

# Enhanced anticancer effects of a mixture of low-dose mushrooms and *Panax ginseng* root extracts in human colorectal cancer cells

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Received February 7, 2017; Accepted June 30, 2017

DOI: 10.3892/or.2017.5796

**Abstract.** Worldwide, colorectal cancer is the third most common cancer in men and the second most common in women. As conventional colorectal cancer therapies result in various side effects, there is a need for adjuvant therapy that can enhance the conventional therapies without complications. In this study, we investigated the anticancer effects of combined mixture of the several medicinal mushrooms and *Panax ginseng* root extracts (also called Amex7) as an adjuvant compound in the treatment of human colorectal cancer. We observed the *in vivo* inhibitory effect of Amex7 (1.25, 6.25, and 12.5 ml/kg, oral administration, twice daily) on tumor growth in a mouse model xenografted with HT-29 human colorectal cancer cells. *In vitro*, at 6, 12, and 24 h after 4% Amex7 treatment, we analyzed cell cycle by flow cytometry and the expression levels of cell cycle progression, apoptosis, and DNA damage repair-related proteins using immunoblotting and immunofluorescence staining in HT-29 cell line. As a result, Amex7 significantly suppressed tumor growth in HT-29 human colorectal cancer cells and xenografts. *In vitro*, Amex7 induced G2/M arrest through the regulation of cell cycle proteins and cell death by apoptosis and autophagy. Additionally, Amex7 consistently induced DNA damage and delayed the repair of Amex7-induced DNA damage by reducing the level of HR repair proteins. In

conclusion, Amex7 enhanced anticancer effects through the induction of G2/M arrest and cell death, including apoptosis and autophagy. Furthermore, Amex7 impaired DNA damage repair. The present study provides a scientific rationale for the clinical use of a combined mixture of medicinal mushrooms and *P. ginseng* root extracts as an adjuvant treatment in human colorectal cancer.

## Introduction

In 2012, colorectal cancer (CRC) was the third most common cancer in men (746,000 case, 10.0% of the total) and the second most common cancer in women (614,000 cases, 9.2% of the total) worldwide (1). The primary treatment for early stage CRC is surgery; however, in more advanced stages of CRC, adjuvant therapy is recommended to increase overall survival rate and prevent recurrence as either local or distant metastasis (2). However, adjuvant chemotherapy harms normal cells, increases treatment resistance, and causes side effects such as vomiting, nausea, diarrhea, and alopecia (3). Several papers have presented candidate drugs from natural products to overcome multidrug resistance in cancer chemotherapy (4-7). Especially, Belcaro *et al* showed that curcumin reduced semi-quantitative evaluation of cancer chemotherapy side effects including vomiting, nausea, diarrhea, and malnutrition (8). Thus, a promising effective and safe strategy to enhance cancer treatment is the development of natural compound-based cancer therapies.

Natural products have been mainly used as functional foods in order to improve health through activation of physiological functions. In some cases, these have been developed as medicines for several diseases, including cancer, diabetes, and hyperlipidemia (9-11). Among the various natural products, mushrooms and ginsengs are good resources of natural medicines that are non-toxic to normal cells but cytotoxic to cancer cells. *Phellinus linteus* has been demonstrated to inhibit tumor proliferation by decreasing expression of cyclin B1 and cyclin-dependent kinases (CDK2, 4, and 6), induce apoptosis through the activation of caspase-2, -3, and -7 and cleaved-PARP in various cancer cells (12), and also suppress tumor growth and pulmonary metastasis in C57BL/6 mice intravenously injected with melanoma cells (13). Furthermore, *Inonotus obliquus* (Chaga mushroom) has been reported to prevent cell proliferation via the induction of G1 cell cycle arrest in HT-29 human

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**Key words:** anticancer effect, G2/M arrest, human colorectal cancer, mushroom, *Panax ginseng*, apoptosis, autophagy, DNA damage repair

colon cancer cells (14). Other mushrooms have been shown to be synergistically adjuvant by exerting immunomodulatory and anti-inflammatory effects (15). Additionally, *P. ginseng* is commonly used by itself or in combination with other medical components. Several studies have demonstrated that the root of *P. ginseng* has anti-inflammatory, anti-diabetes, and anticancer activity (16-18). The previous studies mainly describe a single effect of medicinal mushrooms or *P. ginseng* extract in various cancer cells. However, anticancer effects in a combination of several medicinal mushrooms and/or *P. ginseng* extracts are still unclear.

Our study, which focused on the cell cycle and cell death pathways, investigated whether a combination of seven medicinal mushrooms and *P. ginseng* root extracts (Amex7) enhanced anticancer effects in human colorectal cancer cells when used at a concentration lower than that used in previous studies. Our data may contribute to provide a scientific rationale for the clinical use of the combination of medicinal mushrooms and *P. ginseng* root extracts as an adjuvant compound in human colorectal cancer.

## Materials and methods

**Reagents.** Anti-Rad51 and anti-excision repair cross-complementation group 1 (ERCC1) antibodies were purchased from Abcam (Cambridge, MA, USA). Cyclin B1, cyclin A2, cyclin D1, cleaved-PARP (Asp214), phospho-p53 (Ser15), phospho-cdc2 (Tyr15), LC3A/B, sequestosome 1 (SQSTM1)/p62 and cyclin-dependent kinase 2 (CDK2) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-cyclin-dependent kinase 4 (CDK4) and anti-cyclin-dependent kinase 6 (CDK6) were obtained from Bethyl (Montgomery, TX, USA). Cyclin E, Bcl-2, Bcl-x<sub>L</sub>, Ku70, Ku80, anti-p53, survivin, DNA-PKcs, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Histone H2AX (γ-H2AX, Ser139) was provided by Millipore (Billerica, MA, USA). Alexa Fluor® 488 goat anti-mouse IgG (H+L) and Alexa Fluor 594 goat anti-rabbit IgG (H+L) secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were obtained from Welgene (Daegu, South Korea).

**Preparation of the combined mixture of various mushrooms and *P. ginseng* root extracts (Amex7).** The combined mixture of seven mushrooms and *P. ginseng* root extracts (also called Amex7) used in this study was obtained from Han Kook Shin Yak Pharmaceutical Co., Ltd. In the first step, *P. ginseng* root was extracted with purified water at 80-100°C for 2-3 h. The water extract from *P. ginseng* root was filtered through a 150-μm pore size, and then evaporated in a vacuum below 60°C. In the second step, the extract of *P. ginseng* was mixed with extract powders of *Phellinus linteus*, *Lentinula edodes*, *Trametes versicolor*, *Cordyceps militaris*, *Sparassis crispa*, *Hericium erinaceum*, and *Grifola frondosa* at 200 rpm for 20 min. The mixture was filtered through a 5-μm pore size and

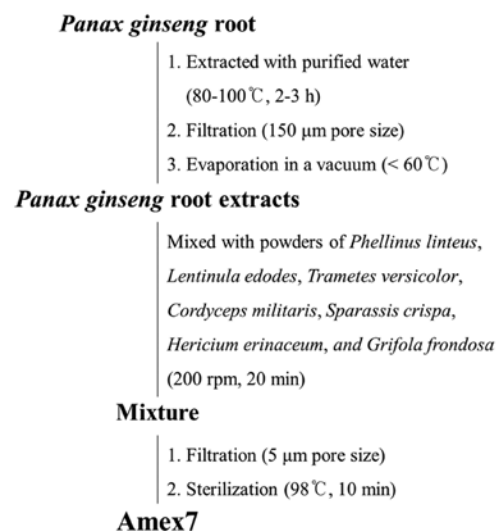


Figure 1. Preparation of a combined mixture of various mushrooms and *P. ginseng* root extracts (Amex7).

Table I. Combined ratio of several compounds in Amex7.

Compound	Combination percentage (%)	Volume (ml)
<i>Phellinus linteus</i>	1.340	1.005
<i>Lentinula edodes</i>	0.400	0.300
<i>Trametes versicolor</i>	0.400	0.300
<i>Cordyceps militaris</i>	0.400	0.300
<i>Sparassis crispa</i>	0.400	0.300
<i>Hericium erinaceum</i>	0.400	0.300
<i>Grifola frondosa</i>	0.400	0.300
<i>Panax ginseng</i> root	0.130	0.098
Pure water	81.464	61.098
Others	14.666	10.999
Total	100.000	75.000

subsequently sterilized at 98°C for 10 min. Fig. 1 shows the preparation process of Amex7 and Table I lists the combined ratio of several compounds in Amex7.

**Cell lines and cell culture.** HT-29 human colorectal cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). HT-29 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in an atmosphere of 5% CO<sub>2</sub> at 37°C.

**Drug treatment.** Amex7, a combined mixture of several medicinal mushrooms and *P. ginseng* extracts, was kindly provided by Han Kook Shin Yak Co., Ltd. For *in vivo* experiments, Amex7 was freshly mixed with drinking water at 1.25, 6.25, and 12.5 ml/kg. For *in vitro* experiments, Amex7 was suspended at a concentration of 1, 2, 4, 8, and 16% in culture medium.

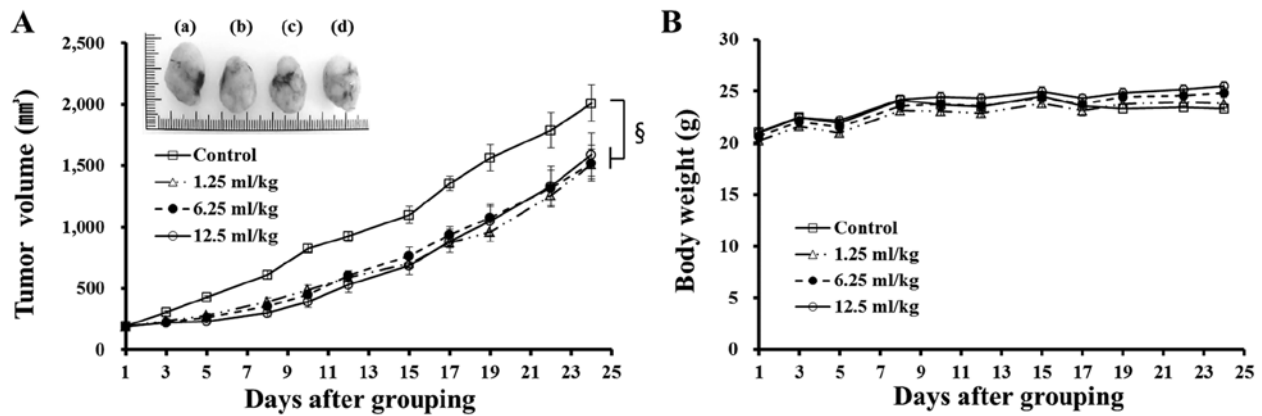


Figure 2. Amex7 inhibits tumor growth in HT-29 human colorectal cancer xenografts. Mice bearing 180–200 mm<sup>3</sup> tumors were administered twice daily with Amex7 (1.25, 6.25, and 12.5 ml/kg) until the mean tumor volume in the control group reached approximately 2,000 mm<sup>3</sup>. The graphs indicate (A) the tumor volumes and (B) body weights for tumors in the control and three concentrations of Amex7-treated groups. Statistically significant differences are shown: §p<0.001 vs. control group.

**Tumor xenografts in Balb/c nude mice.** Balb/c nude mice (5-week-old males) were obtained from Nara Biotech Co. (Seoul, South Korea) and maintained in a laminar airflow cabinet under specific pathogen-free conditions. Mice xenografted with HT-29 cells were obtained by subcutaneous inoculation of 3×10<sup>6</sup> HT-29 cells into the right hind leg. After tumor implantation, we monitored the condition of the animals twice daily and prepared analgesics to minimize animal suffering. When the tumor attained a volume of approximately 180–200 mm<sup>3</sup>, the mice were randomly divided into four groups (n=10): a) control, b) 1.25 ml/kg, c) 6.25 ml/kg, and d) 12.5 ml/kg. The tumor volume (V) was calculated using the standard formula:  $V \text{ (mm}^3\text{)} = \pi/6 \times (\text{shortest diameter})^2 \times (\text{longest diameter})$ . Mice were euthanized by CO<sub>2</sub> inhalation when the average tumor volume of the control group reached 2,000 mm<sup>3</sup>.

**Cell count assay.** Cells were seeded in 60-mm culture dishes at 5×10<sup>4</sup> per well and cultured overnight. Then, the cells were treated with serial concentrations of Amex7 (0, 1, 2, 4, 8, and 16%) for 6, 12, and 24 h. At the indicated time points, cells were washed with phosphate buffered saline (PBS), harvested, and immediately stained with 0.4% trypan blue. Viable cells were quantified using a hemocytometer under an inverted microscope. Cell viability of control group was set at 100% and the effects of Amex7 on cell viability were expressed relative to the control group.

**Cell morphology.** Cells were seeded at a density of 3×10<sup>6</sup> cells/dish in a 100π dish and incubated for 24 h. The cells were treated with 4% Amex7 and incubated for a further 6, 12, and 24 h. The cells were observed by light microscopy (x200) (Motic AE31 microscope, Ted Pella Inc., Redding, CA, USA).

**Cell cycle analysis.** Cells were treated with 4% Amex7, incubated for 6, 12, and 24 h, harvested, stained with propidium iodide (1 mg/ml) according to the manufacturer's protocol, and then analyzed using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). A minimum of 10,000 cells were counted for each sample and data analysis was performed using the CellQuest software.

**Immunoblotting.** The cells were lysed in radioimmuno-precipitation assay (RIPA) buffer and the proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes blots were blocked with 5% (v/v) skim milk in PBS with 0.1% Tween-20, incubated with the indicated primary (1:1,000 dilution) and secondary antibodies (1:1,000 dilution), developed using enhanced chemiluminescence (ECL) immunoblotting substrate (Pierce, Rockford, IL, USA), and imaged with the ImageQuant LAS-4000 mini (GE Healthcare, Fairfield, CT, USA). The signal intensity of the bands was measured with the Multi Gauge image analysis software (Fujifilm, Tokyo, Japan).

**Immunofluorescence and confocal microscopy.** HT-29 cells were seeded on coverslips and grown overnight. After treatment with 4% Amex7 for 6, 12, and 24 h, the cells were washed and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were washed again, permeabilized in PBS/0.4% Triton X-100 for 10 min at room temperature, and blocked in PBS/4% FBS for 1 h at room temperature. The coverslips were stained with primary anti-γ-H2AX (1:500 dilution) antibody, incubated overnight at 4°C, and then stained with Alexa Fluor 488 Goat anti-Mouse IgG (H+L) secondary antibody and DAPI for 1 h at room temperature. The coverslips were washed and mounted in VectaMount™ mounting medium (Vector Laboratory, Inc., Burlingame, CA, USA).

**Ethics statement.** All animal protocols and studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korean Institute of Radiological and Medical Sciences (KIRAMS 2015-0058).

**Statistical analysis.** Results from the *in vivo* study are expressed as the mean ± standard error of the mean (SEM) and those from the *in vitro* study were plotted as the mean ± standard deviation (SD). The statistical analysis was performed using a Student's t-test and one-way ANOVA followed by Tukey's HSD test using the statistical package for the social sciences (SPSS) software (version 23.0; Chicago, IL, USA). The level of statistical significance was set at a p<0.05.

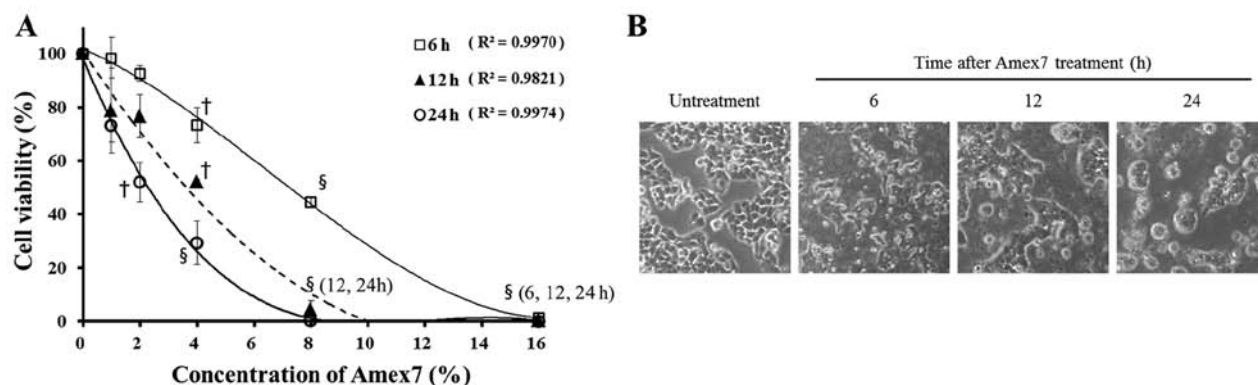


Figure 3. Amex7 decreases cell viability and induced morphological changes in HT-29 human colorectal cancer cells. (A) HT-29 cells were treated with 1-16% Amex7 for 6, 12, and 24 h, and then final cell numbers were measured by cell count assay. The graphs indicate the percentage of cell viability in all groups. (B) After treatment of 4% Amex7 for 6, 12, and 24 h, cell morphology was photographed by light microscopy (x200). Statistically significant differences compared with control are shown:  $^{\dagger}p < 0.01$ ,  $^{\S}p < 0.001$ .

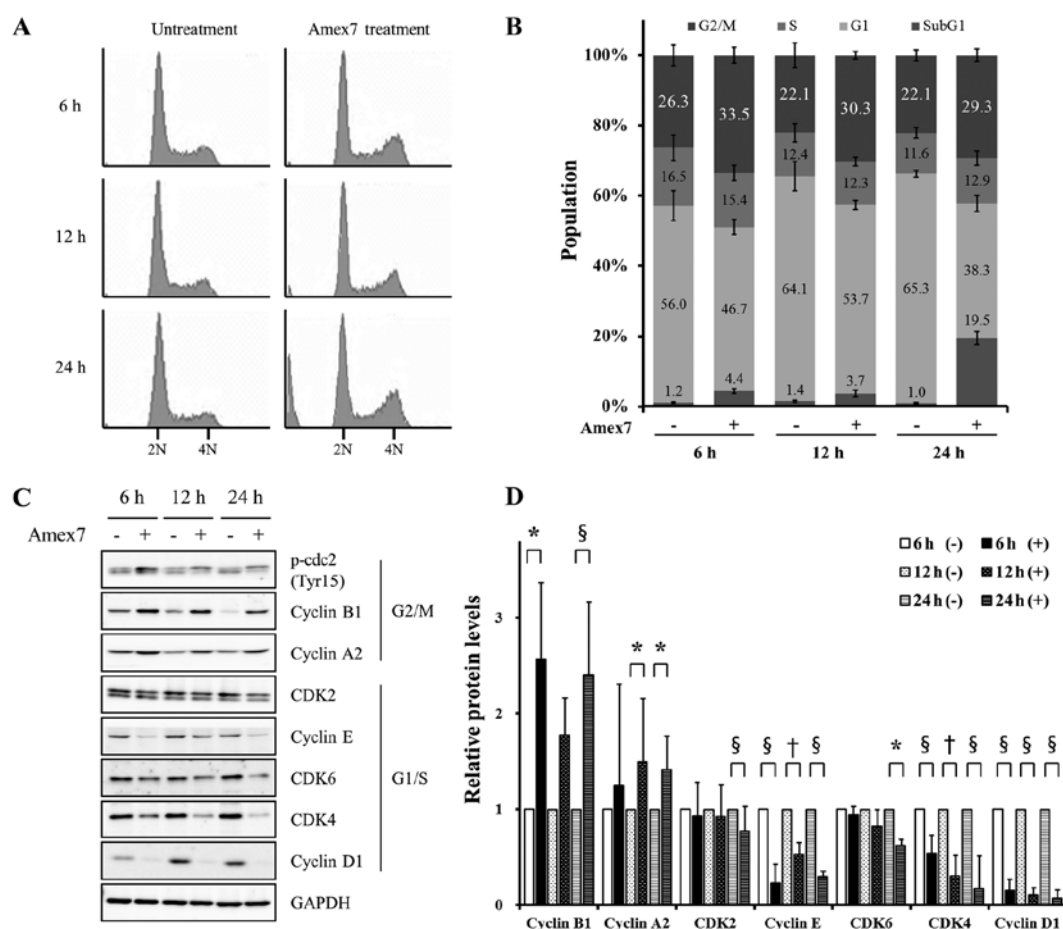


Figure 4. Amex7 prevents cell cycle progression by regulating cell cycle regulatory proteins. HT-29 cells were treated with 4% Amex7 for 6, 12, and 24 h. (A) Cell cycle distribution was analyzed by flow cytometry and (B) was quantified using a FACScan flow cytometer. (C) Expression of G1/S checkpoint regulators (cyclin D1, cyclin E, CDK2, CDK4, and CDK6) and G2/M checkpoint regulators (cyclin A2, cyclin B1, and p-cdc2) were measured by immunoblotting and (D) were quantified using Multi Gauge V3.0 software in HT-29 cells. Densitometric quantification was normalized to GAPDH. (-) indicates the control group and (+) indicates Amex7-treated group. Values are mean  $\pm$  SD of four determination.  $^*p < 0.05$ ,  $^{\dagger}p < 0.01$ ,  $^{\S}p < 0.001$  vs. each control group.

## Results

*Amex7 significantly inhibits tumor growth in HT-29 human colorectal cancer xenografts.* We evaluated the *in vivo* anticancer effect of Amex7 at various concentrations (1.25, 6.25, and 12.5 ml/kg) in mice xenografted with HT-29

cells. When the mean tumor volume in the control group reached approximately 2,000 mm<sup>3</sup>, the tumor volume in the mice treated with Amex7 at 1.25, 6.25, and 12.5 ml/kg was 46.7 $\pm$ 1.5, 46.4 $\pm$ 1.2 and 43.9 $\pm$ 1.8% smaller, respectively, than the control (Fig. 2A). A significant concentration-independent anticancer effect ( $p < 0.001$ ) of Amex7 was observed in the

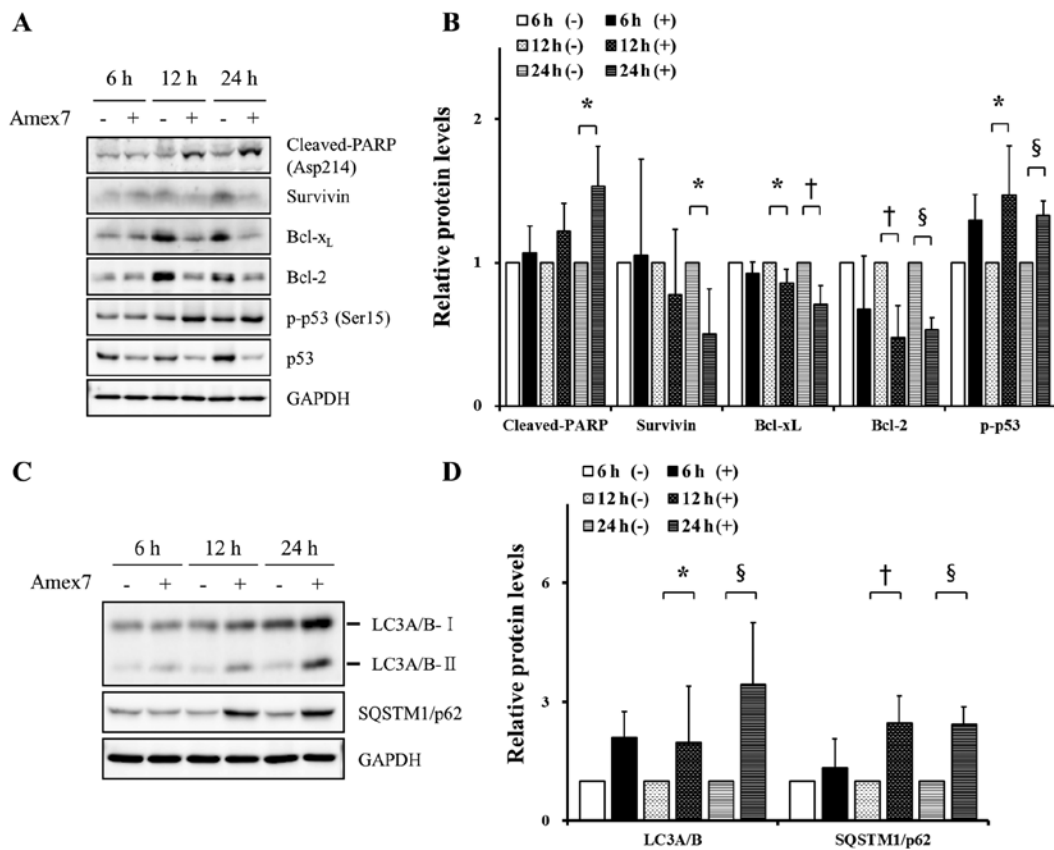


Figure 5. Amex7 induces apoptosis and autophagy in HT-29 cells. Cells were treated with 4% Amex7 for 6, 12, and 24 h. To confirm the induction of apoptosis by Amex7, (A) cells from all groups were immunoblotted with p53, phospho-p53 (Ser15), Bcl-2, Bcl-x<sub>L</sub>, survivin, and cleaved-PARP (Asp214) and (B) protein levels were quantified using Multi Gauge V3.0 software. In addition, (C) to assess induction of autophagy by Amex7, protein levels of SQSTM1/p62 and LC3A/B-II were measured by immunoblotting and (D) protein levels were quantified using Multi Gauge V3.0 software. Densitometric quantification was normalized to GAPDH. (-) indicates the control group and (+) indicates the Amex7-treated group. Values are mean  $\pm$  SD of four determinations, \* $p$ <0.01, † $p$ <0.001 vs. each control group.

mouse model. Moreover, there were no marked changes in body weight between the control and Amex7 treatment groups during the administration period of Amex7 (Fig. 2B).

**Amex7 decreases cell viability and induces morphological changes in HT-29 human colorectal cancer cells.** To assess the anti-proliferative effect of Amex7 on human colorectal cancer cells, we exposed HT-29 cells to serial concentrations of Amex7 (0, 1, 2, 4, 8, and 16%) for 6, 12, and 24 h. By using cell count assay, Amex7 treatment resulted in 99, 93, 73, 45 and 1% cell viability for 6 h; 79, 77, 52, 4 and 0% cell viability for 12 h; and 73, 52, 29, 0 and 0% cell viability for 24 h at 1, 2, 4, 8, and 16%, respectively (Fig. 3A). Furthermore, Amex7 changed cell morphology and increased cell aggregations and cellular debris compared with the control cells in time-dependent manner (Fig. 3B). Our data showed that Amex7 induces concentration- and time-dependent inhibition of HT-29 human colorectal cancer cell lines.

**Amex7 modulates cell cycle progression in HT-29 human colorectal cancer cells.** To determine whether the anti-proliferative effect of Amex7 was associated with regulation of the cell cycle, we used flow cytometry to analyze the cell cycle distribution of HT-29 cells in the presence of Amex7. Fig. 4A and B show that Amex7 significantly decreased the proportion of G0/G1 phase cells ( $p$ <0.001) and accumulation of G2/M

phase cells compared with the control ( $p$ <0.001). Furthermore, Amex7 markedly increased the proportion of sub-G1 phase cells at 24 h, but not at 6 and 12 h after treatment of Amex7 compared with the control. These results suggested that Amex7 inhibited cell proliferation by inducing G2/M phase arrest and apoptosis.

**Amex7 induces G2/M arrest by regulating cell cycle regulatory proteins.** To further confirm the reduction of cells in the G0/G1 phase and the increase of cells in the G2/M phase, the expression levels of cell cycle regulatory proteins such as cyclin A2, cyclin B1, cyclin D1, cyclin E, phospho-cdc2 (Tyr15), CDK2, CDK4, and CDK6 were examined by immunoblotting. At 6, 12, and 24 h after Amex7 treatment, the expression levels of cyclin D1, cyclin E, and CDK4, which are involved in G1/S progression, were significantly decreased ( $p$ <0.01) and CDK6 protein level was slightly decreased. Moreover, Amex7 markedly increased cyclin A2 and cyclin B1 protein levels, which are required for G2/M progression ( $p$ <0.05), but did not change the expression level of phospho-cdc2 (Tyr15) (Fig. 4C and D). In summary, we showed that Amex7 regulated cell cycle progression by significantly reducing G1/S phase-related proteins and increasing G2/M phase-related proteins.

**Amex7 induces apoptosis and autophagy in HT-29 human colorectal cancer cells.** In order to identify the induction

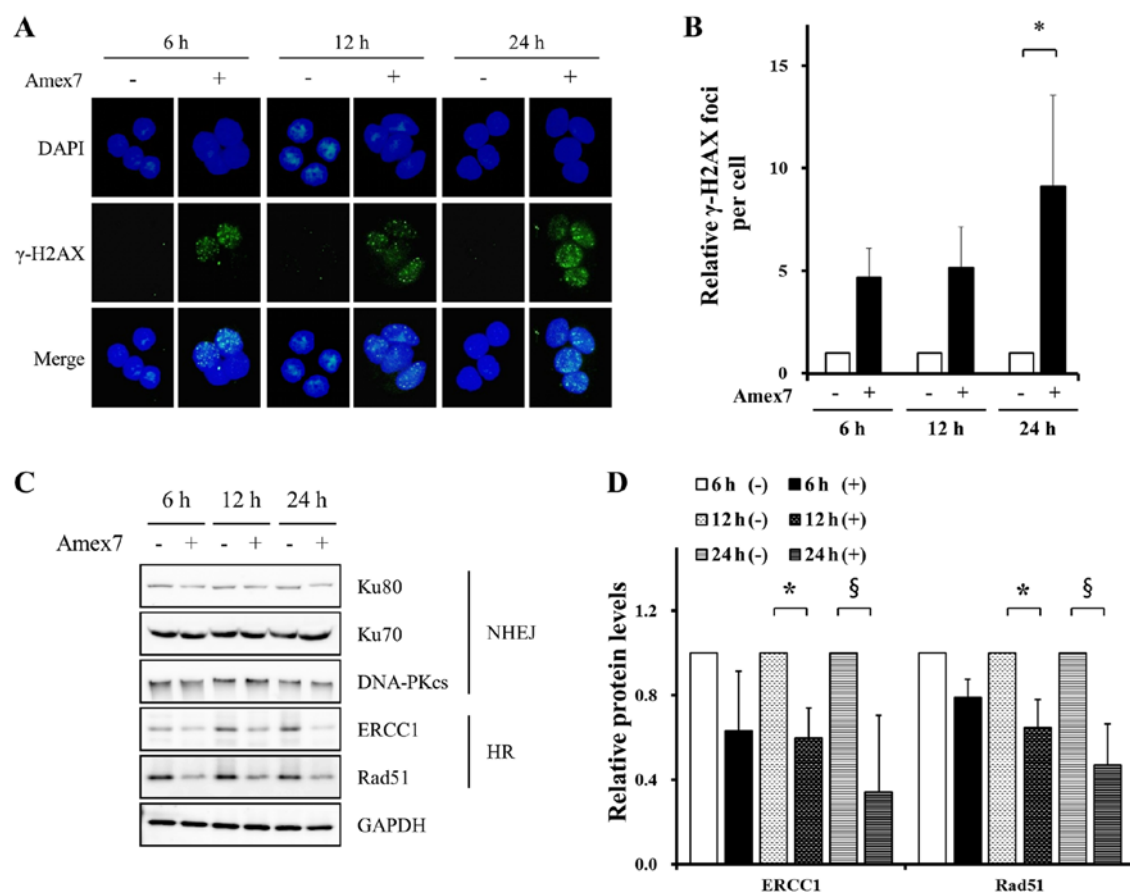


Figure 6. Amex7 regulates DNA damage and repair proteins. HT-29 cells were treated with 4% Amex7 for 6, 12, and 24 h. To observe DNA damage by Amex7, immunofluorescence staining was performed on cells from all groups. (A) Fluorescence photomicrographs show the merged images of cells stained with  $\gamma$ -H2AX (green) and counterstained for the nucleus with DAPI (blue). (B) For quantitative analysis,  $\gamma$ -H2AX foci numbers were counted in 50 cells from randomly captured images. To investigate DNA repair by Amex7, (C) cells from all groups were immunoblotted with Rad51, ERCC1, DNA-PKcs, Ku70, and Ku80 and (D) protein levels were quantified using Multi Gauge V3.0 software. (-) indicates the control group and (+) indicates the Amex7-treated group. Values are mean  $\pm$  SD, \* $p$ <0.05, § $p$ <0.001 vs. each control group.

of apoptosis by Amex7, we observed the expression of p53, phospho-p53 (Ser15), Bcl-2, Bcl-x<sub>L</sub>, survivin, and cleaved-PARP (Asp214) by immunoblotting. At 12 and 24 h after treatment of Amex7, cleaved-PARP protein (Asp214) was increased and anti-apoptotic proteins, including Bcl-2, Bcl-x<sub>L</sub>, and survivin, were decreased. In addition, Amex7 treatment for 12, 24 h reduced total p53 protein level, whereas phosphorylated p53 at Ser15 (Fig. 5A and B). Next, to evaluate the induction of autophagy by Amex7, we showed the conversion of the autophagosome protein LC3A/B-I to LC3A/B-II and the expression of SQTM1/p62 using immunoblotting. Starting 12 h after treatment of Amex7, LC3A/B-II and SQTM1/p62 were consistently induced (Fig. 5C and D). These results suggested that Amex7 induced apoptosis and autophagosome formation in human colorectal cancer cells.

**Amex7 enhances anticancer effect by regulating DNA damage and repair proteins.** To investigate the effect of Amex7 on the kinetics of DNA damage and repair, we examined the immunofluorescent staining for  $\gamma$ -H2AX: a marker for DNA strand breaks (DSBs) and stained nuclear DNA with DAPI. The images were analyzed using two (green/blue) fluorescence channels. At 24 h after treatment of Amex7, we observed an increased number of  $\gamma$ -H2AX foci compared with the control

group ( $p$ <0.05) (Fig. 6A and B). Next, to evaluate whether Amex7 could affect the DNA repair pathway, we examined the expression of homologous recombination (HR) repair proteins and non-homologous end joining (NHEJ) repair proteins using immunoblotting. As shown in Fig. 6C and D, Amex7 treatment for 12 and 24 h significantly decreased HR repair proteins, including Rad51 and ERCC1, in HT-29 cells ( $p$ <0.05). However, Amex7 did not affect NHEJ repair proteins such as the catalytic subunit of DNA-PKcs, Ku70, and Ku80. These results showed that Amex7 enhanced anticancer effects by increasing DNA damage and reducing HR repair proteins.

## Discussion

Among many functional foods, various types of mushrooms and *P. ginseng* have been reported to possess multiple biological effects, including immunomodulatory, anticancer, anti-oxidative, and anti-inflammatory activity (19-21). In particular, recent studies have focused on the anticancer effect of a concentrated substance extracted from a single medicinal mushroom or *P. ginseng*; however, the biological activity of the combination of multiple medicinal mushrooms and/or *P. ginseng* extracts is still unclear. In our study, we investigated whether a combination of seven medicinal mushrooms

and *P. ginseng* root extracts (Amex7), when used at lower concentration than in previous studies, enhanced anticancer effects in human colorectal cancer cells, focusing on multiple mechanisms for the anticancer effect.

Several studies have reported that medicinal mushrooms, including *C. militaris*, *P. linteus*, and *L. edodes* inhibited tumor growth in various human cancer xenografts (22-24), while *in vivo* anticancer studies of *P. ginseng* root are inconclusive. Consistent with previous studies, we confirmed that Amex7, a combined mixture of medicinal mushrooms and *P. ginseng* root, markedly suppressed tumor growth in HT-29 human colorectal cancer xenografts without a loss of body weight (Fig. 2). Of note, although the amount of extract of seven mushrooms and *P. ginseng* root in Amex7 was much lower than that used in previous studies, Amex7 significantly inhibited tumor growth in human colorectal cancer xenografts. Therefore, our results indicated that the mixture of a low-dose medicinal mushrooms and *P. ginseng* root extracts enhanced anticancer effects without complication.

Next, we demonstrated the underlying anticancer mechanisms of Amex7 in HT-29 cells. Many studies have reported that the extract of medicinal mushrooms or *P. ginseng* root resulted in cytotoxicity via cell cycle arrest and apoptosis in various human cancer cells. As shown in Fig. 4, Amex7 induced G2/M arrest by significantly reducing G1/S phase-related proteins (cyclin D1, cyclin E, CDK2, CDK4, and CDK6) and increasing G2/M phase-related proteins (cyclin A2 and cyclin B1), which was consistent with previous studies (23,25). Especially, the present study showed that Amex7 significantly altered the expression levels of cyclin D1, cyclin E, CDK4, and cyclin B1 compared with the control cells. Additionally, Amex7 induced sub-G1 accumulation, which was suggestive of apoptosis, by reducing anti-apoptotic proteins (Bcl-2, Bcl-x<sub>L</sub>, and survivin), increasing apoptotic protein (cleaved-PARP protein (Asp214), and phosphorylating p53 at Ser15 (Fig. 5A and B); these changes were in agreement with previous reports (26,27). Furthermore, we found that Amex7 induced autophagosome formation, as evidenced by LC3A/B-II conversion and SQTM1/p62 accumulation (Fig. 5C and D). Collectively, these data provided that the mixture of medicinal mushrooms and *P. ginseng* root extracts induced G2/M arrest, apoptosis, and autophagy in human colorectal cancer cells.

An interesting observation in our study concerned the effect of Amex7 on the kinetics of DNA damage and repair. Starting early time after Amex7 treatment, Amex7 consistently induced DNA damage (Fig. 6A and B) and also suppressed the expression of DNA repair proteins, including Rad51 and ERCC1, which play a key role in the DNA HR repair pathway (Fig. 6C and D). Therefore, these results suggested that Amex7 enhances cytotoxicity in human colorectal cancer cells by inducing DNA damage and inhibiting DNA repair.

In conclusion, the combined mixture of mushrooms and *P. ginseng* root extracts inhibited tumor growth, increased cytotoxicity via induction of G2/M arrest, and induced apoptosis and autophagy. Moreover, it enhanced anticancer effect by impairing DNA damage repair. Despite the low-dose of extracts, the mixture enhanced the anticancer effect in human colorectal cancer cells. Accordingly, we suggested a scientific rationale for the clinical application of the mixture

of medicinal mushrooms and *P. ginseng* root extracts as an adjuvant compound in treatment of human colorectal cancer that presented no safety concerns.

## Acknowledgements

This study was supported by a grant of the Korea Institute of Radiological and Medical Sciences (KIRAMS), funded by Ministry of Science, ICT and Future Planning, Republic of Korea (no. 1711045548).

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