts compared to treatment with vehicle alone. These results suggest that MHY2256 not only inhibits Ishikawa cell growth in vitro, but also greatly inhibits endometrial tumor cell growth in vivo. To determine the mechanisms underlying this tumor growth inhibition, the effects of MHY2256 on tumor cell proliferation were examined by calculating the proliferative index Ki67 in tumors collected at necropsy from all experiments. MHY2256 markedly reduced the expression of Ki67-positive cells compared to in the control group.

4. Materials and methods

4.1. Chemistry

All reagents were obtained commercially and used without further purification. Mass spectrometry (MS) data were obtained on an Expression CMS (Advion, Ithaca, NY, USA).
Nuclear magnetic resonance (NMR) data were obtained using a Varian Unity INOVA 400 spectrometer or a Varian Unity AS500 spectrometer (Agilent Technologies, Santa Clara, CA, USA) using CDCl₃ and DMSO-d₆, and chemical shifts are reported in parts per million (ppm) versus residual solvent or deuterated peaks (δH 7.24 and δC 77.0 for CDCl₃, δH 2.50, and δC 39.7 for DMSO-d₆). Coupling constants are reported in Hz. All reactions described below were performed under a nitrogen atmosphere and monitored by thin-layer chromatography, which was performed on Merck precoated 60F₂₅₄ plates (Billerica, MA, USA).

4.2. Synthesis of MHY2256

A solution of 2,6-di-tert-butylphenol (10.00 g, 48.47 mmol) and hexamethylenetetramine (6.79 g, 48.43 mmol) in acetic acid (20.0 mL) and water (4.0 mL) was refluxed for 7 h. After cooling, the reaction mixture was filtered and washed with methyl alcohol and water to give pure 3,5-di-tert-butyl-4-hydroxybenzaldehyde (10.40 g, 91.6%) as a solid. ¹H NMR (500 MHz, CDCl₃) δ 9.85 (s, 1 H, CHO), 7.73 (s, 2 H, 2-H, 6-H), 5.85 (s, 1 H, OH), 1.48 (s, 18 H, 2 x tert-C₄H₉); ¹³C NMR (100 MHz, CDCl₃) 192.0 (CHO), 159.9 (4C), 136.7 (3C, 5C), 129.0 (1C), 127.9 (2C, 6C), 34.6 (2 x C(CH₃)₃), 30.3 (2 x C(CH₃)₃). To a stirred solution of 3,5-di-tert-butyl-4-hydroxybenzaldehyde (300 mg, 1.28 mmol) in ethanol (12 mL) and water (12 mL) was added 2-thioxodihydropyrimidine-4,6(1H,5H)-dione (thiobarbiturate, 185 mg, 1.28 mmol) and the reaction mixture was refluxed for 16 h. After evaporating the ethanol, the mixture was filtered and washed with water and methylene chloride to give pure 5-(3,5-di-tert-butyl-4-hydroxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (MHY2256, 378.2 mg, 82%) as a solid. ¹H NMR (400 MHz, DMSO-d₆), 12.21 (s, 2 H, 2 x NH), 8.31 (s, 2 H, 2-H, 6-H), 8.20 (s, 1 H, vinylic H), 1.36 (s, 18 H, 2 x tert-C₄H₉); LMHR(ESI-) 359 (M-H).
4.3. Cell culture

Ishikawa cancer cells were kindly provided by Dr Jacques Simard (CHUL Research Center, Quebec, Canada). The cells were maintained as monolayers at 37°C in DMEM (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) in an atmosphere containing 5% CO₂/air. After incubation for 48 h, the culture medium was replaced with fresh medium containing the desired concentrations of drugs.

4.4. SIRT1 activity assay

SIRT1 activity was measured with the SensoLyte® 520 fluorimetric SIRT1 activity assay kit (AnaSpec, Fremont, CA) according to the manufacturer’s instructions. Nicotinamide was used as a reference compound. Briefly, SIRT1 enzymes were incubated with vehicle or various concentrations of test compound at 37°C in the presence of an SIRT1 fluorimetric substrate. The SIRT1 assay developer, which produces a fluorophore in the reaction mixture, was added, and the fluorescence was measured using VICTOR X2 (Perkin Elmer, Waltham, MA, USA) with excitation at 490 nm and emission at 520 nm. The measured activities were calculated using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

4.5. Cytotoxicity assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL, Sigma, St. Louis, MO, USA). The cultures were initiated in 96-well plates at a density of 2.5 × 10³ cells per well. After incubation for 48 h, the cells were treated with various concentrations of MHY2256 and salermide and then cultured for 48 h. At the end of the treatment period, 15 µL of MTT reagent was added to each well, and the cells were
incubated for 4 h at 37°C in the dark. After incubation, the supernatant was aspirated, and formazan crystals formed were dissolved in 100 µL of DMSO at 37°C for 10 min with gentle agitation. The absorbance per well was measured at 540 nm using a VERSA Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed from 3 independent experiments and then normalized to the absorbance of wells containing media only (0%) and untreated cells (100%). The values of half-maximal inhibitory concentration (IC50) were calculated from sigmoidal dose-response curves using SigmaPlot 10.0 software (Systat Software, Chicago, IL, USA).

4.6. Western blot analysis

Ishikawa cells were treated with MHY2256 (0.2, 1, and 5 μM) or salermide (50 μM) for 48 h. The cells were harvested by trypsinization and washed twice with cold phosphate-buffered saline (PBS). For total protein isolation, the cells were suspended in PRO-PREP™ protein extract solution (iNtRON, Seongnam, Korea), and protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Equivalent amounts of proteins were resolved and subjected to sodium dodecyl polyacrylamide gel electrophoresis on a 6-15% gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and the membranes were blocked with blocking buffer (TNA buffer containing 5% skim milk) for 1 h. Next, the membranes were incubated with different primary antibodies at 4°C overnight. After washing the membranes for 1 h with TNA buffer (10 mM Tris-Cl, pH 7.6; 100 mM NaCl; and 0.5% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:10,000, Santa Cruz Biotechnology, Dallas, TX, USA) for 30 min at room temperature and then washed for 1 h with TNA buffer. The blots were developed using...
an enhanced chemiluminescence (ECL)-plus kit (Amersham Biosciences, Amersham, UK).

4.7. Flow cytometry analysis

The cells were treated with various concentrations of MHY2256 (0.2, 1, and 5 μM) or salermide (50 μM) for 48 h. The total number of cells, both in suspension and those adhered to the walls for sub-G1 phase and only those adhered to the walls for other cell cycle phases, were harvested separately and washed in 1% bovine serum albumin before fixing in 95% ice-cold ethanol containing 0.5% Tween 20 for 1 h at -20°C. The cells ($1 \times 10^6$) were washed in 1% bovine serum albumin, stained with cold propidium iodine (PI) staining solution (10 μg/mL PI and 100 μg/mL RNase in PBS), and incubated in the dark for 30 min at room temperature. Data acquisition and analysis were performed using a flow cytometry system (BD Biosciences, San Jose, CA, USA).

4.8. Annexin V-FITC/PI binding assay

The Annexin V-FITC/PI binding assay was performed according to the manufacturer’s instructions using the annexin V-FITC detection kit I (BD Biosciences). The cells were treated with MHY2256 (0.2, 1 or 5 μM) or salermide (50 μM) for 48 h. The total number of cells was counted following trypsinization and washing twice with cold PBS. The cell pellet was resuspended in 100 μL binding buffer at a density of $1 \times 10^5$ cells/mL and incubated with 5 μL of FITC-conjugated annexin V and 5 μL of PI for 15 min at room temperature in the dark. Next, 400 μL of binding buffer was added to each sample tube, and the samples were immediately analyzed by fluorescence-activated cell sorting (BD Biosciences).

4.9. In vivo tumor xenograft model
Six-week-old female nude mice (BALB-c nu/nu, Charles River Lab, Inc., Wilmington, MA, USA) were housed under controlled temperature (22 ± 2°C) and lighting (12 h light/dark cycle) contains in filtered-air laminar-flow cabinets and manipulated using aseptic procedures. All animal procedures were approved by the Korea Medical Experimental Animal Care Commission (Daejon, Korea) prior to experimentation. Ishikawa cells (2 × 10^7) in 0.1 mL serum-free medium containing 50% Matrigel were injected subcutaneously (s.c.) into the upper flank of each nude mouse. When the tumor size reached 200 mm^3, the mice were randomized to two groups (n = 5). MHY2256 (5 mg/kg) and salermide (30 mg/kg) were injected intraperitoneally to the mice daily for 21 days, while control mice were administered 0.1% DMSO in the same manner. Tumor sizes were measured with calipers and their volumes calculated using the standard formula: width^2 × length × 0.52. The body weights were recorded prior to dosing. The mice were sacrificed at the end of the treatment period. The tumor tissue was excised, weighed, frozen in liquid nitrogen, and stored at -80°C until analysis.

4.9. Statistical methods

The data were expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was performed using one-way analysis of the variance (ANOVA) followed by Bonferroni’s multiple comparison tests. *p <0.05; **p<0.01 were considered statistically significant. All statistical comparisons were performed using SigmaPlot graphing software and Statistical Package for the Social Sciences v.13 (SPSS, Inc., Chicago, IL, USA).

5. Conclusions

Our results reveal the anticancer mechanism of a new HDAC inhibitor in regulating tumor suppressor genes and, consequently, its potential therapeutic role for endometrial cancer. In this
study, the SIRT inhibitor MHY2256 induced anti-proliferative effects in endometrial cancer cells by inducing cell cycle arrest, apoptosis, and autophagic cell death. MHY2256 also induced p53 activation by reducing MDM2 expression. Therefore, MHY2256 is a potential SIRT-targeted agent against female-related cancers, particularly in patients with high p53 expression. Additional studies are needed to evaluate the female-specific effects of SIRT inhibitors in cancer.

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Author Contributions: Hyung Sik Kim, and Hyung Ryong Moon designed the research. Hyung Ryong Moon provided suggestions for optimizing MHY2256. Umasankar De, Ji Yeon Son, Richa Sachan, Yu Jin Park, and Dongwan Kang performed the in vitro experiments. Kyungsil Yoon and In Su Kim analyzed the data. Umasankar De and Byung Mu Lee wrote and revised the paper. All authors read and approved the final manuscript.

Competing Interests: The authors have no competing interests to declare.

References

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Figure Legends

**Figure 1. MHY2256 sensitizes Ishikawa endometrial cancer cells by reducing SIRT activity and protein levels**

(A) Chemical structure of MHY2256 used in the present study. (B) The cells were treated with MHY2256 and salermide at various concentrations (0.1–50 μM) for 48 h. Cell viability was detected using MTT assay and the data are represented as the mean ± SEM of three independent experiments. (C) Effects of MHY2256 and nicotinamide on SIRT1 activity. SIRT1 enzyme activity was measured using the SensoLyte® 520 FRET SIRT1 assay kit. Statistical analysis was performed using one-way analysis of the variance followed by Bonferroni’s multiple comparison tests. *p < 0.05, **p < 0.01 indicate significant differences between control and treatment groups. (D) Effects of MHY2256 on different type of SIRT expression. The cells were treated with MHY2256 and salermide for 48 h, and then western blot analysis was performed.

**Figure 2. MHY2256 increases G1 arrest and reduces p53 levels via MDM2 degradation**

(A) Ishikawa cells were treated with the indicated concentrations for 48 h. Cells stained with propidium iodide (PI) were subjected to flow cytometric analysis to determine the cell distributions in each phase of the cell cycle. (B) Effect of MHY2256 on expression levels of cell cycle regulatory proteins. Cells were treated with the MHY2256 (0, 0.2, 1, and 5 μM) or salermide (50 μM) for 48 h, and then protein levels were detected by western blot analysis. Aliquots of proteins were immunoblotted with specific primary antibodies against cyclin D1, cyclin E, cyclin A, CDK2, CDK4, CDK6, and p21. (C) Basal expression levels of p53 protein in Ishikawa cancer cell. Immunofluorescence for p53 proteins levels and fluorescence detection of p53 using rhodamine red-tagged secondary antibody was observed using confocal microscopy (Magnification x400). (D) Effects of MHY2256 on p53, acetylated p53 (Ac-p53),
and MDM2 expression. Ishikawa cells were treated with MHY2256 and salermide for 48 h, and then western blot analysis was performed.

Figure 3. MHY2256 increased late apoptosis and autophagy in Ishikawa endometrial cancer cells

(A) Cells were treated with MHY2256 or salermide for 48 h at the indicated concentrations. MHY2256-induced apoptosis was examined by Annexin V/7-AAD double staining. Flow cytometry scatter plots indicate the percentage of cells in the early and late phases of apoptosis after drug treatment. (B) To determine the apoptosis-related protein levels, cells were treated with MHY2256 or salermide for 48 h at the indicated concentrations, and western blot analysis was performed. The protein levels were normalized by comparison to the levels of β-actin. (C) To evaluate MHY2256-induced autophagy, western blot analysis was performed to detect LC3-I/II, Beclin-1, Atg5, and Atg7 protein expression. (D) Immunofluorescence microscopy of acridine orange-stained Ishikawa cells treated for 48 h with the indicated drug treatment (magnification, x400). (E) Histogram profiles of control and drug-treated cells analyzed by flow cytometry.

Figure 4. MHY2256 significantly reduces the growth of Ishikawa endometrial cancer cells in nude mice

Mice with pre-established tumors were randomized into 3 groups with 5 mice in each group. Vehicle control, MHY2256 (5 mg/kg, twice/week, i.p.) and selermide (30 mg/kg/week, i.p.) were administered to tumor-bearing mice. (A) During the 30-day treatment, the mean tumor volumes in each treatment group are indicated. The results are represented as the mean ± SEM per group. Statistical analysis was performed using one-way analysis of the variance followed
by Bonferroni’s multiple comparison tests. * $p < 0.05$, **$p < 0.01$ indicate significant differences between control and treatment groups. (B) Each bar represents the inhibition rate (\% of control) of mean tumor weight. The results are presented as the mean ± SEM per group. Statistical analysis was performed using one-way analysis of the variance (ANOVA) followed by Bonferroni’s multiple comparison test. *$p<0.05$, **$p<0.01$ indicate significant differences between control and treatment groups. (C) The tumors were fixed in 10% formalin and embedded in paraffin. Immunohistochemical staining for Ki-67 were measured in tumors. Magnification x200. Scale bar = 50 μm. The representative images were recorded under a 40x objective lens.