Malformin A1 treatment alters invasive and oncogenic phenotypes of human colorectal cancer cells through stimulation of the p38 signaling pathway

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Received April 16, 2017; Accepted July 3, 2017

DOI: 10.3892/ijo.2017.4070

Abstract. Malformin A1 (MA1), a cyclic pentapeptide isolated from *Aspergillus niger*, has been found to possess a range of bioactive properties including antibacterial activity. However, it is unclear whether MA1 exerts an anticancer effect or not. In this study, we conducted *in vitro* experiments to investigate its anticancer properties in human colorectal cancer cells. The effect of MA1 on human colorectal cancer cells, SW480 and DKO1, was examined by the WST-1 cell viability assay, inverted microscopy, 5-bromo-2-deoxyuridine (BrdU) incorporation, flow cytometry, DNA fragmentation, wound healing, Transwell assays, and western blotting. MA1 treatment showed potent cytotoxic activities on human colorectal cancer cells. MA1 treatment induced apoptosis by activating the poly(ADP-ribose) polymerase (PARP), caspase-3, -7, and -9. MA1 treatment led to the increase in p53 upregulated modulator of apoptosis (PUMA) and the decrease in X-linked inhibitor of apoptosis protein (XIAP) and Survivin. In addition, MA1 treatment induced cell cycle arrest in the sub-G1 phase. The pan-caspase inhibitor, Z-VAD-FMK, attenuated these MA1-induced apoptotic effects on human colorectal cancer cells. Moreover, MA1 treatment suppressed tumor cell migration and invasion. The phosphorylation level of p38 was upregulated by MA1 treatment, and the inhibitor of p38, SB203580, attenuated the MA1-induced p38 phosphorylation as well as caspase-3 and PARP activation. These results indicate that MA1 treatment alters invasive and oncogenic phenotypes of human colorectal cancer cells through the stimulation of the p38 signaling pathway.

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Key words: apoptosis, Malformin A1, colorectal cancer cells, p38

Introduction

Colorectal cancer is one of the leading causes of cancer-associated morbidity and mortality worldwide. Despite recent advances in the management, including surgery, chemotherapy, and radiotherapy, the overall survival of patients with advanced colorectal cancer still remains low (1-3). Therefore, the development of clinically effective anticancer agents with minimal side effects is needed to enhance treatment and to improve the survival of patients with advanced colorectal cancer.

Plant-associated microorganisms are known as an important sources of diverse natural compounds with novel structures and biological activities. A substantial body of evidence demonstrates that various compounds and extracts isolated from plant-associated microorganisms present significant pharmacological and biological activities (4-8). For instance, the bioactive compounds isolated from endophytic fungi that reside on medicinal plants have been reported (4-8). Malformins, which are a group of cyclic pentapeptides with a disulfide bond from two cysteine thiols, were originally discovered and isolated from the culture broth of the fungus *Aspergillus niger* (*A. niger*). It typically induces malformations in bean plants and in the curvature of corn roots (9-12). To date, three sub-groups of Malformins have been identified: A, produced by *A. niger* strain 56-39, B, by *A. niger* strain 56-30, and C, by *A. niger* strain AN-1 (9-12). Malformin A mainly consists of Malformin A1, A2, A3, and A4, indicated by both amino acid analyses and molecular formula. Malformin A1 (MA1) is a bicyclic pentapeptide containing five amino acids: L-isoleucine, L-valine, D-leucine, and two D-cysteines (12). MA1 is the most well-studied out of the malformin subtypes, and its various biological activities have been reported previously, including causing malformations in plants, having some antibacterial effects (13-15), enhancing cellular fibrinolytic activity (16-18), preventing IL-1-induced procoagulant reaction (19,20), and inhibiting the cell cycle at G2 checkpoint followed by DNA damage (21,22). Additionally, the anticancer activity of MA1 has been reported in various human cancer cells including colorectal cancer (9,22-24). Therefore, understanding these functional and biological properties of MA1, as

well as its mechanism of action is very important for its application into mainstream of medicine. The aim of this study is to investigate the impact of MA1 on tumor cell behavior and its associated oncogenic signaling pathways in human colorectal cancer cells.

Materials and methods

Cell culture and materials. The SW480 and DKO1 human colorectal carcinoma cell lines were obtained from the American Type Culture Collection (ATCC CRL 1589, Manassas, VA, USA). The SW480 and DKO1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere of 5% CO_2. p38 chemical inhibitor (SB203580) and pan-caspase inhibitor Z-VAD-FMK were purchased from Sigma-Aldrich (St. Louis, MO, USA). MA1 was purchased from AdipoGen Life Sciences (San Diego, CA, USA).

Cell proliferation assay. SW480 and DKO1 cells were plated onto a 96-well plate at a density of $1x10^4$ cells/well and were incubated for 24 h at 37˚C before the MA1 treatment. Cells were then treated with various concentrations of MA1 or DMSO for 24 h. After the treatment, cell viability was determined by EZ-CyTox (tetrazolium salts, WST-1) cell viability assay kit (Daeil Lab Inc., Seoul, Korea), and BrdU cell proliferation assay kit (Cell Signaling Technology, Inc., Danvers, MA, USA). After the application, absorbance at 450 nm was measured using a microplate reader (Infinite M200; Tecan, Austria GmbH, Austria). Each experiment was done in triplicate wells and was repeated at least three times.

Flow cytometric analysis. SW480 and DKO1 cells were plated in a 6-well plate at $5x10^5$ cells/well and were incubated for 24 h at 37˚C before treatment with MA1. MA1 treated cells were collected and were resuspended in binding buffer (BD Biosciences, San Diego, CA, USA). These cells were incubated with 7-amino-actinomycin D (7-AAD) and Annexin V-APC (BD Biosciences) for 20 min at room temperature. To analyze the number of apoptotic cells, FACSCalibur flow cytometer (Becton-Dickinson) and WinMDI version 2.9 (The Scripps Research Institute, San Diego, CA, USA) were used.

DNA fragmentation. MA1-treated cells were collected and incubated with cell lysate buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) for 30 min on ice. The samples were then centrifuged at 12,00 x g for 30 min. RNase A was added to the resulting supernatant and was incubated for 2 h at 56˚C. Proteinase K was then added to the supernatant and was incubated for 2 h at 37˚C. An equal volume of isopropanol was added and was incubated at -80˚C overnight to precipitate the genomic DNA. The genomic DNA was loaded into 2% agarose gel and was stained with ethidium bromide. The DNA was visualized under UV light transilluminator.

Western blotting. MA1-treated cells were lysed in RIPA extraction solution with Halt™ phosphatase inhibitor and Halt™ protease inhibitor cocktails (Thermo Fisher Scientific, Rockford, IL, USA) for 30 min in an ice bath. The protein concentration of each lysate was measured using BCA™ protein assay (Thermo Fisher Scientific). Equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Antibodies against the following proteins were used: cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, -7, and -9, p53 upregulated modulator of apoptosis (PUMA), X-linked inhibitor of apoptosis protein (XIAP), Survivin, extracellular signal-regulated kinase1/2 (ERK1/2), phospho-ERK1/2, p38, phospho-p38, c-Jun NH2-terminal kinase (JNK), phospho-JNK (Cell Signaling Technology, Inc.), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, CA, USA). Protein bands were developed using an ECL reagent (Amersham, Arlington Heights, IL, USA) and the luminescent image analyzer LAS-4000 (Fuji Film, Tokyo, Japan).

Wound healing assay. Cell migration was evaluated with a wound-healing assay using culture inserts (Ibidi, Regensburg, Germany). Cells were plated into culture-inserts and a wound gap was created by removing inserts after 24 h of incubation. Wound widths were measured using images photographed by an inverted microscope at 0, 24 and 48 h time-points.

Transwell invasion assay. SW480 and DKO1 cells were seeded in the upper well of Transwell filter chambers (8.0-μm pore size; Costar, Cambridge, MA, USA) with gelatin coating. After 24 h of incubation, invading cells on the lower surface of the upper chamber were fixed with 70% ethanol and stained with Hemacolor® Rapid staining solution (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol. The stained cells were counted under a light microscope.

Results

MA1 inhibits the growth of human colorectal cancer cells. We first investigated the effect of MA1 on the growth of the human colorectal cancer cell lines, SW480 and DKO1. Human colorectal cancer cells were exposed to different concentrations of MA1 (0-2 μM) for 24 h. The effect of MA1 on SW480 and DKO1 cells was determined by the WST-1 cell viability assay, inverted microscopy and the BrdU incorporation assay. The WST-1 assays and inverted microscopy showed that MA1 treatment inhibited the growth of SW480 and DKO1 cells, as compared to control cells (Fig. 1A and B). Moreover, the BrdU assays also showed similar results, as 1 and 1.25 μM of MA1 treatment resulted in a slower BrdU incorporation rate for SW480 and DKO1 cells as compared to control cells ($p<0.05$, $p<0.01$) (Fig. 1C). These results indicate that MA1 inhibits the proliferation of human colorectal cancer cells.

MA1 induces apoptosis and cell cycle arrest in human colorectal cancer cells. In order to determine whether MA1-induced human colorectal cancer cell death is related to apoptosis and cell cycle arrest, we performed flow cytometric analyses and a DNA fragmentation assay. Human colorectal cancer cells were exposed to different concentra-

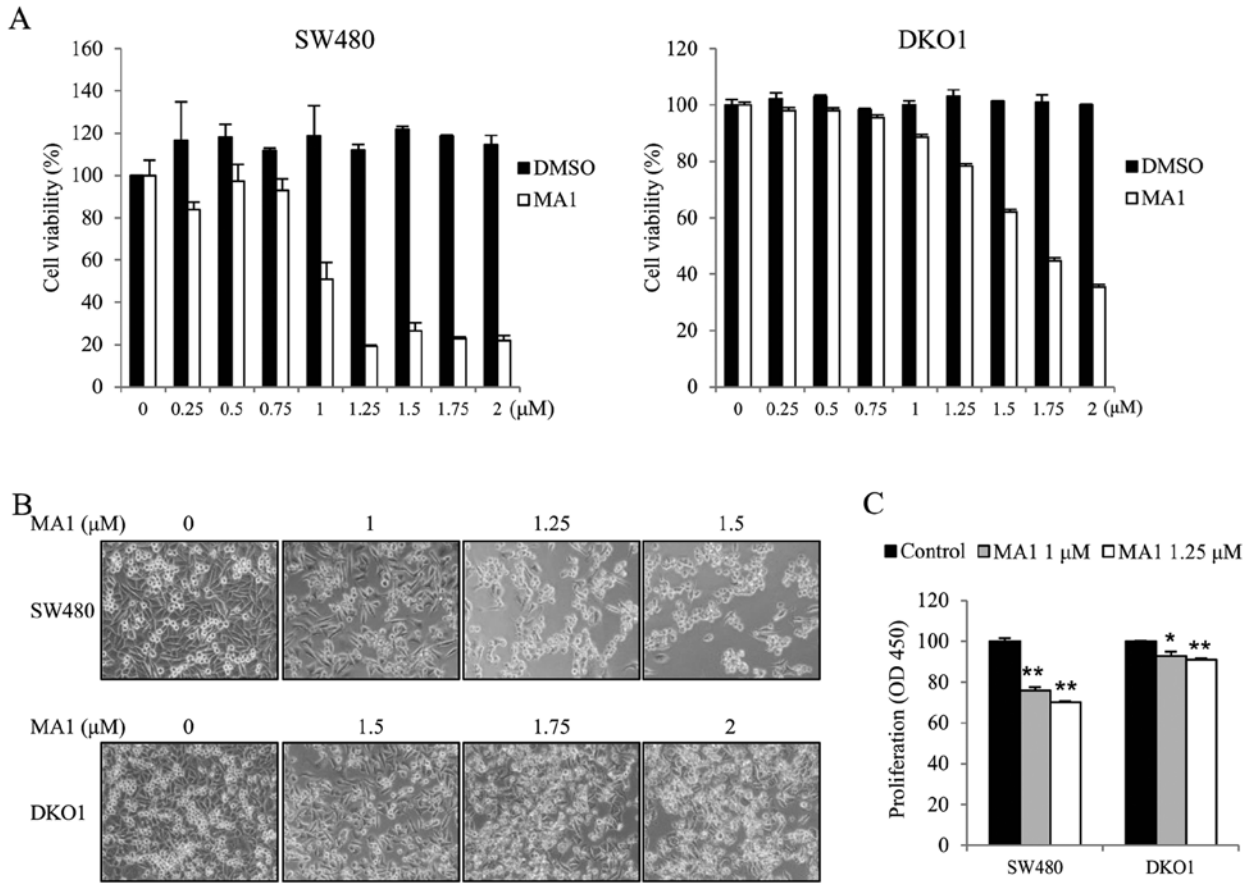

Figure 1. Impact of Malformin A1 on the viability of human colorectal cancer cells. (A) Human colorectal cancer cell lines, SW480 and DKO1, were cultured with or without 0-2 μM MA1 for 24 h. Thereafter, cell viability was measured by the WST-1 procedure. (B) SW480 and DKO1 cells were treated with the indicated concentrations of MA1 for 24 h. Cell morphology was observed under an inverted microscope and images were obtained. (C) SW480 and DKO1 cells were cultured with or without MA1 (0, 1 and 1.25 μM) for 24 h. Thereafter, cell viability was measured by the BrdU proliferation assay procedure (*p<0.05, **p<0.01 vs control). MA1, Malformin A1.

tions of MA1 (0-1.5 μM) for 24 h. The proportion of early and late apoptotic cells were dose-dependently greater in MA1-treated SW480 and DKO1 cells compared to non-treated control cells (Fig. 2A). Additionally, MA1 treatment induced cell cycle arrest at the sub-G1 phase in SW480 and DKO1 cells (Fig. 2B). MA1 treatment (0.75-1.5 μM) induced an increase in DNA fragmentation in human colorectal cancer cells as compared to the non-treated control cells (Fig. 2C). To determine the activation of caspases, key enzymes in apoptosis, we further investigated caspase-specific activities. The cleaved PARP, caspase-3, -7, and -9 expressions were upregulated dose-dependently in SW480 and DKO1 cells after MA1 treatment (Fig. 2D). We further examined whether MA1 treatment-induced apoptosis is associated with the modulation of apoptosis regulatory proteins. As shown in Fig. 2D, MA1 treatment led to the increase in the pro-apoptotic protein, PUMA and the decrease in anti-apoptotic proteins, XIAP and Survivin in SW480 and DKO1 cells.

Pan-caspase inhibitor attenuates the MA1-induced apoptotic effect on human colorectal cancer cells. The pan-caspase inhibitor, Z-VAD-FMK (10 μM), was used to determine whether the apoptosis was induced by MA1 treatment. The increase in early and late stage apoptotic cells by MA1 treatment was decreased in SW480 and DKO1 cells when treated with Z-VAD-FMK (Fig. 3A). Furthermore, Z-VAD-FMK abrogated the MA1-induced caspase-3 and PARP activation (Fig. 3B). Therefore, MA1 treatment works by inducing apoptosis by directly activating caspases in human colorectal cancer cells.

MA1 inhibits the migration and invasion of human colorectal cancer cells. To investigate the effect of MA1 on the migration ability of human colorectal cancer cells, SW480 and DKO1 cells were treated with different concentrations of MA1 (0-1.25 μM) for 24 and 48 h, followed by the scratch wound healing motility assay. The results showed that SW480 and DKO1 cells treated with MA1 for 24 and 48 h migrated significantly slower than non-treated control cells (p<0.05, p<0.01) (Fig. 4A), suggesting that MA1 could significantly decrease cell migration. The effect of MA1 on the invasiveness of SW480 and DKO1 cells was further detected by using a Transwell invasion assay. Compared to the non-treated control cells, the number of invasive SW480 and DKO1 cells treated with 0.1 and 0.5 μM MA1 decreased significantly (p<0.05) (Fig. 4B), indicating that MA1 could significantly decrease the invasiveness of human colorectal cancer cells.

Impact of MA1 on oncogenic signaling pathways in human colorectal cancer cells. To examine whether MA1 affects

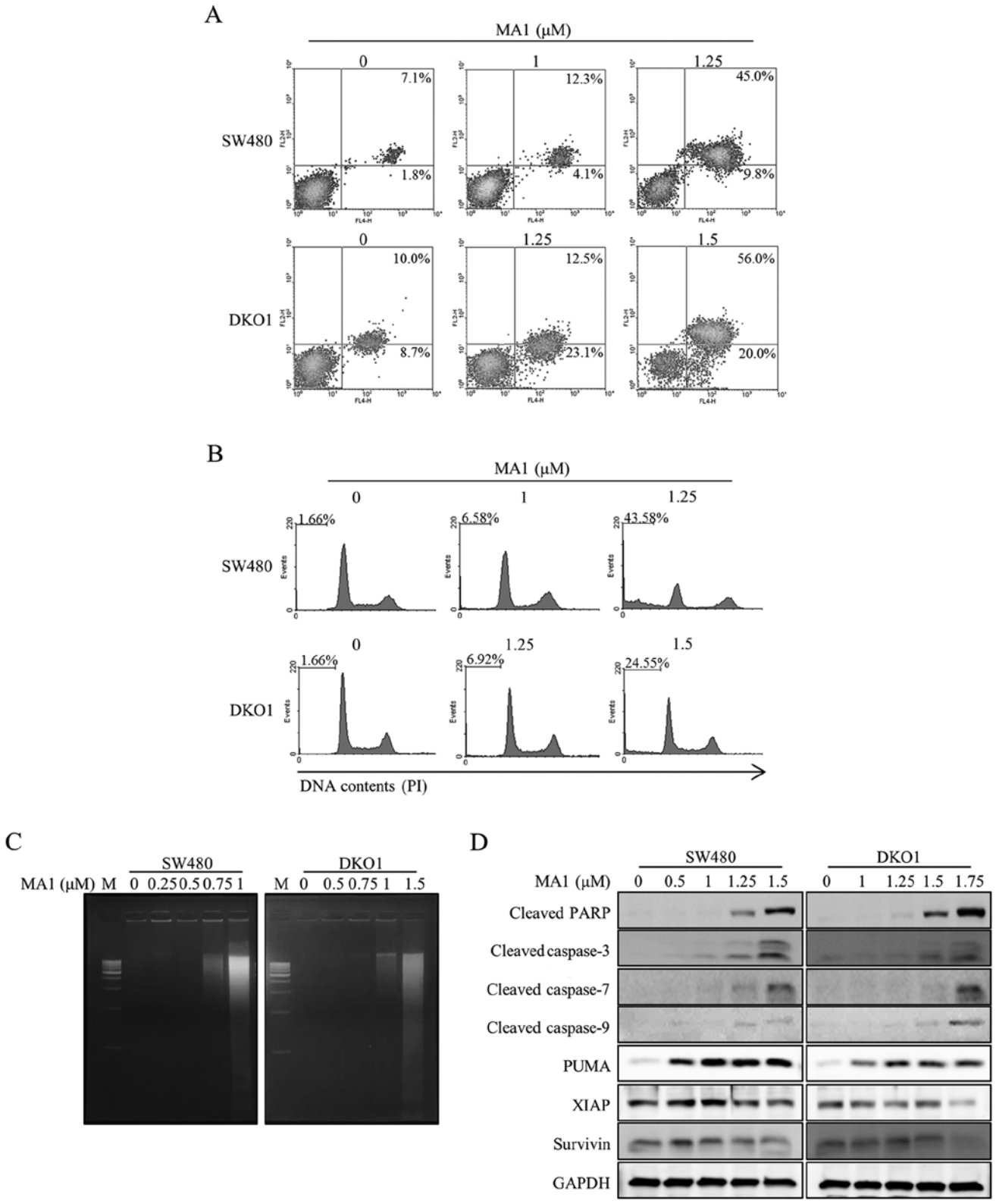

Figure 2. MA1 induces apoptosis in human colorectal cancer cells. SW480 and DKO1 cells were treated with the indicated concentrations of MA1 for 24 h. (A) Treated cells were stained with Annexin V and then the number of apoptotic cells were determined. (B) Treated cells stained with propidium iodide (PI). Flow cytometric analysis was performed to evaluate the cells in the sub-G1 phase of the cell cycle. (C) The cells were harvested and genomic DNA was extracted from the cells of each treatment. Genomic DNA was separated on a 2% agarose gel, stained with ethidium bromide, and visualized under a UV light transilluminator. (D) Protein levels were detected by western blotting. MA1, Malformin A1; PARP, poly(ADP-ribose) polymerase; PUMA, p53 upregulated modulator of apoptosis; XIAP, X-linked inhibitor of apoptosis protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

oncogenic signaling pathways in human colorectal cancer cells, we determined the phosphorylation levels of MAPK signaling proteins. For these experiments, the human colorectal cancer cells were exposed to MA1 (1.5 μM) for different times (0-6 h).

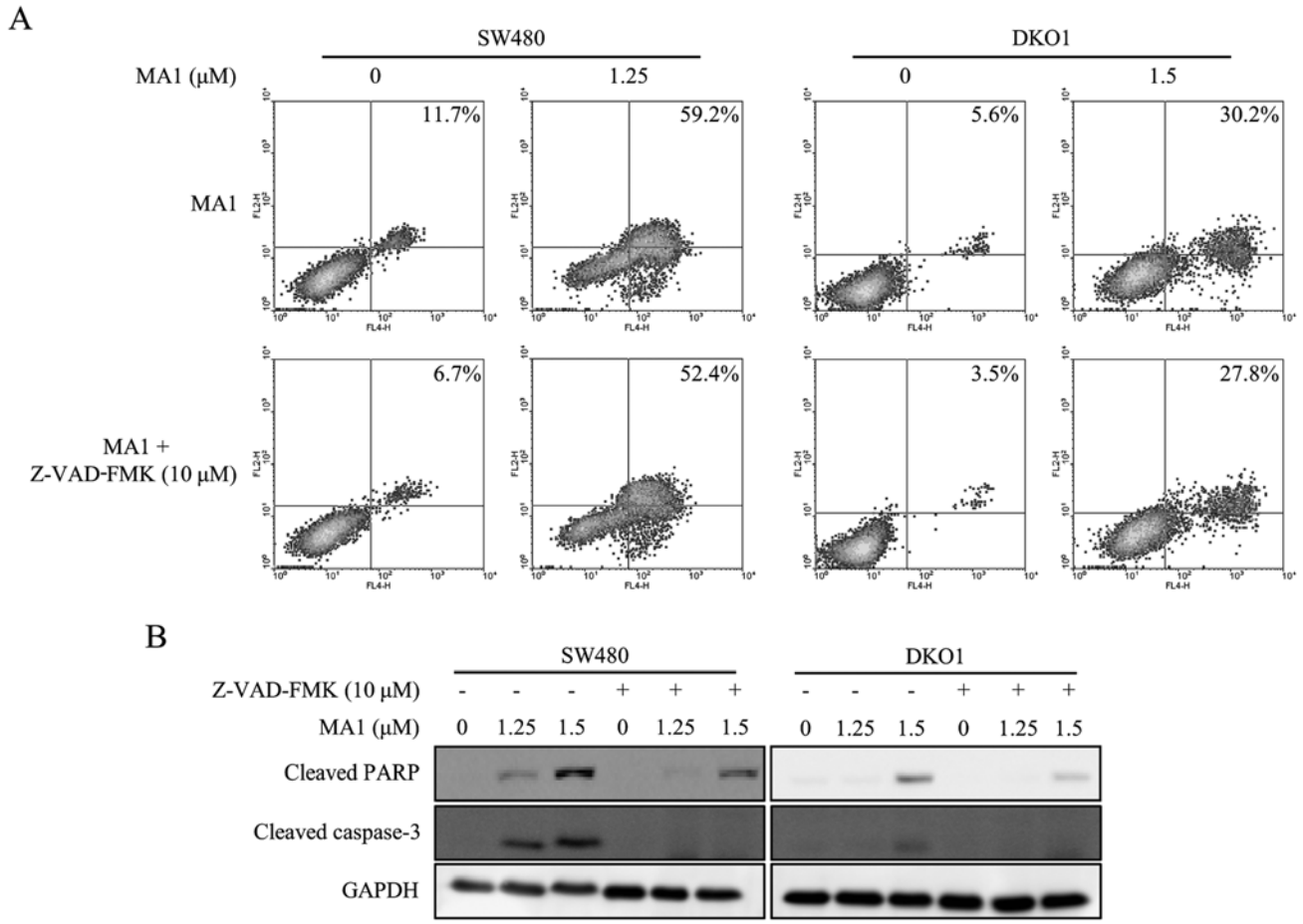

Figure 3. Apoptosis induced by MA1 is inhibited by the pan-caspase inhibitor Z-VAD-FMK in human colorectal cancer cells. SW480 and DKO1 cells were pretreated with 10 μM of the pan-caspase inhibitor Z-VAD-FMK for 2 h and then exposed to the indicated concentrations of MA1 for 24 h. (A) Treated cells stained with Annexin V and then analyzed for the number of apoptotic cells. (B) Protein levels were detected by western blotting. MA1, Malformin A1; PARP, poly(ADP-ribose) polymerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The phosphorylation levels of p38 was upregulated by MA1 treatment in SW480 and DKO1 cells but the phosphorylation levels of JNK and ERK1/2 were not changed by MA1 treatment (Fig. 5A). Subsequently, the inhibitor of the p38 signaling pathway, SB203580, was used to determine whether this pathway was involved in MA1 action. SB203580 attenuated the MA1-induced p38 phosphorylation, as well as caspase-3 and PARP activation (Fig. 5B). We also examined whether SB203580 affects the modulation of apoptosis regulatory proteins. As shown in Fig. 5B, SB203580 led to the decrease in the pro-apoptotic protein, PUMA and increase in the anti-apoptotic proteins, XIAP and Survivin. These results suggest that the apoptotic effect of MA1 on human colorectal cancer cells is mediated by the activation of p38 signaling pathway.

Discussion

Plant-associated microorganisms are known to produce a variety of metabolites with novel structures and interesting biological activities (4-8). MA1 isolated from the fungus *A. niger* has been found to possess a range of bioactive properties, which includes enhancing fibrinolytic activity, and acting as an antibacterial/viral/fungal agent (13-18). Furthermore, MA1 exhibits a strong cytotoxic effect against various human cancer cell lines (9,22-24). This activity is believed to be related to its anticancer properties such as inhibiting cell proliferation, inducing apoptosis, arresting the cell cycle and inhibiting cell migration and invasion (25,26). Therefore, searching for the antitumor targets of MA1 and its signal transduction mechanisms is crucial for the development of natural drugs and the treatment of cancer.

In our study, MA1 inhibited cell proliferation and induced apoptosis and cell cycle arrest in human colorectal cancer cells. These results suggest that MA1 can alter the oncogenic phenotypes of human colorectal cancer cells.

Apoptosis is a type of cell death that results in the orderly and efficient removal of damaged cells. However, deregulation of apoptosis is responsible for cancer development, progression, and treatment resistance (27,28). Apoptosis is a complex process that results from multiple genetic alterations in apoptotic regulatory genes, and thus, we studied the effects of MA1 on apoptosis in human colorectal cancer cells. Our study showed that caspase-3, -7, -9, and PARP in human colorectal cancer cells were activated by MA1 treatment. Furthermore, MA1 treatment led to the increase in the pro-apoptotic protein, PUMA and a decrease in anti-apoptotic protein, XIAP and Survivin. Additionally, we used the pan-caspase inhibitor, Z-VAD-FMK, to determine whether MA1-induced apoptosis

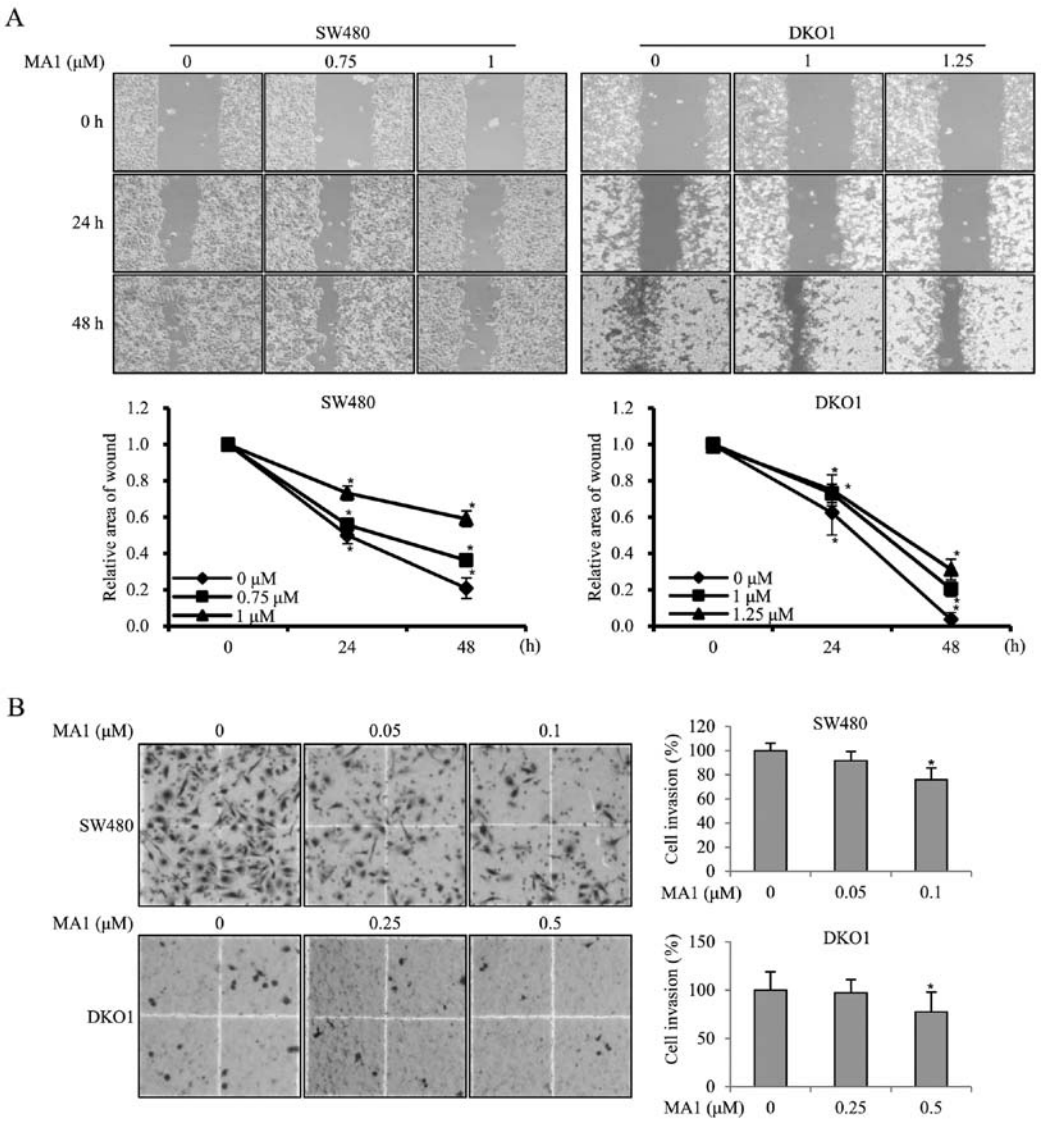

Figure 4. MA1 inhibits the migration and invasion of human colorectal cancer cells. (A) SW480 and DKO1 cells were treated with the indicated concentrations of MA1 for 24 and 48 h. Migration was analyzed using the wound healing assay. Graphs of cell migration display relative healing distances (mean ± SE, *p<0.05). (B) Cells which penetrated the membrane were fixed and stained after 24 h (mean ± SE, *p<0.05). MA1, Malformin A1.

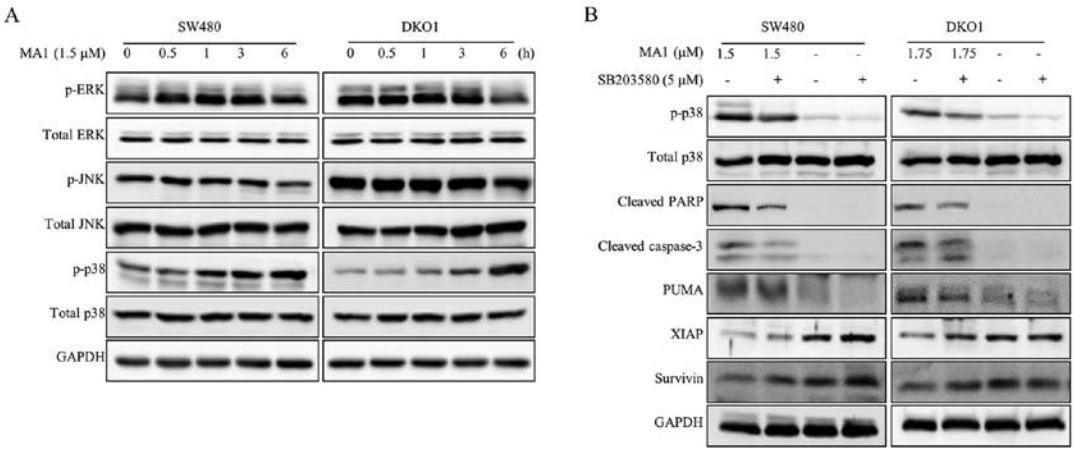

Figure 5. Impact of MA1 on oncogenic signaling pathways in human colorectal cancer cells. (A) SW480 and DKO1 cells were treated for the indicated times (0-6 h) with 1.5 μM of MA1. Cell lysates were prepared and subjected to western blotting using phospho-ERK1/2, phospho-p38, and phospho-JNK antibodies. (B) SW480 and DKO1 cells were pretreated with SB203580 (a p38 inhibitor) and then exposed to MA1 for 24 h. Cell lysates were prepared and subjected to western blotting using cleaved PARP, cleaved caspase-3, PUMA, XIAP, and Survivin antibodies. MA1, Malformin A1; ERK1/2, extracellular signal-regulated kinase1/2; JNK, c-Jun NH2-terminal kinase; PARP, poly(ADP-ribose) polymerase; PUMA, p53 upregulated modulator of apoptosis; XIAP, X-linked inhibitor of apoptosis protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

was related to caspase activation. Our study showed that the pan-caspase inhibitor abrogated the MA1-induced increase in early and late stage apoptotic cells, as well as the activation of caspase-3 and PARP. Therefore, MA1 treatment exerts its effect on apoptosis by directly inducing caspase activity in human colorectal cancer cells. Our study also demonstrated that MA1 treatment induced cell cycle arrest at the sub-G1 phase in human colorectal cancer cells. Previously, MA1 and Malformin C were found to abrogate bleomycin-induced G2 arrest, resulting in a drastic decrease in cells at the G2 phase and increase in cells in the sub-G1 phase (21,22).

The regulation of cell migration and invasion is crucial for various physiological processes including embryonic development, angiogenesis, tissue repair, and the immune response. Its loss is also a principal hallmark of cancer cells (25,26). Our study showed that MA1 treatment suppressed tumor cell migration and invasion in human colorectal cancer cells.

Given the effects of MA1 on cancer cell behavior, we studied the effects of MA1 treatment on the activation of intracellular signaling pathways involved in the alteration of the oncogenic phenotype of colorectal cancer cells. Previously, MA1 induced apoptosis, necrosis, and autophagy through the activation of AMPK/mTOR signaling pathway in prostatic cancer cells (24). Furthermore, the inhibitor of the phosphatidylinositol 3-kinase signaling pathway abolished the enhancement of cellular fibrinolytic activity by MA1 in human leukemia U937 cells. That said, the inhibitor of MAPK signaling showed minimal effects on MA1 treated leukemia cells (13). MAPK signaling pathways, including ERK1/2, JNK, and p38 MAPK, play a critical role in many physiologic processes, including cell growth, differentiation, migration, proliferation, apoptosis, and cell cycle progression (29,30). Moreover, MAPK signaling pathways play important roles in cancer progression, and particularly in determining the outcome and sensitivity to anticancer therapies (29,30). Many chemotherapeutic agents such as cyclophosphamide and oxaliplatin induce apoptosis through activating p38 MAPK pathway (31,32). We observed that MA1 treatment increased the phosphorylation level of p38, but the phosphorylation levels of JNK and ERK1/2 were unchanged. The inhibitor of the p38 signaling pathway, SB203580, was used to determine whether this pathway was involved in MA1 action. SB203580 abrogated the MA1-induced p38 phosphorylation, as well as the activation of caspase-3 and PARP. Moreover, SB203580 led to a decrease in the pro-apoptotic protein, PUMA and an increase in the anti-apoptotic proteins, XIAP and Survivin. Thus, these results confirm that MA1 promotes apoptosis by caspase activation and the modulation of apoptosis regulatory proteins through the stimulation of the p38 signaling pathway in human colorectal cancer cells.

Taken together, our results indicate that MA1 treatment suppresses tumor progression by the inhibition of proliferation and induction of apoptosis through the activation of the p38 signaling pathway in human colorectal cancer cells.

Acknowledgements

This study was supported by a grant (GRI16075-3) of the Chonnam National University Hospital-Gwangju Institute of Science and Technology (CNUH-GIST).

References

1. Kim ER and Kim YH: Clinical application of genetics in management of colorectal cancer. Intest Res 12: 184-193, 2014.
2. Park SH, Song CW, Kim YB, Kim YS, Chun HR, Lee JH, Seol WJ, Yoon HS, Lee MK, Bhang CS, et al: Clinicopathological characteristics of colon cancer diagnosed at primary health care institutions. Intest Res 12: 131-138, 2014.
3. Lin OS, Kozarek RA and Cha JM: Impact of sigmoidoscopy and colonoscopy on colorectal cancer incidence and mortality: An evidence-based review of published prospective and retrospective studies. Lancet 383: 1490-1502, 2014.
4. Gunatilaka AAL: Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69: 509-526, 2006.
5. Schulz B, Boyle C, Draeger S, Römmert AK and Krohn K: Endophytic fungi: A source of novel biologically active secondary metabolites. Mycol Res 106: 996-1004, 2002.
6. Strobel G, Daisy B, Castillo U and Harper J: Natural products from endophytic microorganisms. J Nat Prod 67: 257-268, 2004.
7. Tan RX and Zou WX: Endophytes: A rich source of functional metabolites. Nat Prod Rep 18: 448-459, 2001.
8. Chomcheon P, Wiyakrutta S, Sriubolmas N, Ngamrojanavanich N, Isarangkul D and Kittakoop P: 3-Nitropropionic acid (3-NPA), a potent antimycobacterial agent from endophytic fungi: Is 3-NPA in some plants produced by endophytes? J Nat Prod 68: 1103-1105, 2005.
9. Li XB, Xie F, Liu SS, Li Y, Zhou JC, Liu YQ, Yuan HQ and Lou HX: Naphtho-γ-pyrones from Endophyte *Aspergillus niger* occurring in the liverwort *Heteroscyphus tener* (Steph.) Schiffn. Chem Biodivers 10: 1193-1201, 2013.
10. Curtis RW, Stevenson WR and Tuite J: Malformin in *Aspergillus niger*-infected onion bulbs (Allium cepa). Appl Microbiol 28: 362-365, 1974.
11. Kim KW, Sugawara F, Yoshida S, Murofushi N, Takahashi N and Curtis RW: Structure of malformin B, a phytotoxic metabolite produced by *Aspergillus niger*. Biosci Biotechnol Biochem 57: 787-791, 1993.
12. Kim KW, Sugawara F, Yoshida S, Murofushi N, Takahashi N and Curtis RW: Structure of Malformin A, a phytotoxic metabolite produced by *Aspergillus niger*. Biosci Biotechnol Biochem 57: 240-243, 1993.
13. Ma YM, Liang XA, Zhang HC and Liu R: Cytotoxic and antibiotic cyclic pentapeptide from an endophytic *Aspergillus tamarii* of *Ficus carica*. J Agric Food Chem 64: 3789-3793, 2016.
14. Zhou X, Fang W, Tan S, Lin X, Xun T, Yang B, Liu S and Liu Y: Aspernigrins with anti-HIV-1 activities from the marine-derived fungus *Aspergillus niger* SCSIO Jcsw6F30. Bioorg Med Chem Lett 26: 361-365, 2016.
15. Tan QW, Gao FL, Wang FR and Chen QJ: Anti-TMV activity of malformin A1, a cyclic penta-peptide produced by an endophytic fungus *Aspergillus tubingensis* FJBJ11. Int J Mol Sci 16: 5750-5761, 2015.
16. Koizumi Y, Nagai K, Hasumi K, Kuba K and Sugiyama T: Structure-activity relationship of cyclic pentapeptide malformins as fibrinolysis enhancers. Bioorg Med Chem Lett 26: 5267-5271, 2016.
17. Koizumi Y, Fukudome H and Hasumi K: Fibrinolytic activation promoted by the cyclopentapeptide malformin: Involvement of cytoskeletal reorganization. Biol Pharm Bull 34: 1426-1431, 2011.
18. Koizumi Y and Hasumi K: Enhancement of fibrinolytic activity of U937 cells by malformin A1. J Antibiot (Tokyo) 55: 78-82, 2002.
19. Herbert JM, Savi P, Lalé A, Laplace MC, Baudry N, Pereillo JM and Emonds-Alt X: Malformin-A1 inhibits the binding of interleukin-1 beta (IL1 beta) and suppresses the expression of tissue factor in human endothelial cells and monocytes. Biochem Pharmacol 48: 1211-1217, 1994.
20. Bannon PG, Dawes J and Dean RT: Malformin A prevents IL-1 induced endothelial changes by inhibition of protein synthesis. Thromb Haemost 72: 482-483, 1994.
21. Kojima Y, Sunazuka T, Nagai K, Julfakyan K, Fukuda T, Tomoda H and Omura S: Total synthesis of malformin C, an inhibitor of bleomycin-induced G2 arrest. J Antibiot (Tokyo) 61: 297-302, 2008.
22. Hagimori K, Fukuda T, Hasegawa Y, Omura S and Tomoda H: Fungal malformins inhibit bleomycin-induced G2 checkpoint in Jurkat cells. Biol Pharm Bull 30: 1379-1383, 2007.

23. Zhan J, Gunaherath GM, Wijeratne EM and Gunatilaka AA: Asperpyrone D and other metabolites of the plant-associated fungal strain *Aspergillus tubingensis*. Phytochemistry 68: 368-372, 2007.
24. Liu Y, Wang M, Wang D, Li X, Wang W, Lou H and Yuan H: Malformin A1 promotes cell death through induction of apoptosis, necrosis and autophagy in prostate cancer cells. Cancer Chemother Pharmacol 77: 63-75, 2016.
25. Chambers AF, Groom AC and MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2: 563-572, 2002.
26. Brábek J, Mierke CT, Rösel D, Veselý P and Fabry B: The role of the tissue microenvironment in the regulation of cancer cell motility and invasion. Cell Commun Signal 8: 22, 2010.
27. Kiechle FL and Zhang X: Apoptosis: Biochemical aspects and clinical implications. Clin Chim Acta 326: 27-45, 2002.
28. Thompson CB: Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456-1462, 1995.
29. Cargnello M and Roux PP: Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75: 50-83, 2011.
30. Haagenson KK and Wu GS: Mitogen activated protein kinase phosphatases and cancer. Cancer Biol Ther 9: 337-340, 2010.
31. Pang H, Cai L, Yang Y, Chen X, Sui G and Zhao C: Knockdown of osteopontin chemosensitizes MDA-MB-231 cells to cyclophosphamide by enhancing apoptosis through activating p38 MAPK pathway. Cancer Biother Radiopharm 26: 165-173, 2011.
32. Chiu SJ, Chao JI, Lee YJ and Hsu TS: Regulation of gamma-H2AX and securin contribute to apoptosis by oxaliplatin via a p38 mitogen-activated protein kinase-dependent pathway in human colorectal cancer cells. Toxicol Lett 179: 63-70, 2008.