MHY451 induces cell cycle arrest and apoptosis by ROS generation in HCT116 human colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is the third most frequently diagnosed cancer and cause of cancer-related deaths. Despite advancements in conventional therapeutic approaches to CRC, most patients with CRC die of their disease. There is a need to develop novel therapeutic agents for this malignancy. Therefore, the present study aimed to examine the anticancer effects and elucidate the underlying mechanism of MHY451 in HCT116 human colorectal cancer cells. Treatment with MHY451 inhibited cell growth in a time- and concentration-dependent manner. MHY451 increased the accumulation of cell cycle progression at the G2/M phase. This agent decreased the protein level of cyclin B1 and its activating partners, Cdc25c and Cdc2, whereas it increased the cell cycle inhibitor p21WAF/CIP. The induction of apoptosis was observed by decreased viability, cleavage of poly(ADP-ribose) polymerase (PARP), alteration in the ratio of Bax/Bcl-2 protein expression and reduction of procaspase-8 and -9. Pretreatment with Z-VAD-FMK, a pan-caspase inhibitor, inhibited MHY451-induced apoptosis, indicating that apoptotic cell death by MHY451 was mediated through caspases. Moreover, the apoptotic effect of MHY451 was reactive oxygen species (ROS)-dependent, evidenced by the inhibition of MHY451-induced PARP cleavage and ROS generation by N-acetylcysteine-induced ROS scavenging. Taken together, these results demonstrate that MHY451 exerts anticancer effects by regulating the cell cycle, inducing apoptosis through caspase activation and generating ROS. These results suggest that MHY451 has considerable potential for chemoprevention or treatment of CRC or both.

Introduction

Colorectal cancer (CRC) primarily occurs in the colon and rectum. CRC is the third most commonly diagnosed cancer in both men and women in the US based on the American Cancer Society, 2016. According to the National Cancer Center of Korea, it is the second most diagnosed cancer in men and the third in women. Currently, the effective methods for treating CRC include radiation, surgery, chemotherapy, and a combination of chemotherapy with radiotherapy; however, most patients died from their disease (1,2). If CRC is diagnosed and treated early, then patients will have a higher survival rate, thus timely and precise treatment is important (2,3). There is continuous need to investigate and develop potential preventive treatment approaches for CRC.

Carcinogenesis is a multistep process that involves tumor initiation during which normal cells are genetically altered into malignant cells (4,5). In addition, tumor promotion occurs where the growth progression of small groups of malignant cells is stimulated, and thereby the growing tumor becomes more aggressive (4,5). The definition of chemoprevention is the use of specific agents to interfere in the multistage carcinogenesis (5,6). Furthermore, chemoprevention also involves numerous types of cell death including apoptosis (7).

Apoptosis (programmed cell death), a mechanism that is important for all multicellular organisms, controls cell proliferation and maintains tissue homeostasis as well as eliminates harmful or unnecessary cells from organisms (8). The evasion of apoptosis contributes to carcinogenesis and tumor progression (9). There are two main apoptosis pathways, including the extrinsic pathway, mediated by the activation of cell surface receptors, which are death receptors. The death receptor families are integrated into the plasma membrane and become activated following ligation by their ligands (10). This leads to the formation of aggregates of a series of proteins called death-inducing signaling complexes that activate caspase-8.

Once caspase-8 is activated, its downstream effector, caspase-3, directly cleaves and initiates the mitochondrial pathway (intrinsic) of apoptosis by proteolytic cleavage of BH3 interacting-domain death agonist (Bid) to initiate t-Bid (10).
The intrinsic mitochondrial pathway is initiated by loss of mitochondrial transmembrane potential and release of cytochrome c into the cytosol, which then activates procaspase-9. This pathway is initiated in the cells and regulated by proteins of the B-cell lymphoma-2 (Bcl-2) family (11). The members of the Bcl-2 family, which are key regulators of apoptosis and overexpressed in numerous malignancies, are the most crucial regulators of the intrinsic pathway (12). The extrinsic and intrinsic pathways are regulated by influencing each other. Initiator caspases (caspase-8 and -9) and active executioner caspases (caspase-3, -6 and -7) subsequently cleave key structural proteins and active other enzymes (13).

The anthracycline doxorubicin (DOX) is one of the most active drugs used in the treatment of a wide range of cancers (14). This drug intercalates into the DNA of living cells and causes cell death by inhibiting topoisomerase II as well as the generation of reactive oxygen species (ROS) and free radicals by redox reactions (15). DOX has been reported to induce apoptosis through the intrinsic pathway by disrupting the mitochondrial membrane potential with the release of cytochrome c and subsequent activation of the caspase cascade (16,17). Although DOX has strong anticancer activity and is approved by the US Food and Drug Administration (FDA), it causes serious side-effects in most of the major organs, especially the heart (18-20). Numerous novel anthracycline derivatives have been synthesized to overcome these side-effects (21,22). Therefore, we studied a newly synthesized DOX derivative, MHY451 [1-hydroxy-3-(oxiran-2-ylmethoxy)-5-(oxiran-2-ylmethyl)benzo[b]acridin-12(5H)-one], which is designed to investigate the effects of MHY451 on cell viability was determined using the MTT assay.

**Materials and methods**

**Chemicals.** The simplified code name and structure of MHY451 [1-hydroxy-3-(oxiran-2-ylmethoxy)-5-(oxiran-2-ylmethyl)benzo[b]acridin-12(5H)-one] used in the present study is shown in Fig. 1. MHY451 was dissolved in dimethyl sulfoxide (DMSO), stored at -20°C before the experiments, and working dilutions were prepared in culture medium. The maximum concentration of DMSO did not exceed 0.1% (v/v) in the treatment range of 1.25-20 μM, which did not affect cell growth. Antibodies specific for Bcl-2-associated X protein (Bax), Bcl-2, cyclin-dependent kinase 2 (Cdcd2), Cdcd25c, cyclin B1, p21, p53, poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP), caspase-3, caspase-8 and caspase-9 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

**Cell culture and viability assay.** The human colorectal cancer cell lines HCT116 and HT29 were cultured in Roswell Park Memorial Institute (RPMI)-1640 (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 units/ml penicillin (GE Healthcare Life Sciences) at 37°C in a humidified atmosphere of 5% CO₂. The effect of MHY451 on cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, Solon, OH, USA) assay. For the MTT assay, HCT116 cells were seeded in a 24-well culture plate at a density of 5.5x10⁴ cells/well, cultured for 24 h in the growth medium, and then treated with or without various reagents at the indicated concentrations. The cells were incubated with 0.5 mg/ml MTT at 37°C for 2 h. The formazan granules generated by the live cells were dissolved in DMSO, and the absorbance at 540 nm was monitored using a multi-well reader (Thermo Fisher Scientific).

**Western blot analysis.** After MHY451 treatment, the cells were harvested by trypsinization and washed once with PBS. After centrifugation, the cells were fixed in 70% ethanol at 4°C overnight. The fixed cells were pelleted and stained in cold propidium iodide (PI) solution (50 µg/ml in PBS) at room temperature. The cells were harvested and washed with cold PBS. For the Western blot analysis, the supernatant was collected, and protein concentration was measured using protein assay reagents (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were denatured by boiling at 100°C for 5 min in 4X Laemmli sample buffer (Bio-Rad Laboratories). Equal amounts of protein were separated using 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF). The membranes were blocked with 5% non-fat dry milk in Tris-buffere 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 with different primary antibodies at 4°C overnight. After thorough washing with TBS-T buffer, the membranes were incubated for 1 h with horseradish peroxiside-conjugated secondary antibody (1:5000 dilution; Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Membranes were washed with TBS-T buffer and blocked with 5% non-fat dry milk in TBS-T for 1 h before applying primary antibody. After washing, the membranes were incubated with goat anti-rabbit/horseradish peroxiside (1:1000 dilution; PerkinElmer, Waltham, MA, USA) for 2 h. After washing, peroxiside reaction was developed with enhanced chemiluminescence (ECL) reagent (Amersham, Arlington Heights, IL, USA) and visualized using X-ray film (Kodak, Rochester, NY, USA).
peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The membranes were washed with TBS-T buffer, and the antigen-antibody complexes were detected using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Sciences).

Annexin V staining. Annexin V-fluorescein isothiocyanate (FITC) was used to quantitatively investigate the percentage of cells that underwent apoptosis. Cells were seeded in a 6-well culture plate at a density of 2.7x10^5 cells/well, cultured for 24 h, harvested, trypsinized and washed once with cold PBS, and then suspended in 1X binding buffer (Becton-Dickinson, San Jose, CA, USA). The cells were stained with PI and Annexin solution (Becton-Dickinson) for 15 min at room temperature in the dark. The stained cells were analyzed using an Accuri C6 (BD Biosciences, Ann Arbor, MI, USA) within 1 h.

Intracellular ROS measurement. For the assessment of intracellular ROS generation, cells were seeded in a 96-well culture plate at a density of 0.5x10^4 cells/well and cultured for 24 h. After 1 day, the cells were treated with MHY451 (2.5 µM) for 2, 4 and 6 h; the medium was replaced with ROS buffer; and then 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR, USA) was added. After 30 min, changes in fluorescence intensity were measured seven times for 30 min using a fluorescence plate reader (GENios; Tecan Instruments, Salzburg, Austria) and the absorbance was measured at 485 and 530 nm.

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

Results

MHY451 suppresses the growth of HCT116 cells. To investigate the effect of MHY451 on the viability of HCT116 and HT29 cells, the MTT assay was performed. Cell viability was reduced by treatment with MHY451 in a concentration- and time-dependent manner. As shown in Fig. 2, the half-maximal inhibitory concentrations (IC_{50}) of MHY451 at 24 and 48 h were 3.14 and 2.87 µM in HCT116 cells (Fig. 2A) and 4.47 and 3.14 µM in HT29 cells (Fig. 2B), respectively. These results indicate that the HCT116 cell line was more sensitive to treatment with MHY451 than HT29 cells. Thus, HCT116 cells were chosen for further experiments.

MHY451 modulates cell cycle progression in HCT116 cells. To investigate whether MHY451 influenced cell cycle distribution, HCT116 cells were treated with various concentrations of MHY451 for 24 h and then the cell cycle was analyzed using flow cytometry. As shown in Fig. 3A, cells exposed to MHY451 exhibited G2/M cell cycle accumulation. A total of 43.47% of the cells cultured with 5 µM MHY451 were in the G2/M phase compared to 29.93% of control cells in the G2/M phase. In addition, sub-G1 populations were increased from 2.09 in control to 10.54% in cells treated with 10 µM MHY451. Next, we examined whether MHY451 modulated the expression of G2/M cell cycle regulators. Cells were treated with various concentrations of MHY451 for 24 h, and then the level of G2/M cell cycle regulatory proteins was examined using western blot analysis. As shown in Fig. 3B, the expression levels of cyclin B1, its regulatory protein Cdc2 and Cdc25c were decreased in HCT116 cells by MHY451 treatment in a concentration-dependent manner. The induction of p21^{WAF/CIP1} causes arrest in the G1/G0 or G2/M phase of the cell cycle binding of the cyclin-cyclin-dependent kinase (CDK) complex. Thus, further studies were performed to determine whether p21^{WAF/CIP1} was induced by MHY451 either by p53-dependent or p53-independent pathways in HCT116 cells. The results showed that the expression level of p53 was slightly increased and consequently the level of p21^{WAF/CIP1} was also increased by MHY451 treatment in HCT116 cells. These data suggest that the MHY451-induced G2/M phase arrest was mediated by a p53-dependent pathway.

MHY451 treatment induces apoptosis in HCT116 cells. To determine whether the growth inhibitory effects of MHY451 were due to apoptosis, microphotographs were acquired. HCT116 cells treated with MHY451 showed distinct morphological changes compared with the control cells (Fig. 4A, upper panel). They were round-shaped, which is a typical feature of apoptosis. Furthermore, morphological changes in
Figure 3. Effects of MHY451 on cell cycle and levels of cell cycle regulator proteins in HCT116 cells. (A) Exponentially growing cells at 60-70% confluence were treated with MHY451 (0-10 µM) for 24 h. Cells were fixed and digested with RNase and then DNA was stained with PI. The percentage of cells in Sub G1, G1, S and G2/M phases of cell cycle was calculated and displayed within each histogram. (B) Cells were prepared and treated with indicated concentration of MHY451 for 24 h. Whole cell lysates were subjected to 10-15% SDS-PAGE and electrophoretically transferred to PVDF membrane. The membranes were probed with ECL kits. Representative results from three independent experiments are shown.

Figure 4. Effect of MHY451 on the induction of apoptosis in HCT116 cells. (A) Morphological changes in MHY451-treated cells. Nuclei of HCT116 cells stained with fluorescent DNA-binding dye (Hoechst 33342). (B) Effect of MHY451 on cell death in cells stained with Annexin V-FITC/PI and analyzed using flow cytometry. (C) Immunoblot analysis with pro-caspase-3, -8, -9, Bax, Bcl-2, Bid and PARP (116 kDa) following treatment with MHY451. Actin was used as a protein loading control. (D) Cells stained with PI and analyzed using flow cytometry (**P<0.01 vs. MHY451-treated cells). (E) Effects of 40 µM Z-VAD-FMK (Z-VAD) in total cell lysate using immunoblot analysis with actin as loading control. Representative results of three independent experiments are shown. FITC, fluorescein isothiocyanate; PI, propidium iodide; Bid, BH3-interacting domain death agonist; Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2; PARP, poly(ADP-ribose) polymerase.
the cellular structures were assessed using Hoechst 33342. As shown in Fig. 4A (lower panel), after MHY451 exposure, HCT116 cells showed distinct morphological changes such as cell shrinkage and compaction of the nuclei compared with control cells. In addition, to confirm the apoptotic effect of MHY451 in the HCT116 cells, flow cytometry was performed. As shown in Fig. 4B, the early apoptotic proportion (lower right quadrant) increased from 3.5 to 25.4%, and the late apoptotic proportion (upper right quadrant) increased from 3.0 to 27.8% after treatment with 10 µM MHY451. The effect of MHY451 on apoptosis was further verified using western blot analysis. As shown in Fig. 4C, treatment with MHY451 caused proteolytic degradation of PARP, a molecular marker of apoptosis. In addition, pro-caspase-9 decreased after treatment with MHY451. Moreover, the expression level of pro-apoptotic Bax was upregulated while the anti-apoptotic Bcl-2 protein was downregulated in HCT116 cells. These results suggest that MHY451 induced apoptosis of HCT116 cells.

Caspases are involved in MHY451-induced apoptosis in HCT116 cells. To confirm the role of caspase in MHY451-induced apoptosis, the effects of Z-VAD-FMK, a pan caspase inhibitor, were examined in MHY451-treated cells. As shown in Fig. 4D, pretreatment with Z-VAD-FMK reduced the proportion of cells that underwent MHY451-induced apoptosis in the HCT116 cells. These results indicate that Z-VAD-FMK partially inhibited the apoptotic effect of MHY451 in HCT116 cells. To further confirm this result, western blot analysis was performed. As shown in Fig. 4E, pretreatment with Z-VAD-FMK markedly inhibited the MHY451-induced downregulation of pro-caspase-3 and PARP cleavage. These results demonstrate that activation of the caspase cascade is involved in the induction of apoptosis by MHY451.

**MHY451 induces apoptosis in HCT116 cells via ROS generation.** The regulation of intracellular ROS levels is vital to cellular homeostasis and different ROS levels can cause different biological responses. At low and moderate levels, ROS act as signaling molecules while at high levels they induce cell damage and death. Cancer cells show elevated ROS levels compared to those of normal cells due to the accumulation of intrinsic or environmental factors or both (23). It is currently becoming increasingly clear that the generation of ROS can be used therapeutically in the treatment of cancer (24). Therefore, we examined the generation of ROS by MHY451 to explore whether this was its mechanism of apoptosis induction. The intracellular ROS level was measured using the ROS-detecting fluorescent dye DCFDA in HCT116 cells. The cells were treated with 2.5 µM of MHY451 for various times. As shown in Fig. 5A, the cells exposed to MHY451 showed increased levels of intracellular ROS in a time-dependent manner. Next, HCT116 cells were pretreated with N-acetylcysteine (NAC), a well-known ROS scavenger, and then treated with MHY451. At the end of the incubation period, the levels of intracellular ROS were measured. As shown in Fig. 5B, pretreatment with NAC considerably
blocked ROS generation in MHY451-treated HCT116 cells. To further identify the relationship between ROS generation and apoptosis, the effects of NAC were examined in MHY451-treated cells. As shown in Fig. 5C, the presence of cells with sub-G1 DNA content following treatment with MHY451 with or without NAC was evaluated using flow cytometry to quantify the onset of apoptosis. Pretreatment of cells with NAC significantly inhibited cell death in MHY451-treated cells (P<0.01). In agreement with these observations, sequestration of ROS by NAC effectively suppressed the decrease in pro-caspase-3 levels and prevented MHY451-induced PARP cleavage in HCT116 cells (Fig. 5D). These results demonstrate that ROS generation plays an important role in the MHY451-mediated apoptotic pathway in HCT116 cells.

Discussion

The present study was conducted to investigate the anticancer effects of MHY451 in HCT116 cells. MHY451 is a novel molecule based on DOX, which has anticancer mechanisms that might be mediated by DNA intercalation, cell cycle distribution, or apoptosis. In the present study, MHY451 induced apoptosis and cell cycle arrest in HCT116 cells. Flow cytometric analysis revealed that MHY451 induced G2/M cell cycle arrest. G2/M transition is regulated by cyclin B/Cdc2, which has been identified as a principal component of the mitosis-promoting factor (25).

p21^{WAF/CIP1}, a member of the CDK interacting protein kinase inhibitory protein (CIP/KIP) family, inhibits a variety of CDK proteins and is controlled by p53 (26). Furthermore, p53, a tumor suppressor gene, induces cell cycle regulation and apoptosis. In this study, the protein level of p53 was not significantly changed while that of p21^{WAF/CIP1} increased in a concentration-dependent manner in HCT116 cells. These results demonstrate that MHY451 increased p21^{WAF/CIP1} levels and decreased cyclin B1. Furthermore, its activating partners, Cdc25c and Cdc2 (Fig. 3B), may be involved in the p53-independent pathway by which MHY451 inhibited HCT116 cell growth and induced G2/M arrest.

Apoptosis, which is programmed cell death that occurs after exposure to specific stimuli, is an important component of cell growth control (27). There are two main apoptotic pathways, which are the intrinsic and extrinsic pathways that are initiated by the cleavage of caspase-3 and the action of caspase-9 and -8, respectively (28). The intrinsic pathway involves mitochondrial disruption by pro-apoptotic Bcl-2 family members and Bax oligomerization, which consequently releases cytochrome c. Expression of Bcl-2 decreased whereas that of Bax increased in MHY451-treated HCT116 cells. In addition, Bid was remarkably decreased.

MHY451 effectively inhibited HCT116 cell proliferation and induced apoptosis in a concentration-dependent manner. To confirm the MHY451-induced apoptosis, cell cycle and Annexin V/PI double staining was performed. MHY451 also increased the sub-G1 populations in HCT116 cells (Fig. 3A). As shown in Fig. 4B, the increase in early and late apoptosis was clearly observed in a concentration-dependent manner. Further studies indicated that MHY451, in the presence of Z-VAD-FMK, a pan-caspase inhibitor, prevented the cleavage of PARP. Therefore, MHY451-induced apoptosis is likely mediated, at least in part, by the caspase cascade.

This study is first to investigate the ROS-mediated apoptotic effects of MHY451 in HCT116 human colorectal cancer cells. In the biological system, ROS are constantly generated and eliminated and play crucial roles in both normal and abnormal conditions. ROS, which are produced in the mitochondria, play a crucial role in the regulation of cell death when accumulated in excessive amounts (29). In cancer, increased ROS serves as an endogenous source of DNA-damaging agents that facilitate genetic instability and development of drug resistance. Despite the negative impact of elevated ROS in cancer cells, it is possible to exploit this characteristic to develop novel therapeutic strategies to preferentially kill cancer cells through an ROS-mediated mechanism (30). Several anticancer agents such as cisplatin, oxaliplatin, and mitomycin C exert their effects, at least in part, by the induction of ROS generation (31). In the present study, to reveal if MHY451 affected the level of ROS, MHY451-treated HCT116 cells were stained with DCFDA. MHY451-induced ROS generation increased in a time-dependent manner (Fig. 5A). Moreover, pretreatment with the ROS scavenger NAC significantly decreased MHY451-induced ROS levels (Fig. 5B) and inhibited MHY451-induced activation of caspase-3 and PARP cleavage, as well as subsequent cell death. This observation suggests that ROS were involved in the upstream events of caspase induced by MHY451 in HCT116 cells. Although the effect of NAC on the MHY451-induced modulation of pro-apoptotic proteins have not been elucidated, the apoptotic effect of MHY451 is closely related to the generation of ROS.

In conclusion, MHY451 suppressed the growth of HCT116 cells in a concentration-dependent manner by inducing G2/M phase arrest. The present data also suggest that MHY451 induced apoptosis through caspase and ROS-mediated pathways (Fig. 6). Moreover, the mechanism of action of MHY451 should be studied further. Overall, these results demonstrate that MHY451 may be a promising therapeutic agent for treating colorectal cancer.
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