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Amygdalin induces apoptosis in human cervical cancer cell line HeLa cells

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Abstract

Amygdalin, a naturally occurring substance, has been suggested to be efficacious as an anticancer substance. The effect of amygdalin on cervical cancer cells has never been studied. In this study, we found that the viability of human cervical cancer HeLa cell line was significantly inhibited by amygdalin. 4,6-Diamino-2-phenyl indole (DAPI) staining showed that amygdalin-treated HeLa cells developed typical apoptotic changes. The development of apoptosis in the amygdalin-treated HeLa cells were confirmed by double staining of amygdalin-treated HeLa cells with annexin V-FITC and propidium iodide (PI) along with increase in caspase-3 activity in these cells. Further studies indicated that antiapoptotic protein Bcl-2 was downregulated whereas proapoptotic Bax protein was upregulated in the amygdalin-treated HeLa cells implying involvement of the intrinsic pathway of apoptosis. *In vivo*, amygdalin administration inhibited the growth of HeLa cell xenografts through a mechanism of apoptosis. The results in the present study suggest that amygdalin may offer a new therapeutic option for patients with cervical cancer.

Keywords: Amygdalin, cervical cancer, HeLa cells, apoptosis, xenograft

Introduction

Cervical cancer is currently one of the most common malignancies⁽¹⁾ and the second leading cause of death in women worldwide, killing ~280,000 women each year.⁽²⁾ Eighty percent of cervical cancer occurs in developing countries, and it is also the second leading cause of mortality in women aged 21–39 in the United States.⁽¹⁾ Despite advances in radiotherapy and chemotherapy, problems related to these therapies such as side effects and development of drug resistance remained unsolved.⁽³⁾ Meanwhile, the high rate of mortality in cervical cancer has remained relatively static.⁽⁴⁾ One of the strategies to solve these problems is to develop novel therapies and add them to the regimen of current anticervical cancer approaches. In the search for the alternative therapies for cancer treatment, we have noticed that a natural cyanide-containing substance, amygdalin, is gaining reputation as a complementary substance for cancer treatment due to its effectiveness in inhibiting the growth of cancer cells^(5,6) and easy availability. Therefore, the anticancer

mechanisms of amygdalin and its application *in vivo* are worthy of further study.

Amygdalin is abundant in the seeds of apricots, almonds, peaches, apples, and other rosaceous plants^(5,7) and is composed of two molecules of glucose, one molecule of benzaldehyde and one molecule of hydrocyanide. Amygdalin was reported to have an anti-cancer effect via inducing apoptosis in prostate cancer cells⁽⁵⁾ and downregulating cell cycle-related genes in SNU-C4 human colon cancer cells.⁽⁶⁾ It is believed that the benzaldehyde in amygdalin is able to induce an analgesic effect and the hydrocyanide in amygdalin is able to induce an anti-neoplastic effect.⁽⁸⁾ Amygdalin could be a new therapeutic substance for patients with cancers although there has been controversy surrounding the use of amygdalin as a cancer drug due to concerns of cyanide toxicity.^(9–11)

There is a growing interest in reports of amygdalin as a cancer treatment drug. The anticancer effect and mechanism of amygdalin in cervical cancer has never

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(Received 30 September 2012; revised 07 October 2012; accepted 08 October 2012)

been reported. The present study was designed to study the apoptotic effect of amygdalin on human cervical cancer HeLa cells *in vitro* and *in vivo* in order to provide rationale for its use in the treatment of cervical cancer.

Materials and methods

Reagents

Amygdalin, dimethyl sulfoxide (DMSO), 4,6-diamino-2-phenyl indole (DAPI), and triton X-100 were purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse anti- β -actin, mouse anti-Bcl-2, and rabbit anti-Bax primary antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary rabbit anti-mouse or goat anti-rabbit antibodies were purchased from Rockland Inc. (Philadelphia, PA, USA).

Cell culture

Human cervical cancer HeLa cell line and human embryonic amnion FL cell line were purchased from the Tumor Center of Chinese Academy of Medical Sciences. The cells were cultured in 75 cm² tissue culture flasks under humidified 5% CO₂ atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), and 1% penicillin-streptomycin (100 U/mL penicillin and 100 μ g/mL streptomycin). During the course of the experiment, the cells were maintained under the same conditions described above except using 0.2% FBS instead of 10% FBS.

MTT cytotoxicity assay

MTT cytotoxicity assay was used to determine the cytotoxicity of amygdalin on HeLa cells and FL cells. HeLa cells and FL cells were grown in a final volume of 100 μ l DMEM medium containing 10% FBS per well in 96-well plates at a density of 1×10^5 cells/mL. After 12 h, amygdalin was added at concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL in the DMEM medium with 0.2% FBS for 24 h. After adding 5 μ l of MTT labeling reagent (MTT Cell Proliferation Assay Kit; Trevigen, USA) to each well, the plate was incubated for 4 h before 100 μ l solubilization solution DMSO was added to the wells. Absorbance at 570 nm⁽¹²⁾ was then measured in a microtiter plate reader (Bio-Tek, Winooski, VT, USA). The percentage of cell viability was calculated by a formula of (OD of drug-treated sample/control OD) \times 100%. The assay was performed at least three times.

Morphology of HeLa and FL cells

HeLa and FL cells were seeded in 96-well culture plates. After 12 h, amygdalin was added to the wells at concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL in DMEM medium containing 0.2% FBS for 24 h. The morphologic changes of the cells were then observed under an inverted optical microscope (CKX41 Olympus; Olympus, Japan).

DAPI staining

DAPI staining was used to observe the morphological changes of the nucleus in apoptotic cells. 2×10^5 /well of HeLa cells were plated in 6-well plates and cultured in DMEM medium containing 10% FBS overnight. The medium was then replaced with DMEM medium containing 0.2% FBS and treated with amygdalin at concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL for 24 h. After removing the medium, the cells were washed twice with a cold phosphate-buffered saline (PBS), fixed with 100% ethanol for 20 min at room temperature, and washed twice again with cold PBS. The cells were observed under a fluorescence microscopy (IX70-SIF2 Olympus; Olympus).

Double staining with annexin V-FITC and propidium iodide

The induction of apoptosis by amygdalin was evaluated by double staining of annexin V-FITC and propidium iodide (PI). After HeLa cells were incubated with 1.25, 2.5, 5, 10, and 20 mg/mL amygdalin in DMEM medium containing 0.2% FBS in 6-well plates for 24 h, the cells were harvested, washed twice with cold PBS, and assayed for apoptosis by the double staining of annexin V-FITC and PI (Annexin V-FITC Apoptosis Detection Kit; KeyGEN, Nanking, China). Briefly, 5×10^5 cells were resuspended in a binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, 2.5 mM CaCl₂), stained with 5 μ l of annexin V-FITC for 10 min, and then stained with 5 μ l of PI for another 15 min. The cells were then immediately analyzed with a flow cytometer (FACScan; BD Biosciences, Milano, Italy). On the image from the flow cytometer, cells in the upper-right portion (Q2), the lower-left portion (Q3), and the lower-right portion (Q4) represent late-apoptotic cells, viable cells, and early apoptotic cells, respectively.

Western blot

Western blot was used to evaluate the expressions of apoptosis related proteins. After incubated with 1.25, 2.5, 5, 10, and 20 mg/mL amygdalin in DMEM medium containing 0.2% FBS for 24 h, HeLa cells were harvested and washed twice with the PBS and dissolved in a lysis buffer [150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM dithiothreitol (DTT), 5 mM Na₃VO₄, 1 mM phenylmethanesulfonyl fluoride, 10 μ g/mL trypsin, 10 μ g/mL aprotinin, 5 μ g/mL leupeptin; pH 7.4] for 2 h at 4°C. The lysate was centrifuged at 12,000g for 15 min at 4°C. The protein concentration of the lysate was measured with a Bio-Rad colorimetric protein assay kit (Bio-Rad, USA). Protein (30 μ g) from each sample was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was then blocked with PBS containing 5% nonfat milk at 4°C for 1 h and incubated with mouse anti- β -actin, mouse anti-Bcl-2, and rabbit anti-Bax primary antibodies (1:200) at 4°C for 12 h. The membrane was washed with TBST buffer (20 mM Tris-HCl, 150 mM

NaCl 0.05% (vol/vol) Tween 20) for three times. The membrane was then incubated with horseradish peroxidase-conjugated secondary rabbit anti-mouse or goat anti-rabbit antibodies (1:500) for 1 h at room temperature. After incubation, the membrane was covered completely with an equal amount of enhancer and peroxide solution from the ECL Plus kit (Beyotime, Shanghai, China) for 2 min. The membrane was then exposed to a film (Kodak, USA) and developed. The experiment was repeated at least three times.

Caspase-3 enzyme activity assay

Caspase-3 is a key biomarker for apoptosis. *In vitro* caspase-3 protease activity was measured using a colorimetric assay kit (Genscript, NJ, USA). As per the manufacturer's instruction, HeLa cells incubated in DMEM medium containing 0.2% FBS were treated with amygdalin at concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL for 24 h. The cells were then lysed to allow for detection of chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The absorbance was measured at wavelength of 405 nm with a microtiter plate reader. The relative increase in caspase-3 activity was determined by comparing the absorbance of pNA from the amygdalin-treated HeLa cells with that from the nontreated control.

In vivo anticancer activity of amygdalin

A model of nude mice implanted with HeLa cells was used to evaluate the effect of amygdalin on the tumor formation and morphology. Male BALB/c nude mice (4–6 weeks old, 16–20 g) were purchased from the Beijing Vital River Experimental Animals Technology (Beijing, China). To establish the HeLa cell tumor xenograft in mice, a cultured 5×10^6 of HeLa cells were collected and injected into the right flank of the mice. When the size of the tumors reached 30–50 mm³, the mice were randomly divided into three groups with six mice in each group. The control group mice received a daily intraperitoneal injection of 0.9% NaCl in 0.2 mL for 14 days. The positive control group mice received a daily intraperitoneal injection of 30 mg/kg 5-fluorouracil (5-FU) in 0.2 mL for 14 days. In the treatment group, the mice received 300 mg/kg amygdalin in 0.2 mL for 14 days. The mice in each group were weighed and the tumor sizes were recorded with a Vernier caliper once every 2 days. The tumor volume (TV) was calculated by a formula of $0.5 \times \text{long axis} \times (\text{short axis})^2$.⁽²⁾ One day after the last injection, the tumors were removed carefully, fixed in 10% neutral formalin in PBS buffer, and then embedded in paraffin. All procedures were conducted in accordance with the guidelines established by the National Science Council of Republic China.

TUNEL assay

Sections 5- μm thick obtained from the tumor paraffin-embedded tumor tissues were used to identify apoptosis with a terminal deoxynucleotidyl transferase dUTP

nick end-labeling (TUNEL) kit (KeyGEN). The extent of apoptosis was evaluated by counting the TUNEL-positive cells. The apoptotic index was determined by a formula of number of TUNEL-positive cells/total number of cells in five randomly selected high-power fields (magnification, $\times 400$).

Statistical analyses

The data was expressed as mean values and SD for all experiments. Statistically significant differences were determined between the control and amygdalin-treated groups using Student's t-test (SPSS 16.0 software). Significance of difference is indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Effect of amygdalin on viability of HeLa and FL cells

The viability of the HeLa cells treated with amygdalin decreased in a dose-dependent manner. At concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL, the viability of HeLa cells treated with amygdalin were $93.38 \pm 4.15\%$ ($p > 0.05$), $91.67 \pm 5.29\%$ ($p > 0.05$), $83.14 \pm 5.46\%$ ($p < 0.01$), $70.67 \pm 2.59\%$ ($p < 0.001$), and $48.56 \pm 2.86\%$ ($p < 0.001$) of the control value, respectively. Interestingly, amygdalin has no effect on the viability of FL cells (Figure 2).

Morphologic changes of HeLa cells treated with amygdalin

Increasing concentrations of amygdalin caused a trend of decreasing in the numbers of the HeLa cells but did not cause significant change in the numbers of FL cells (data not shown). The HeLa cells became round in shape after amygdalin treatment especially at higher concentrations of amygdalin (Figure 3A). DAPI staining of the amygdalin-treated HeLa cells revealed nuclear condensation and fragmentation (Figure 3B).

Analysis of apoptosis by double staining HeLa cells with annexin V-FITC and PI

HeLa cells became apoptotic after they were treated with amygdalin. The distribution of apoptotic HeLa cells measured by flow cytometry showed that the numbers of early

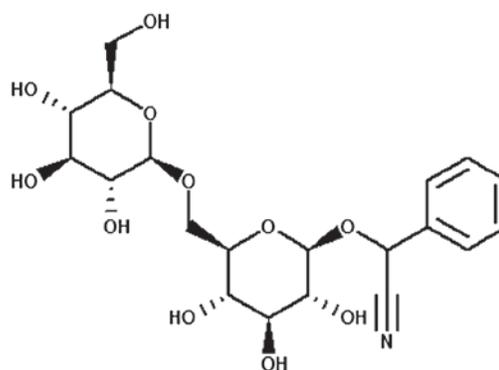


Figure 1. Chemical structure of amygdalin.

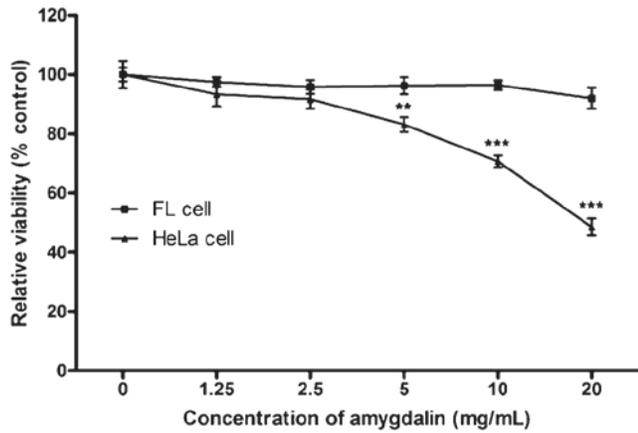


Figure 2. Effect of amygdalin on the viability of HeLa cells and FL cells. HeLa and FL cells in Dulbecco's modified Eagle's medium (DMEM) medium containing 0.2% fetal bovine serum (FBS) were treated with different concentrations of amygdalin for 24 h. After adding 5 μ l of MTT-labeling reagent, the cells were incubated for 4 h before 100 μ l solubilization solution was added. The experiments were repeated at least three times. The results are presented as the mean \pm SE. ** p < 0.01, *** p < 0.001 vs. untreated control.

(Q4) and late-apoptotic (Q2) HeLa cells were significantly increased in comparison with the control group (Figure 4A). To be specific, increases in the concentrations of amygdalin at 0, 1.25, 2.5, 5, 10, and 20 mg/mL were correlated with an increase in the ratio of apoptotic to total HeLa cells as follows: $8.8 \pm 0.7\%$, $14.3 \pm 1.6\%$, $15.6 \pm 2.9\%$, $21.4 \pm 1.8\%$, $25.4 \pm 2.3\%$, and $33.7 \pm 2.6\%$ (Figure 4B).

Expression of Bcl-2 and Bax proteins

The expression of Bax protein was increased and the expression of Bcl-2 protein was decreased in a dose-dependent manner in the HeLa cells treated with amygdalin. After 24 h of exposure to amygdalin at concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL, the corresponding ratios of Bax protein expression to Bcl-2 protein expression were as follows: 0.17 ± 0.05 , 0.25 ± 0.04 , 0.41 ± 0.09 , 0.68 ± 0.1 , 1.36 ± 0.13 , and 1.69 ± 0.11 (Figure 5A and B).

Effect of amygdalin on caspase-3 activity in HeLa cells

Caspase-3 in HeLa cells was activated by amygdalin in a dose-dependent manner. Increased concentrations

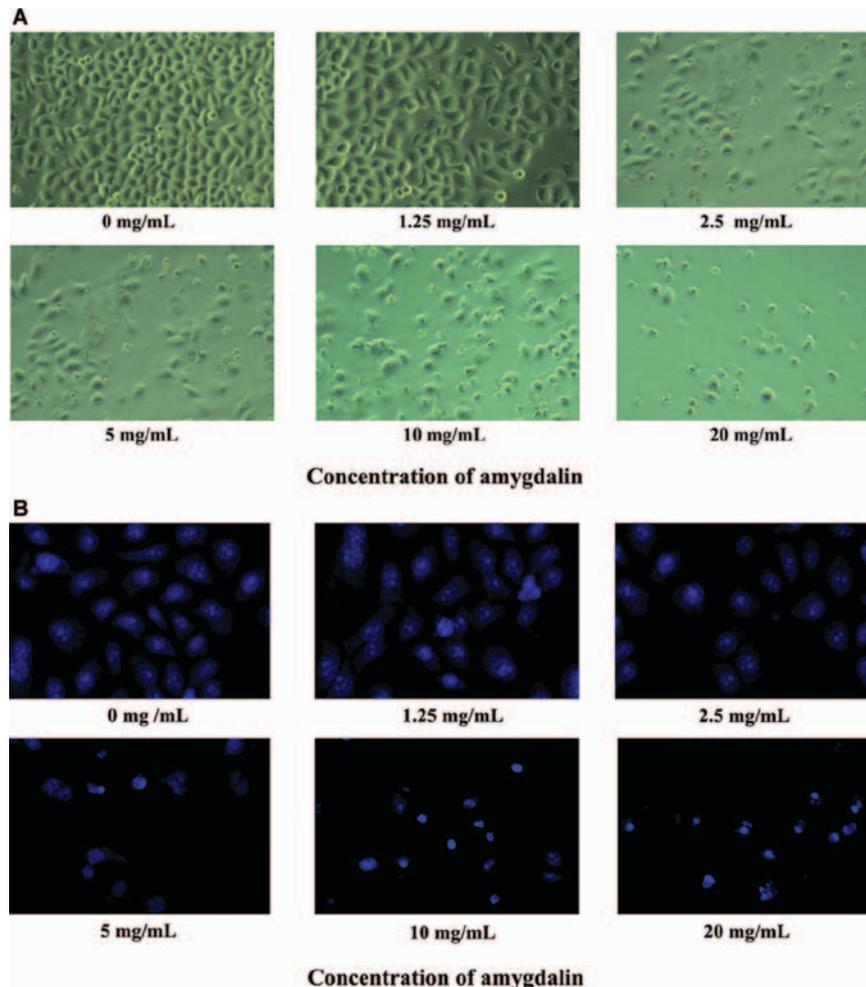


Figure 3. Morphology of the HeLa cells. (A) HeLa cells under an optical microscope. HeLa cells in Dulbecco's modified Eagle's medium (DMEM) medium with 0.2% fetal bovine serum (FBS) were treated with different concentrations of amygdalin for 24 h. The HeLa cells were observed under an inverted microscope. (B) DAPI staining. HeLa cells in DMEM medium containing 0.2% FBS were treated with different concentrations of amygdalin for 24 h. The morphological changes of the HeLa cells were observed by fluorescence microscopy ($\times 400$). The experiments were repeated at least three times.

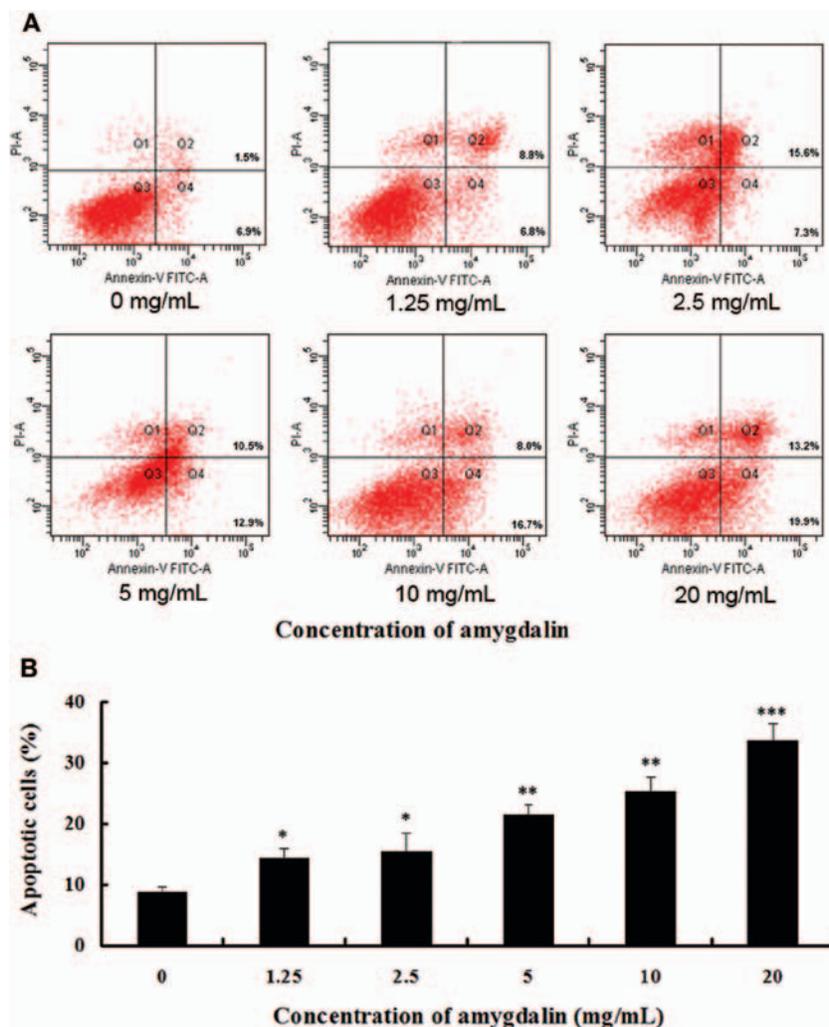


Figure 4. Apoptosis analyzed by flow cytometry. (A) HeLa cells sorted by flow cytometry. The HeLa cells were incubated with different concentrations of amygdalin in Dulbecco's modified Eagle's medium (DMEM) medium containing 0.2% fetal bovine serum (FBS) for 24 h. The cells were harvested, washed with a cold PBS, and double stained with Annexin V-FITC and propidium iodide. The cells were sorted with a flow cytometer. The experiments were repeated at least three times. (B) Percentage of apoptotic HeLa cells. The apoptotic HeLa cells were calculated as the percentage of apoptotic cells in the portions of Q2 and Q4 to the total number of the cells. The data are expressed as mean \pm SD of three experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated control).

of amygdalin at the amounts of 0, 1.25, 2.5, 5, 10, and 20 mg/mL, resulted in corresponding increases of caspase-3 activity as follows: 0.139 ± 0.02 , 0.149 ± 0.02 , 0.164 ± 0.01 , 0.29 ± 0.01 , 0.394 ± 0.01 , 0.482 ± 0.04 . Furthermore, caspase-3 inhibitor DEVD-fmk aborted the activation of caspase-3 activated by amygdalin. At concentration of 20 mg/mL of amygdalin, the caspase-3 activity in the HeLa cells treated with DEVD-fmk was only 25.7% of those without treatment of DEVD-fmk (Figure 6).

Effect of amygdalin on growth of HeLa cells *in vivo*

Tumor xenografts transplanted by the HeLa cells were used to evaluate the antitumor effect of amygdalin *in vivo*. Amygdalin significantly inhibited the tumor growth. The average TV in the control mice was $453.2 \pm 132 \text{ mm}^3$ 14 days after the injection of saline. In contrast, the TV in the mice injected with 300 mg/kg

amygdalin or 30 mg/kg 5-FU was 255.4 ± 134.8 or $269.5 \pm 101.3 \text{ mm}^3$ (Figure 7A and B). There were no significant changes in body weight between the control, 5-FU, and amygdalin groups (Figure 7C). Amygdalin inhibited the tumor growth in the xenografts tumor model through a mechanism of apoptosis. The percentage of the TUNEL positive cells in the groups of control (saline), 5-FU (30 mg/kg), and amygdalin (300 mg/kg) were 2.1 ± 0.7 , 36.3 ± 2.1 , and 33.8 ± 3.5 , respectively (Figure 7D and E).

Discussion

The major goal of the present study was to test the hypothesis that amygdalin induces apoptosis in human cervical cancer cell line HeLa cells in attempt to evaluate it as an addition to the current regimen for treatment of cervical cancer. There are three key findings in the present

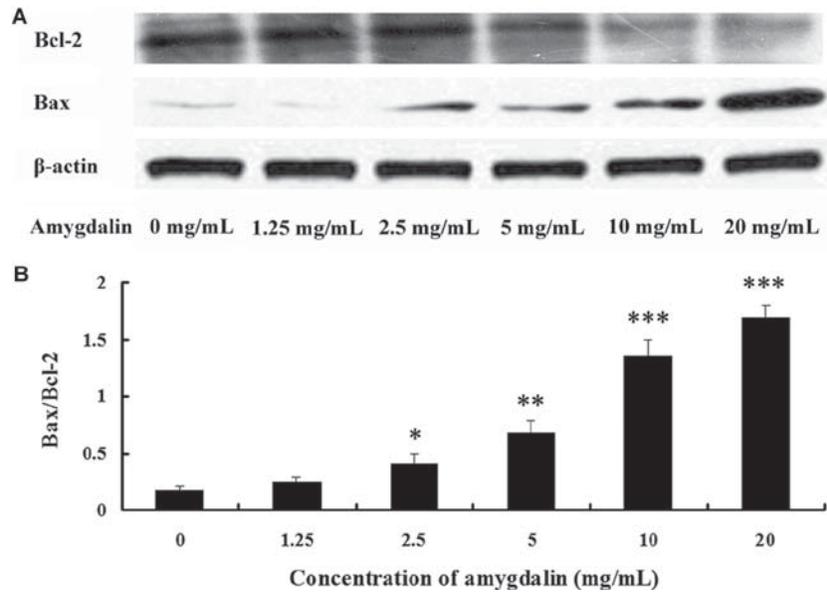


Figure 5. Effect of amygdalin on the expression of Bcl-2 and Bax protein in HeLa cells. (A) Western blot. The protein levels of Bcl-2 and Bax in the HeLa cells were determined by western blot. The expression of β -actin was set as a sample loading control. (B) Ratio of Bax to Bcl-2. The levels of Bcl-2 and Bax protein from the Western blot were quantitatively analyzed with an image analyzer and expressed as the ratio of Bax/Bcl-2. Data are presented as mean \pm SD ($n = 3$). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated control).

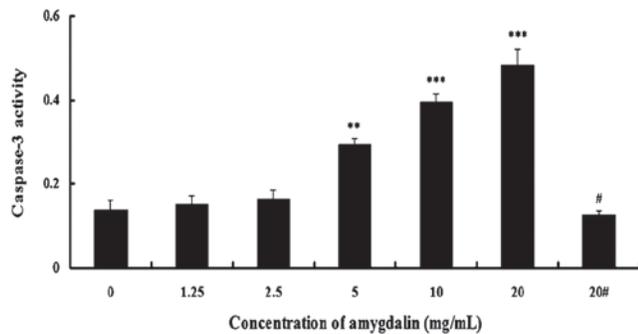


Figure 6. Caspase-3 enzyme activity. HeLa cells in Dulbecco's modified Eagle's medium (DMEM) medium containing 0.2% fetal bovine serum (FBS) were treated with different concentrations of amygdalin for 24 h. 20# represents the HeLa cells treated with 20 mg/mL amygdalin and 1 μ g of DEVD-fmk a caspase inhibitor. The experiments were repeated at least three times. The results are presented as the mean \pm SD. *** $p < 0.001$ vs. control. # $p < 0.001$ vs. the 20 mg/mL amygdalin-treated group.

study: (1) the viability and numbers of HeLa cells were decreased after treatment with amygdalin; (2) induced apoptosis in HeLa cells might be through the intrinsic pathway of apoptosis; (3) *in vivo*, amygdalin administration inhibited the growth of tumor of HeLa cells through a mechanism of apoptosis.

MTT assay can accurately determine the count of live cells and is indispensable for the assessment of cytotoxicity relevant to screening of anticancer drugs.⁽¹³⁾ By using the assay in the present study, we found that amygdalin reduced the viability of HeLa cells in a dose-dependent manner. The effect of amygdalin on cell viability appeared to be cell type dependent as the viability of the amygdalin-treated FL cells did not show significant changes in comparison with

non-amygdalin-treated FL cells (Figure 2). A similar phenomenon was demonstrated by observation of cultured cells under a microscope: The numbers of amygdalin-treated HeLa cells (Figure 3A) were markedly less than those of non-amygdalin-treated control HeLa cells, whereas the numbers of FL cells were not affected by amygdalin treatment. Mechanisms underlying the difference between HeLa and FL cells responding to amygdalin described above remains elusive. Some unique features in HeLa or FL cells must contribute to the differences. Some studies suggest that cancer cells are rich in β -glucosidase which can break down amygdalin in order to release cyanide, exerting toxicity upon the cancer cells.⁽¹⁴⁻¹⁶⁾ Some other studies suggest that rhodanese, which has the ability to detoxify cyanide, is present in normal tissues but deficient in cancer cells. The combined action of the two enzymes may be responsible for inducing cyanide-related toxicity in cancer cells treated with amygdalin-poison cancer cells by cyanide, while normal cells remain undamaged.^(6,17) The HeLa cell-line is derived from human cervical cancer cells and FL cells from benign cells of human embryonic amnion. Further studies are required to determine whether or not β -glucosidase is enriched in HeLa cells and rhodanese in FL cells and whether these features are responsible for the specificity of amygdalin on the proliferation of HeLa cells vs. FL cells.

The present study demonstrated that amygdalin was able to induce apoptosis in HeLa cells. Therefore, we believe that the amygdalin reducing viability of HeLa cells was through a mechanism of apoptosis. We tested the apoptotic effects of amygdalin on HeLa cells with various techniques from different angles including DAPI staining and analyzing the morphological alterations of

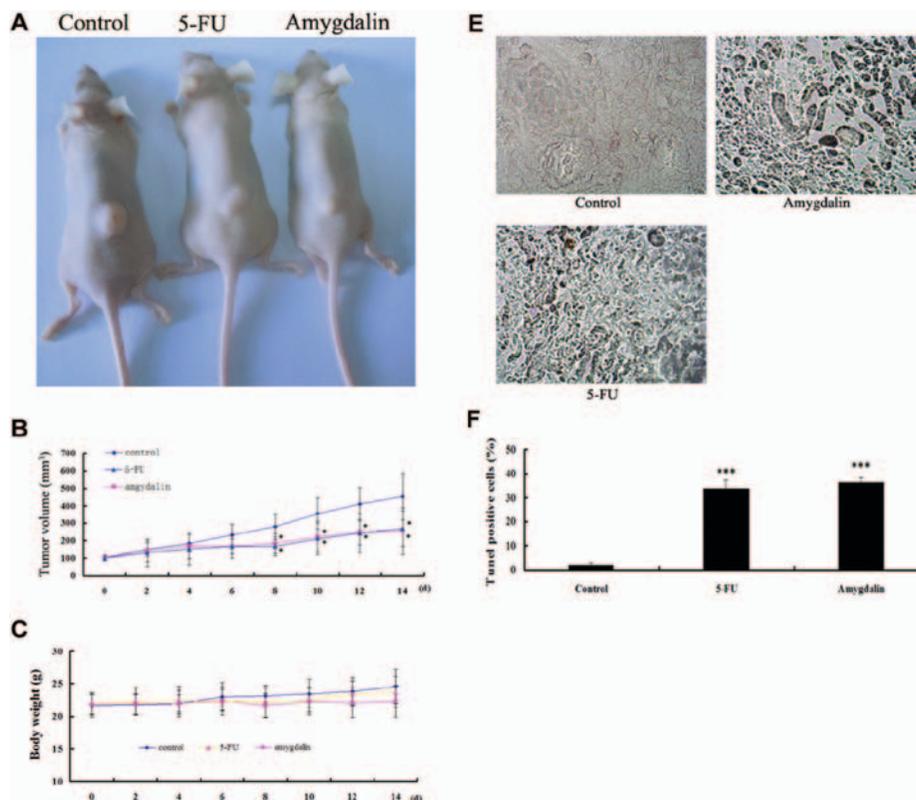


Figure 7. Effect of amygdalin on the growth of HeLa cell xenografts *in vivo*. HeLa cells were injected subcutaneously into the right flank of nude mice. After solid tumor was formed, the mice were randomly divided into three groups of control, 5-fluorouracil (5-FU) and amygdalin with six mice in each group. The mice were injected intraperitoneally daily with 0.9% NaCl, 30 mg/kg of 5-FU and 300 mg/kg of amygdalin in corresponding groups. Mice were sacrificed on the day 15 after the injection. (A) Visual observation of implanted tumors. (B) Tumor volume after the injection. Data are represented as means \pm SD. * $p < 0.05$ vs. control on the same day. (C) Growth curve. (D) Apoptosis measured by TUNEL assay. (E) TUNEL positive cells counted under a light microscope. The values represent means \pm SD from five slides. * $p < 0.05$ vs. control.

cellular nuclei under a fluorescent microscope (Figure 3B).^(18,19) Annexin V-FITC and PI double staining was used to measure the amount of early and late-apoptotic cells by flow cytometry (Figure 4).^(20–22) Caspase-3 activity, known to mediate integral parts of the apoptotic pathway^(23–27) and the ratio of Bax to Bcl-2⁽²⁸⁾ were also shown to be increased by amygdalin treatment. The results from above studies demonstrated the apoptotic effect of amygdalin on HeLa cells. The induction of apoptosis by amygdalin in several other cell types has been reported recently including prostate cancer cells⁽⁵⁾ and promyelocytic leukemia cells,⁽¹⁸⁾ which are consistent with the findings in our study except the type of cell. There are two pathways mediating the development of apoptosis, the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is mediated by death receptors.⁽²⁹⁾ The mitochondria play a central role in the intrinsic pathway,^(30,31) which are regulated by the Bcl-2 family of proteins.^(32–35) The results in the present study showed that the ratio of Bax protein to Bcl-2 protein was increased in the HeLa cells treated with amygdalin in a dose-dependent manner suggesting an involvement of the intrinsic pathway in the amygdalin-induced apoptosis. In addition to apoptosis, another mechanism by which amygdalin reduced the viability of HeLa cells might be cell cycle arrest. To test the possibility

of cell cycle involvement, amygdalin-treated HeLa cells were stained and the distribution of the cells at G0/G1, S, and G2/M phases was measured by flow cytometry. It was shown that the distribution pattern of amygdalin and non-amygdalin-treated HeLa cells at G0/G1, S and G2/M phases remain the same indicating no cell cycle arrest was involved in amygdalin reducing the viability of HeLa cells (data not shown). The mechanisms by which amygdalin reduces the viability of a cell might vary in different cell types with different genetic background: the reduced viability caused by amygdalin in SNU-C4 human colon cancer cells has been shown to be mediated by a mechanism of downregulation of cell cycle-related proteins rather than apoptosis.⁽⁶⁾

The results from the present study demonstrated that amygdalin significantly inhibited the growth of the xenograft of HeLa cells in nude BALB/c mice by a mechanism of inducing apoptosis (Figure 7). The results of the *in vivo* study are novel as we have not seen similar report in publication so far. There were no obvious side effects found in the nude mice after amygdalin administration. This may hold true as amygdalin is kind of herb or vitamin occurring naturally. Although it contains a hydrocyanic group which is toxic to living cells, it is safe as long as the molecule of amygdalin remains intact without the

hydrocyanic group being released enzymatically from the molecule of amygdalin.

Conclusion

In this study, our results demonstrate that amygdalin is able to inhibit the growth of human cervical cancer cell line HeLa cells both *in vitro* and *in vivo* through a mechanism of inducing apoptosis. We suggest that amygdalin may serve as a potentially effective therapy for cervical cancer.

Acknowledgment

This work is partly supported by a grant from the Key Grant Project of the Ministry of Education of the P.R. China (No 707020).

Declaration of interest

The authors report no declarations of interest.

References

- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C., Thun, M.J. Cancer statistics, 2006. *CA Cancer J Clin* 2006, 56, 106-130.
- Young, J.L., Jazaeri, A.A., Darus, C.J., Modesitt, S.C. Cyclooxygenase-2 in cervical neoplasia: a review. *Gynecol Oncol* 2008, 109, 140-145.
- Rogers, L., Siu, S.S., Luesley, D., Bryant, A., Dickinson, H.O. Radiotherapy and chemoradiation after surgery for early cervical cancer. *Cochrane Database Syst Rev* 2012, 16, 5-9.
- Costa, L.S., Telles, C.B., Oliveira, R.M., Nobre, L.T., Dantas-Santos, N., Camara, R.B., Costa, M.S., Almeida-Lima, J., Melo-Silveira, R.F., Albuquerque, I.R., Leite, E.L., Rocha, H.A. Heterofucan from *Sargassum filipendula* induces apoptosis in HeLa cells. *Mar Drugs* 2011, 9, 603-614.
- Chang, H.K., Shin, M.S., Yang, H.Y., Lee, J.W., Kim, Y.S., Lee, M.H., Kim, J., Kim, K.H., Kim, C.J. Amygdalin induces apoptosis through regulation of Bax and Bcl-2 expressions in human DU145 and LNCaP prostate cancer cells. *Biol Pharm Bull* 2006, 29, 1597-1602.
- Park, H.J., Yoon, S.H., Han, L.S., Zheng, L.T., Jung, K.H., Uhm, Y.K., Lee, J.H., Jeong, J.S., Joo, W.S., Yim, S.V., Chung, J.H., Hong, S.P. Amygdalin inhibits genes related to cell cycle in SNU-C4 human colon cancer cells. *World J Gastroenterol* 2005, 11, 5156-5161.
- Fukuda, T., Ito, H., Mukainaka, T., Tokuda, H., Nishino, H., Yoshida, T. Anti-tumor promoting effect of glycosides from *Prunus persica* seeds. *Biol Pharm Bull* 2003, 26, 271-273.
- Shin, M.C., Jang, M.H., Chang, H.K., Kim, Y.J., Kim, E.H., Kim, C.J. Modulation of cyclooxygenase-2 on glycine- and glutamate-induced ion currents in rat periaqueductal gray neurons. *Brain Res Bull* 2003, 59, 251-256.
- Flora, K.P., Craddock, J.C., Ames, M.M. A simple method for the estimation of amygdalin in the urine. *Res Commun Chem Pathol Pharmacol* 1978, 20, 367-378.
- Khandekar, J.D., Edelman, H. Studies of amygdalin (laetrile) toxicity in rodents. *JAMA* 1979, 242, 169-171.
- Moertel, C.G., Ames, M.M., Kovach, J.S., Moyer, T.P., Rubin, J.R., Tinker, J.H. A pharmacologic and toxicological study of amygdalin. *JAMA* 1981, 245, 591-594.
- Yi-Chin, F., Wei-Hong, Y., Sheng-Feng, H., Horng-Chaung, H., Kuo-Fung, T., Chin-Jung, H., Chun-Yi, L., Sean, S. 2-Methoxyestradiol induces apoptosis and cell cycle arrest in human chondrosarcoma cells. *J Orthop Res* 2007, 25, 1106-1114.
- Al-Hazzani, A.A., Alshatwi, A.A. Catechin hydrate inhibits proliferation and mediates apoptosis of SiHa human cervical cancer cells. *Food Chem Toxicol* 2011, 49, 3281-3286.
- Dorr, R.T., Paxinos, J. The current status of laetrile. *Ann Intern Med* 1978, 89, 389-397.
- Greenberg, D.M. The case against laetrile: the fraudulent cancer remedy. *Cancer* 1980, 45, 799-807.
- Herbert, V. The nutritionally and metabolically destructive "nutritional and metabolic antineoplastic diet" of laetrile proponents. *Am J Clin Nutr* 1979, 32, 96-98.
- Newmark, J., Brady, R.O., Grimley, P.M., Gal, A.E., Waller, S.G., Thistlethwaite, J.R. Amygdalin (Laetrile) and prunasin beta-glucosidases: distribution in germ-free rat and in human tumor tissue. *Proc Natl Acad Sci USA* 1981, 78, 6513-6516.
- Kwon, H.Y., Hong, S.P., Hahn, D.H., Kim, J.H. Apoptosis induction of *Persicae Semen* extract in human promyelocytic leukemia (HL-60) cells. *Arch Pharm Res* 2003, 26, 157-161.
- Kikuchi, T., Ishii, K., Noto, T., Takahashi, A., Tabata, K., Suzuki, T., Akihisa, T. Cytotoxic and apoptosis-inducing activities of limonoids from the seeds of *Azadirachta indica* (neem). *J Nat Prod* 2011, 74, 866-870.
- Luo, K.W., Sun, J.G., Chan, J.Y., Yang, L., Wu, S.H., Fung, K.P., Liu, F.Y. Anticancer effects of imperatorin isolated from *Angelica dahurica*: induction of apoptosis in HepG2 cells through both death-receptor- and mitochondria-mediated pathways. *Chemotherapy* 2011, 57, 449-459.
- Ratha, J., Majumdar, K.N., Mandal, S.K., Bera, R., Sarkar, C., Saha, B., Mandal, C., Saha, K.D., Bhadra, R. A sphingolipid rich lipid fraction isolated from attenuated *Leishmania donovani* promastigote induces apoptosis in mouse and human melanoma cells *in vitro*. *Mol Cell Biochem* 2006, 290, 113-123.
- Xiang, S., Sun, Z., He, Q., Yan, F., Wang, Y., Zhang, J. Aspirin inhibits ErbB2 to induce apoptosis in cervical cancer cells. *Med Oncol* 2010, 27, 379-387.
- Chou, C.C., Yang, J.S., Lu, H.F., Ip, S.W., Lo, C., Wu, C.C., Lin, J.P., Tang, N.Y., Chung, J.G., Chou, M.J., Teng, Y.H., Chen, D.R. Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. *Arch Pharm Res* 2010, 33, 1181-1191.
- Asselin, E., Mills, G.B., Tsang, B.K. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res* 2001, 61, 1862-1868.
- Fox, R., Aubert, M. Flow cytometric detection of activated caspase-3. *Methods Mol Biol* 2008, 414, 47-56.
- Kang, Y.H., Yi, M.J., Kim, M.J., Park, M.T., Bae, S., Kang, C.M., Cho, C.K., Park, I.C., Park, M.J., Rhee, C.H., Hong, S.I., Chung, H.Y., Lee, Y.S., Lee, S.J. Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. *Cancer Res* 2004, 64, 8960-8967.
- Ola, M.S., Nawaz, M., Ahsan, H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol Cell Biochem* 2011, 351, 41-58.
- Liu, R.M., Zhong, J.J. Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. *Phytomedicine* 2011, 18, 349-355.
- Boatright, K.M., Salvesen, G.S. Mechanisms of caspase activation. *Curr Opin Cell Biol* 2003, 15, 725-731.
- Sharma, C., Sadrieh, L., Priyani, A., Ahmed, M., Hassan, A.H., Hussain, A. Anti-carcinogenic effects of sulforaphane in association with its apoptosis-inducing and anti-inflammatory properties in human cervical cancer cells. *Cancer Epidemiol* 2011, 35, 272-278.
- Fauzi, A.N., Norazmi, M.N., Yaacob, N.S. Tualang honey induces apoptosis and disrupts the mitochondrial membrane potential of

- human breast and cervical cancer cell lines. *Food Chem Toxicol* 2011, 49, 871-878.
32. Kim, K.Y., Seol, J.Y., Jeon, G.A., Nam, M.J. The combined treatment of aspirin and radiation induces apoptosis by the regulation of bcl-2 and caspase-3 in human cervical cancer cell. *Cancer Lett* 2003, 189, 157-166.
 33. Debatin, K.M. Apoptosis pathways in cancer and cancer therapy. *Cancer Immunol Immunother* 2004, 53, 153-159.
 34. Fulda, S., Debatin, K.M. Targeting apoptosis pathways in cancer therapy. *Curr Cancer Drug Targets* 2004, 4, 569-576.
 35. Daniel, P.T., Schulze-Osthoff, K., Belka, C., Güner, D. Guardians of cell death: the Bcl-2 family proteins. *Essays Biochem* 2003, 39, 73-88.