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Dihydromyricetin induces cell apoptosis via a p53-related pathway in AGS human gastric cancer cells

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ABSTRACT. The aim of the present study was to determine the anti-proliferative and pro-apoptotic effects of dihydromyricetin (DHM) on the AGS human gastric cancer cells and their underlying mechanisms. The effects of DHM on AGS cells were evaluated by using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase, and Annexin V/propidium iodide (PI) double-staining assays. The underlying mechanisms were determined by using quantitative real-time polymerase chain reaction. The results demonstrated that DHM significantly ($P < 0.05$) inhibited AGS cell proliferation and induced cell cytotoxicity in a dose- and time-dependent manner. Additionally, Annexin V/PI double-staining assay showed that DHM promoted cell apoptosis in both, early and late stages. Furthermore, DHM also regulated the expression of apoptotic genes such as *p53* and B-cell lymphoma-2 (*bcl-2)* in a dose- and time-dependent manner. In conclusion, this is the first report demonstrating the anticancer and pro-apop-

tosis effects of DHM on AGS human gastric cancer cells. The results strongly suggest that DHM may be a potential therapeutic candidate for the treatment of gastric cancer.

Key words: Dihydromyricetin; Gastric cancer; Cytotoxicity; Apoptosis; p53

INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related deaths worldwide, particularly in developing countries (Alberts et al., 2003). The incidence and mortality rates of gastric cancer are very high, accounting for about one million deaths each year (Ji and Ji, 2014). Currently, surgery and chemotherapy are considered as the primary methods of treatment for gastric cancer. However, the outcome of these anticancer therapies is often unsatisfactory, requiring more active approaches. Numerous pharmacological studies have demonstrated that large amounts of natural ingredients found in plants and fungi show diverse anticancer activities and may be potential candidates for treating cancer (Yang et al., 2013; Dong et al., 2014).

The *p53* gene, a known transcription factor, is also a well-known tumor suppressor gene that plays an important role in many cellular processes, including apoptosis, cell cycle modulation, and cell signaling pathways (Ryu et al., 2014). Once cellular DNA is challenged by extracellular stress, *p53* is activated and translocated into the nucleus. These changes further activate numerous genes involved in apoptosis and suppression of cell division, which results in the induction of apoptosis and suppression of cell division (Vazquez et al., 2008). During apoptosis, the anti-apoptotic factors in the cell are inhibited, including downregulation of B-cell lymphoma-2 (Bcl-2), upregulation of pro-apoptotic factors such as Bcl-2-associated x protein (Bax), and activation of apoptosis-inducing proteins such as caspase (Tsujimoto, 2002).

Dihydromyricetin (DHM) is a natural flavonoid commonly found in the *Ampelopsis grossedentata* species (Du et al., 2002) and exhibits numerous biological properties, including antimicrobial, anti-inflammatory, and antioxidative activities (Zhang et al., 2003; Yin et al., 2010; Shen et al., 2012). Recent reports also demonstrate potent anticancer activity of DHM (Wu et al., 2013; Zhang et al., 2014). Specifically, DHM has been found to inhibit cell proliferation in human hepatoma cells via p53-related pathways (Wu et al., 2013). However, it is still unknown whether DHM is effective in gastric cancer. Therefore, the objective of this study was to evaluate the antiproliferative and pro-apoptotic effects of DHM on AGS human gastric cancer cells and to determine their underlying mechanisms.

MATERIAL AND METHODS

Chemicals and reagents

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and DHM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin (PS), and real-time quantitative polymerase chain reaction (qPCR) kits were purchased from Invitrogen Gibco BRL (Grand Island, NY, USA). Lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Beyotime (Shanghai, China). Annexin V and trypsin

were obtained from Life Technologies (USA). All other chemicals used were of reagent grade.

Cell culture

AGS human gastric cancer cells were obtained from the Cell Storehouse of the Chinese Academy of Science (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% FBS and 1% PS. The cells were incubated in a humidified incubator at 37°C with 5% CO_2.

Cell viability assay

MTT assay was used to investigate the anticancer effects of DHM on cell viability. Briefly, AGS cells were seeded on 96-well plates at a density of 5 x 10^4 cells/mL. The cells were incubated with different concentrations of DHM (25, 50, and 100 μM) or vehicle solution (0.1% DMSO) for 48 or 72 h. Cell-free supernatants (80 μL) were collected from each well and stored at -20°C for LDH leakage assay. Then, 30 μL MTT (5 mg/mL) solution was added into each well and further incubated for 4 h. After replacing the culture medium with 100 μL DMSO, the optical density (OD) was measured at a wavelength of 570 nm by using a microplate reader (TECAN, Austria).

LDH leakage assay

Cell cytotoxicity was tested by LDH leakage assay, which was performed according to manufacturer instructions. Briefly, 50 μL supernatant was pipetted onto a 96-well plate, and 50 μL CytoTox 96® non-radioactive cytotoxicity assay reagents was added into each well. The plate was gently shaken to mix the contents and then incubated for 30 min in the dark at room temperature. The reaction was terminated by adding 50 μL stop solution into each well. The OD at 492 nm was measured by using a microplate reader (TECAN).

Annexin V/PI double-staining assay

To quantify the apoptotic cells induced by DHM, Annexin V/PI double-staining assay and flow cytometry (FACSalibur, BD) were performed, according to manufacturer instructions. Briefly, AGS cells were seeded on 6-well plates for 24 h at a density of 1 x 10^5 cells/mL. The cells were treated with DHM (50 and 100 μM) or DMSO for 48 or 72 h. The cells were harvested, washed twice with PBS, and resuspended in binding buffer. Fluorescein isothiocyanate-conjugated Annexin V (5 μL) and PI (5 μL) were added to the cells and allowed to stand for 15 min at room temperature in the dark. The cells were then washed with PBS, and data were collected by flow cytometry.

Real-time q-PCR

After treatment with DHM, the cells were collected and the total RNA was extracted with TRIzol® reagent (Life Technologies), according to manufacturer instructions. Approximately 1 μg RNA was used for the reverse transcription reaction by using Oligo dT (18T) (Omega, USA). The cDNA products were amplified for *bcl-2*, *bax*, and *p53* gene expression

via qRT-PCR by using specific primers as shown in Table 1. PCR was carried out in triplicate with SYBR Green PCR Master Mix by using a 7900HT qPCR system thermal cycler (Applied Biosystems, USA). GAPDH mRNA was used as internal control for each sample. The Ct values for each sample were normalized to GAPDH mRNA. Results were obtained from 4 independent experiments.

Table 1. Sequences of primers used for real-time quantitative PCR.

Gene	Forward	Reverse
Bax	5'-TGGAGCTGCAGAGGATGATTG-3'	5'-GAAGTTGCCGTCAGAAAACATG- 3'
Bcl-2	5'-CTGCACCTGACGCCCTTCACC-3'	5'-CACATGACCCCACCGAACTCAAAGA-3'
p53	5'-TAACAGTTCCTGCATGGGCGGC-3'	5'-CGGAGGCCCATCCTCACCATCATCA-3'
GAPDH	5'-AACGGGAAGCTTGTCATCAATGGAAA-3'	5'-GCATCAGCAGAGGGGGCAGAG-3'

Statistical analysis

All data are reported as means ± standard deviation (SD) from 4 indepedent tests. The Student *t*-test was performed for statistical analyses by using the GraphPad PRISM software version 5.0 (GraphPad Software, USA), and $P < 0.05$ was considered to be statistically significant.

RESULTS

DHM inhibited cell proliferation in AGS human gastric cells

To examine the effects of DHM on AGS human gastric cell viability, the cells were treated with various concentrations (25, 50, and 100 μM) of DHM for 48 or 72 h, and cell viability was evaluated by the MTT assay. The results showed that DHM significantly inhibited AGS cell growth in a dose- and time-dependent manner (Figure 1). The results indicate that DHM exerted an obvious antiproliferative effect on AGS human gastric cells.

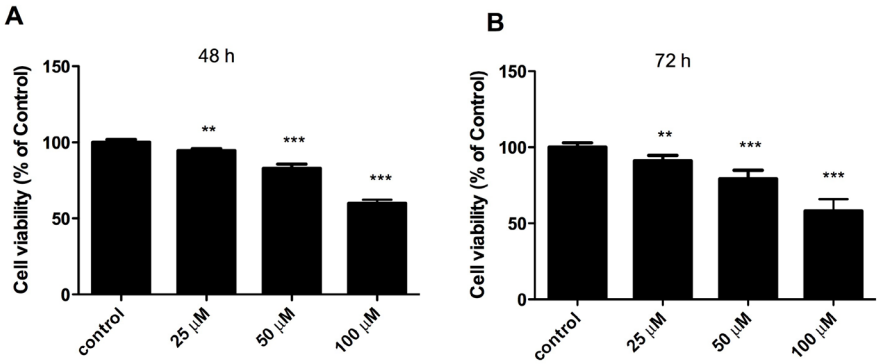

Figure 1. DHM inhibited cell proliferation in AGS human gastric cells. Cell viability was measured by MTT assays in AGS cells after incubation with various concentrations of DHM or DMSO for (**A**) 48 h or (**B**) 72 h. Values are reported as means ± standard deviation of four independent experiments. The differences between mean values were assessed by the Student *t*-test. **$P < 0.01$, ***$P < 0.001$ versus the DMSO treatment control.

DHM induced cell cytotoxicity in AGS human gastric cells

To investigate whether DHM induced cell cytotoxicity, AGS cells were treated with various concentrations (25, 50, and 100 μM) of DHM for 48 or 72 h, and cell cytotoxicity was measured by the LDH leakage assay. Interestingly, DHM was toxic to AGS cells in a dose- and time-dependent manner (Figure 2). These observations strongly suggest that the antiproliferative activity of DHM was mediated by apoptotic cell death in the AGS cells induced by DHM.

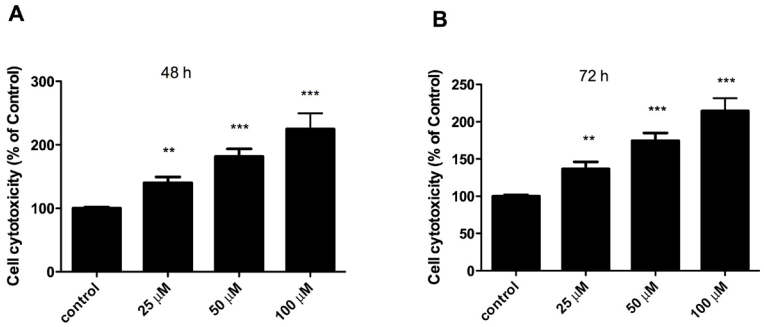

Figure 2. DHM induced cell cytotoxicity in AGS human gastric cells. The cell cytotoxicity was tested by LDH assay in AGS cells after incubation with various concentrations of DHM or DMSO for (**A**) 48 h or (**B**) 72 h. Values are reported as means ± standard deviation of four independent experiments. The differences between mean values were assessed by the Student *t*-test. **P < 0.01, ***P < 0.001 versus the DMSO treatment control.

DHM promoted AGS cells apoptosis

In order to investigate whether DHM can induce cell apoptosis, Annexin V and PI double-staining assays were carried out and evaluated by flow cytometry. The results showed that when AGS cells were treated with DHM (100 μM) apoptotic cells were significantly increased in a time-dependent manner, in both, the early and late stages (Figure 3A). Besides, apoptotic cells also significantly increased in a dose-dependent manner when treated with DHM for 72 h (Figure 3B).

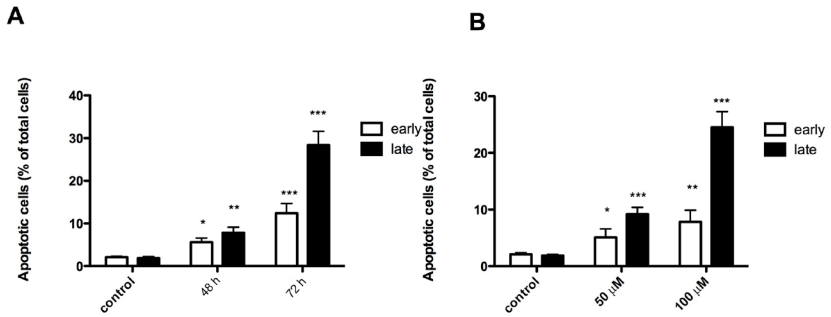

Figure 3. DHM promoted AGS cells apoptosis. **A.** Apoptotic cells in AGS cells analyzed by flow cytometry, which were treated with DHM at 100 μM for 48 and 72 h. **B.** Apoptotic cells in AGS cells analyzed by flow cytometry, which were treated with DHM at 50 and 100 μM for 72 h. Values are reported as means ± standard deviation of four independent experiments. The differences between mean values were assessed by the Student *t*-test. *P < 0.05, **P < 0.01, ***P < 0.001 versus the DMSO treatment control.

DHM regulated the expression of apoptotic genes

In order to determine the underlying pathway by which DHM induced cell apoptosis in AGS human gastric cancer cells, mRNA expression of *p53*, *bcl-2*, and *bax* was evaluated by qPCR analysis. As shown in Figure 4, DHM promoted the expression of *p53* mRNA and inhibited the expression of *bcl-2* mRNA. In addition, the expression of *p53* mRNA was upregulated in a dose- and time-dependent manner, whereas the expression of *bcl-2* mRNA decreased in DHM-treated AGS cells. However, administration of DHM did not affect the expression of *bax* mRNA in AGS cells as compared to that in the control.

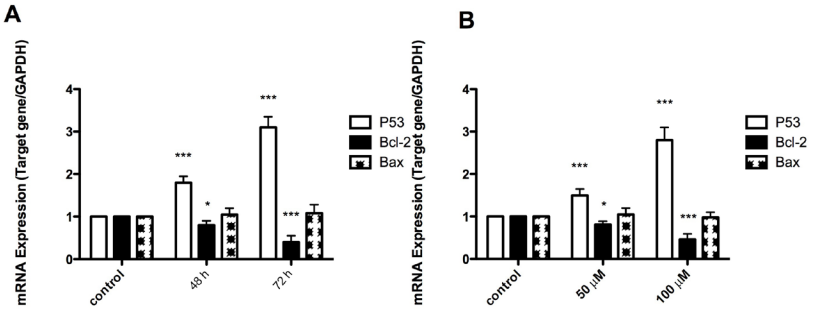

Figure 4. DHM regulated the apoptotic gene expression. **A.** Gene expressions of P53, Bcl-2 and Bax in AGS human gastric cancer cells tested by RT-PCR assay, which were treated with DHM for 48 and 72 h. **B.** Gene expressions of P53, Bcl-2 and Bax in AGS human gastric cancer cells tested by RT-PCR assay, which were treated with different concentrations of DHM for 72 h. Values are reported as means ± standard deviation of four independent experiments. The differences between mean values were assessed by the Student *t*-test. *P < 0.05, ***P < 0.001 versus the DMSO treatment control.

DISCUSSION

DHM is a natural plant component, isolated from *A. grossedentata*. In this study, we investigated its antitumor activity in gastric cancer and found that DHM exerted antiproliferative and pro-apoptotic effects on AGS human gastric cancer cells. We also demonstrated that at concentrations of 25 to 100 μM, DHM inhibited gastric cancer cell (AGS) proliferation and induced cell apoptosis. The antitumor effects of DHM were consistent with previous reports using liver cancer cells (Wu et al., 2013; Zhang et al., 2014). These results suggested that DHM is a potent antitumor compound that can regulate specific genes responsible for tumor cell proliferation and apoptosis.

Apoptosis is a form of programmed cell death, which plays an important role in maintaining cellular function in various tissues and organs (Ryu et al., 2014). In this study, the characteristics of apoptosis in AGS cells treated with DHM were evaluated by Annexin V/PI double-staining and flow cytometry. As shown in Figure 3, the early apoptosis rate (12.4%) of AGS cells treated with 100 μM DHM for 72 h was higher than that of the control (2.1%), while the late apoptosis rate (28.4%) of these cells was also higher than that of the control (1.9%). In addition, DHM caused an increase in the total apoptosis rate in a dose- and time-dependent manner and decreased the survival rate of AGS cells.

The transcription factor, *p53* is also a tumor suppressor gene, which is activated in the cytoplasm and is translocated into the nucleus when DNA is challenged by extracellular stress.

Activation of *p53* increases the induction of targeted genes, including those causing suppression of cell division and encoding apoptotic proteins that induce inhibition of cell division and promote apoptosis in cells (Fisher, 2001). There are 2 major apoptotic pathways activated by *p53* activation, namely the extrinsic death-receptor pathway and the intrinsic mitochondrial pathway (Nagata, 1999; Sheikh and Fornace Jr, 2000). The mitochondrium-related apoptosis is mediated by apoptotic proteins through the inhibition of the anti-apoptotic protein, Bcl-2 and upregulation of the pro-apoptotic protein, Bax (Nagata, 1999). In this study, DHM caused an increase in the expression of *p53* mRNA in a dose- and time-dependent manner (Figure 4). Furthermore, *bcl-2* mRNA, encoding the anti-apoptotic protein Bcl-2 was also inhibited by DHM treatment in a dose- and time-dependent manner. However, *Bax* mRNA, encoding the pro-apoptotic protein, Bax, was not affected by DHM treatment. These results indicate that the intrinsic mitochondrial pathway was activated in DHM-treated AGS cells (Willis and Adams, 2005).

This is the first report to demonstrate anticancer and pro-apoptotic effects of DHM in AGS human gastric cancer cells. Our data also strongly suggest that DHM can induce cell apoptosis possibly through a p53-related pathway, which should be investigated further as DHM can be considered a potential therapeutic candidate for the treatment of gastric cancer.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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