Original Research

The soy-derived peptide Vglycin inhibits the growth of colon cancer cells *in vitro* and *in vivo*

Chang Gao[1,*], Rui Sun[1,*], Ya-Rong Xie[1], An-Li Jiang[2], Mei Lin[1], Min Li[1], Zheng-Wang Chen[3], Ping Zhang[4], Honglin Jin[5] and Jue-Ping Feng[1]

[1]Department of Oncology, PuAi Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430034, P.R. China; [2]Medical College, Wuhan University of Science and Technology, Wuhan 430065, China; [3]Key Laboratory of Molecular Biophysics of Ministry of Education, School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China; [4]Shandong TianJiu Biotechnology Company, HeZe 274108, Shandong, China; [5]Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

*These authors contributed equally to this work

Corresponding author: Jue-Ping Feng. Email: fengjuepingpuai@163.com

Impact statement

The antidiabetic properties and the capability of inducing differentiation of human colon adenocarcinoma cells of Vglycin have been reported in our previous studies. However, the anticancer potential of Vglycin on colon cancer cells and its possible related mechanisms were still unknown. In this study, we found that Vglycin could reduce growth, viability, and colony formation or colony size of CT-26, SW480, and NCL-H716 colon cancer cells. Moreover, Vglycin decreased tumor volume by 38% in xenograft mice transplanted with CT-26 cells. The mechanisms of these phenomena may be due to the down-regulated CDK2 and Cyclin D1, G1/S phase cell cycle arrest, and the dysregulated expression of Bax, Bcl-2, and Mcl-1.

The findings highlight the anticancer potential of Vglycin against colon cancer cells, and suggest Vglycin may be another colon cancer potential suppressive component of plant-derived peptides.

Abstract

Vglycin, a novel natural polypeptide isolated from pea seeds, possesses antidiabetic properties. Our previous studies have shown that Vglycin can induce the differentiation of human colon adenocarcinoma cells. We aimed to determine the anticancer activity of Vglycin against colon cancer cells and to elucidate related apoptosis-inducing mechanisms. Treatment with purified Vglycin significantly reduced growth, viability, and colony formation of CT-26, SW480, and NCL-H716 colon cancer cells in a dose-dependent manner while down-regulating the expression of proliferating cell nuclear antigen. Mouse xenograft studies showed a 38% inhibition of colon cancer growth in mice treated with Vglycin (20 mg/kg/day) at day 21. Furthermore, the potential mechanisms involved in Vglycin-induced cell apoptosis were examined using cell cycle studies, ultrastructural examination, as well as apoptosis-associated pathway analysis. The results showed that Vglycin significantly promoted apoptosis and G1/S phase cell cycle arrest. As revealed by Western blot, the expression of CDK2 and Cyclin D1 was down-regulated in all three Vglycin-treated colon cancer cells, indicating that the CDK2/Cyclin D1 cell cycle pathway involved in the initiation and progression of colon cancer. Moreover, the inhibition of Vglycin-induced cell proliferation in colon cancer cells was accompanied by alteration of the expression levels of the apoptosis-related proteins Bax, Bcl-2 and Mcl-1, and an increase of caspase-3 activity. Together, our results suggest that Vglycin may be another plant-derived peptide that suppresses colon cancer, supporting the continued investigation of Vglycin as therapeutic agent for colon cancer.

Keywords: Vglycin, colon cancer, soybean peptide, antiproliferation, apoptosis, cell cycle

***Experimental Biology and Medicine* 2017; 242: 1034–1043. DOI: 10.1177/1535370217697383**

Introduction

Epidemiological evidence strongly supports that soybean consumption is related to a reduced risk of developing certain cancer[1] and lower mortality rates of particularly colon, breast, and prostate cancers.[2] Numerous studies have shown that soybean contains certain anticancer phytochemicals, such as proteins and peptides, which are suggested to contribute to cancer prevention and treatment.[3,4] Recently, many studies have demonstrated that certain plant-derived peptides exhibit anticancer properties. It includes cell cycle arrest, suppression of cancer cell growth, and invasion.[2,5,6] This led to a more detailed inquiry into the potential of soybean extracts in disease prevention and treatment.

Vglycin, a 37-residue polypeptide purified from pea seeds, consists of six half-cysteine residues interconnected

by three pairs of disulfide bonds. Vglycin is resistant to digestive enzymes and has antidiabetic potential.[7] As we have previously reported, Vglycin is a member of the pea albumin 1b (PA1b) subfamily and can improve insulin sensitivity and glucose tolerance of diabetic rats.[7] Moreover, Vglycin did not exhibit any toxicity on major organ in normal mice.[7] In addition, we found that Vglycin induced differentiation of the poorly differentiated human colon adenocarcinoma cell line NCI-H716.[7] As far as we know, the anticancer effect of Vglycin from pea seeds has not been studied yet, and no information about the effects of Vglycin on colon cancer cells (CCCs) is available by far.

In this study, the proliferation inhibitory effects of Vglycin on CT-26, SW480, and NCL-H716 were investigated *in vitro*. The underlying mechanisms of Vglycin in promoting apoptosis, referring to the expression of Bax, Bcl-2, myeloid cell leukemia 1 (Mcl-1), and the activation of caspase-3 were examined for the first time. A mouse xenograft model by subcutaneous implantation of CT-26 cells was used to assess the anticancer efficacy of Vglycin *in vivo*. Our findings may shed new insight into the understanding of the antitumor effect of Vglycin, potentially providing an alternative drug for colon cancer.

Materials and methods

Reagents and antibodies

Vglycin was isolated from pea seeds (*Pisum sativum* L), purified, and identified as previously described.[7] High-performance liquid chromatographic analysis revealed that the purity of Vglycin was above 95%.[7] The first antibody of proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, Mcl-1, CDK2, Cyclin D1, and β-actin were purchased from Protein Tech Group (10205-2-AP, 50599-2-Ig, 12789-1-AP, 16225-1-AP, 10122-1-AP, 60186-1-Ig; Chicago, IL). Caspase-3 activity kit and radioimmunoprecipitation assay (RIPA) lysis buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China); Cell Counting Kit-8 was purchased from Dojindo (Japan). Cell cycle detection kit and AnnexinV/propidium iodide (PI) apoptosis detection kit were purchased from KeyGen Biotech (NanJing, China).

Cell culture

Murine CCC (CT-26) and human CCCs (SW480 and NCI-H716) were purchased from the Chinese Type Culture Collection (CTCC). Colon epithelial cell (NCM460) was obtained from the ATCC (VA, USA). CT-26 and NCI-H716 cells were maintained in RPMI 1640 medium. SW480 was cultured in Leibovitz's L-15 medium. All cells were incubated with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Cell counting

After trypsin digestion, cells were collected in centrifuge tubes. By using a hemocytometer, 5×10^6 cells were planted in six-well plates for 24 h and treated with Vglycin (2.5, 5.0, 10.0 or 20.0 μmol/L) for another 72 h. Subsequently, cells were counted with a hemocytometer.

Cell viability assay

CT-26, SW480, or NCI-H716 cells were seeded for 24 h in 96-well plates (5×10^3/per well), and then treated with Vglycin (5.0 μmol/L) for 72 h. Cell Counting Kit-8 was used to detect the cell viability. Control wells were considered as 100% survival. All experiments were conducted in triplicate.

Cell viability $(\%) = (A - A_0)/(A_{\text{control}} - A_0) \times 100$ (A: experimental wells with addition of the indicated Vglycin concentration, CCK8 and cells; A_0: blank wells with addition of CCK8 but without cells; A_{control}: control wells with addition of CCK8, cells but without Vglycin).

Colony formation assay

Two hundred single viable CT-26 cells were seeded in a six-well plate. After treated with indicated Vglycin for 10 days, for SW480 cells, 500 single viable cells were seeded in a six-well plate, and stimulated with Vglycin (0.5, 1.0 or 5.0 μmol/L) for additional two weeks. The colony number was counted after stained with crystal violet.

Soft agar colony formation assay

For the clonogenic assay, agarose was added to 2 × RPMI-1640 with 20% fetal bovine serum (FBS), reaching final concentration of 1.2%. Then this mixture was quickly added to a six-well plate and cooled down to room temperature. Subsequently, NCI-H716 cells (2000 single viable cells per well) were mixed with 0.6% agarose containing indicated concentration of Vglycin, and were quickly added to the lower agarose layer. After observation for additional 21 days, 0.5 mg/mL MTT (Sigma-Aldrich, USA) was added to stain the cells and colonies were counted.

Electron microscope analysis

CCCs were treated with Vglycin for 24 h, after digested by trypsin and washed twice, fixed by 2.5% glutaraldehyde for 24 h in dark, cells were observed by transmission electron microscopy.

Apoptosis assay

The cells were plated in six-well plates for 12 h. starved for additional 12 h (cultured in serum-free medium), cells were stimulated with 10 μmol/L Vglycin for additional 24 h. Apoptosis was determined by AnnexinV/PI apoptosis detection kit. Flow cytometry analysis of the apoptotic rate was performed with the EXPO 32 software.

Cell cycle assay

Cells were starved for 12 h (cultured in serum-free medium), and treated with 10 μmol/L Vglycin for 24 h. After digestion with EDTA-free trypsin, collected cells were washed with ice-cold 2% bovine serum albumin–phosphate-buffered saline (BSA–PBS) solution for three times and fixed with 70% ice-cold ethanol for 2 h. Subsequently, 100 μL RNase A was added to the cells. Then 400 μL PI was added to the cells for 30 min in the dark. The cell cycle distribution was examined by

Beckman counter Epics XL. Cell cycle was analyzed by flow cytometry.

Caspase-3 activity analysis

Caspase-3 activity was examined using a caspase-3 activity kit (Beyotime Institute of Biotechnology). Briefly, trypsin digested cells were re-suspended in 100 μL lysis buffer and incubated for 15 min on ice. After centrifugation for 15 min at 20,000 *g* and 4°C, supernatants were collected in ice-cold centrifuge tubes. Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) was added into a 100 μL reaction volume, 50 μL samples were incubated for 2 h at 37°C, and then optical density (OD) value was measured at 405 nm.

Analysis of PCNA, CDK2, Cyclin D1, Bax, Bcl-2, and Mcl-1 by Western blotting

Exponentially growing cells (about 80% confluence) were starved for 12 h. For drug treatment, CCCs were stimulated with Vglycin for 24 h. Then CCCs were lysed using RIPA lysis buffer. Western blot analysis was performed following previously described protocols.[8] Densitometry analysis was conducted using the Gel capture software and Image J software (NIH).

In vivo tumor growth

All animal studies were designed and performed in compliance with approved protocol by the Hubei provincial Animal Care and Use Committee. Male BALB/c mice (5- to 6-weeks old) were supplied by Animal Experimental Center of Tongji Medical College. The CT-26 cells (1×10^5) were re-suspended in PBS (50 μL) and subcutaneously injected into mice for colon tumor generation.

Then, mice were randomly assigned to two groups ($n = 6$ mice/group): a vehicle control and a Vglycin treatment group. The Vglycin-treated group was injected intraperitoneally with Vglycin (20 mg/kg/day) starting upon initiation of the grafts. Control mice were injected with PBS. During the study, body weight and tumor growth of mice were monitored every other day. Tumors were dissected after 21 days of inoculation. The volume of tumor was calculated: tumor volume = $(\text{width})^2 \times (\text{length})/2$.

Statistical analysis

Statistical analyses were performed using the SPSS 13.0 software. Data are expressed as mean values ± standard deviation (SD). One-way analysis of variance (ANOVA) tests were performed for qualitative comparative analysis. Differences were considered to be statistically significant when $P < 0.05$.

Results

Vglycin selectively inhibits the proliferation and colony formation of CCCs

To explore whether Vglycin could suppress the growth of CCCs, three types of CCCs were studied. Vglycin inhibited the proliferation of CT-26, SW480, and NCI-H716 cells at a concentration of 2.5 μmol/L (Figure 1(a)). At this concentration, Vglycin caused a reduction of viable CT-26 cells ($P < 0.05$), resulting in a proliferation inhibition of 21.22%. At the highest concentration of 20 μmol/L, Vglycin caused a 71.51% inhibition ($P < 0.01$) in CT-26 cells, as for SW480 and NCI-H716 cells, corresponding inhibition rates were 80.51% and 87.20%, respectively. Half maximal inhibitory concentration (IC_{50}) values of CT-26, SW480, and NCI-H716 cells were 4.21, 3.68, and 3.62 μmol/L, respectively (Supplemental Figure S1). In contrast, this Vglycin-induced cytotoxic effect was not observed in the normal colon epithelial cell line (NCM460) when the cells were treated with Vglycin at concentrations of 2.5–20.0 μmol/L (data not shown).

We then confirmed the results obtained for a concentration of 5.0 μmol/L by performing CCK-8 analysis on the three cell lines. This confirmed that Vglycin suppresses the growth of CT-26, SW480, and NCI-H 716 cells ($P < 0.05$) (Figure 1(b)).

As colony formation was difficult when the Vglycin concentration was higher than 5.0 μmol/L (data not shown), colony formation assay was conducted at a relatively lower concentration. In the anchorage-dependent colony formation assay, Vglycin decreased the colonogenicity of SW480 and CT-26 cells compared to the control depending on the Vglycin concentration (Figure 1(c)). In the anchorage-independent colony formation assay, Vglycin effectively inhibited colony formation of NCI-H716 cells (Figure 1(c)).

Vglycin reduces tumors growth *in vivo*

In order to evaluate the effect of Vglycin *in vivo*, CT-26 cells were implanted subcutaneously into BALB/c mice, and Vglycin was injected intraperitoneally into the tumor-bearing mice (20 mg/kg/day; $n = 6$ per control and treatment group, respectively). Tumor-bearing mice and dissected tumor tissues were macroscopically observed in Vglycin and control groups, respectively. By day 21 post initiation, tumor volume averaged at 431.7 and 267.8 mm^3 in the control and Vglycin-treated groups, respectively. The tumor volume in the Vglycin-treated group decreased about 38% (Figure 1(d)).

Vglycin caused ultrastructural changes and formation of apoptotic body of CCCs

Characteristic morphological features of apoptosis include chromatin condensation, cell shrinkage, vacuole formation,[9] and mitochondria swelling.[10] We detected ultrastructural changes in these morphological features after Vglycin treatment at the concentration of 10 μmol/L in CT-26, SW480, and NCI-H716 CCCs. At a relatively higher Vglycin concentration of 20 μmol/L, apoptosis became predominant ultrastructural feature. Especially, apoptotic bodies appeared in the 10 μmol/L group (Figure 2).

Vglycin induces a G1-phase arrest of CCCs

Furthermore, we studied the effect of Vglycin on cell cycle distribution of CCCs. We found that Vglycin treatment of CT-26, SW480, and NCI-H716 cells resulted in G1 cell cycle arrest (Figure 3). For CT-26 cells, the cell amount in the G1

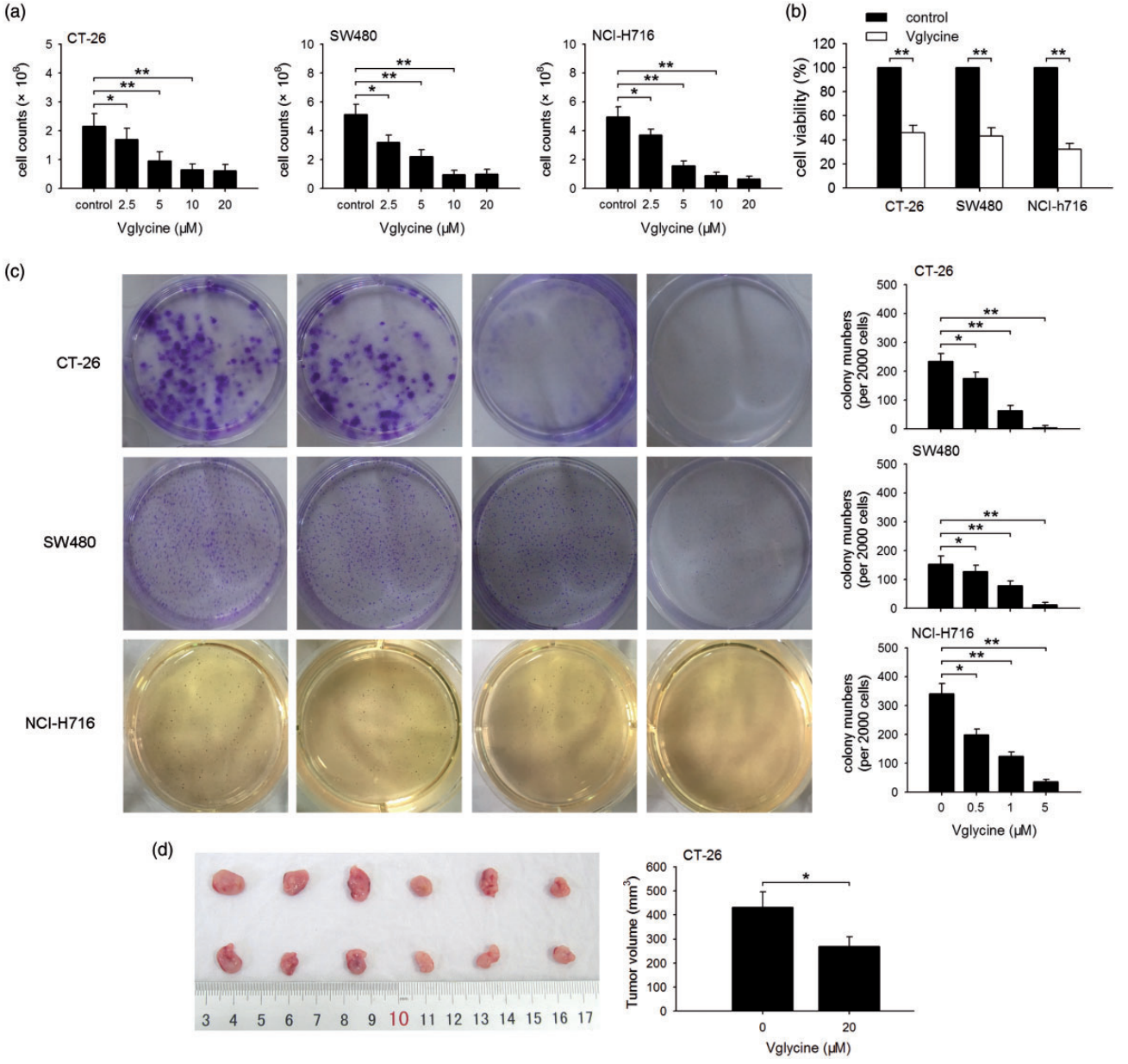

Figure 1 Vglycin caused inhibition of proliferation and colony formation of colon cancer cells. (a) CT-26, SW480, and NCI-H716 cells were treated with 0, 2.5, 5, 10, or 20 μmol/L Vglycin for 72 h. Cells were counted using a hemocytometer. (b) CT-26, SW480, and NCI-H716 cells were treated with 5 μmol/L Vglycin for 72 h and the cell viability was detected. (c) Colony formation assay was conducted and colonies were counted as descript in methods. (d) CT-26, SW480, and NCI-H716 cells were inoculated subcutaneously into BALB/c mice, then Vglycin was injected intraperitoneally, a representative CT-26 tumor is shown (left) and tumor volumes were calculated as described in ''Materials and methods'' section, each group contains six mice. Data are pooled from three independent experiments (a; b), or representative of three independent experiments (c, left; d, left). $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

phase increased from 18.24% for the untreated cells to 51.40% for the treated ones with 20 μmol/L Vglycin. For SW480 and NCI-H716 cells, the cell amount G1 phase increased from 18.62% to 55.36% and from 14.13% to 43.60%, respectively. This cell increase in G1 phase also caused a significant cell reduction in the S-phase (Figure 3).

Vglycin down-/up-regulates protein expression involving cell cycle progression of cancer cells

In order to unveil the mechanism by which Vglycin causes a G1/S cell cycle arrest, the expression of the cell cycle regulatory proteins Cyclin D1, PCNA, and CDK2 in the three cell lines were measured.

Results indicated that Vglycin leads to a dose-dependent decrease of Cyclin D1 and PCNA. The expression of Cyclin D1 decreased by 15.71%, 61.69%, and 57.85% in CT-26 cells, by 25.00%, 56.25%, and 62.50% in SW480 cells, and by 14.29%, 57.14%, and 64.28% in NCI-H716 cells when CCCs were treated with 5, 10, and 20 μmol/L Vglycin, respectively (Figure 4). Similarly, although no statistically significant difference was observed in SW480 cells, the PCNA expression decreased by 14.53%, 57.26%, and 54.42% in CT-26, and 9.09%, 27.27%, and 54.55% in

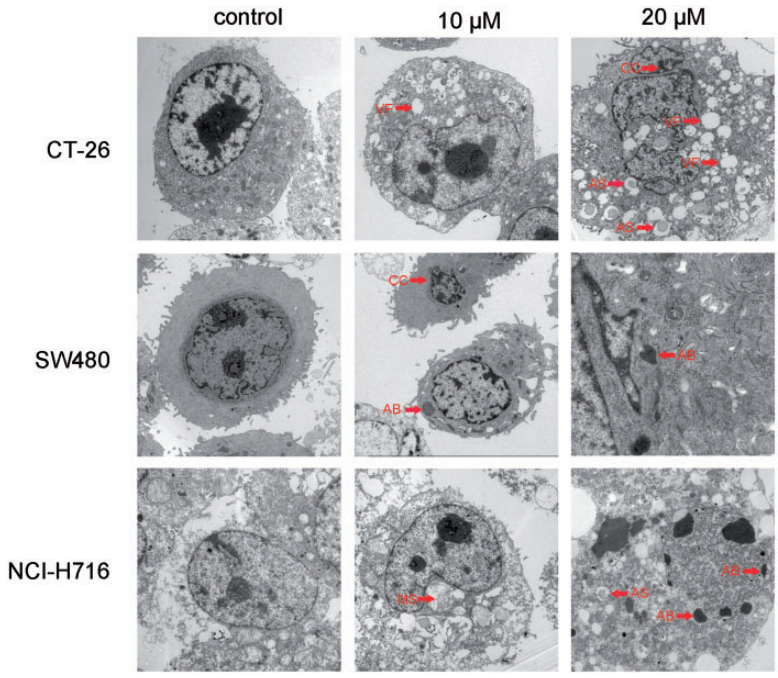

Figure 2 Vglycin caused ultrastructural changes and the formation of apoptotic body of colon cancer cells. CT-26, SW480, and NCI-H716 cells were treated with 5 μmol/L Vglycin for 72 h and fixed in 2.5% glutaraldehyde at 4°C overnight in the dark. Representative images from electron microscopy were shown. Data are representative of three independent experiments. VF: vacuole formation; MS: mitochondria swelling; AB: apoptotic bodies; CC: chromatin condensation; AS: autophagosome. (A color version of this figure is available in the online journal.)

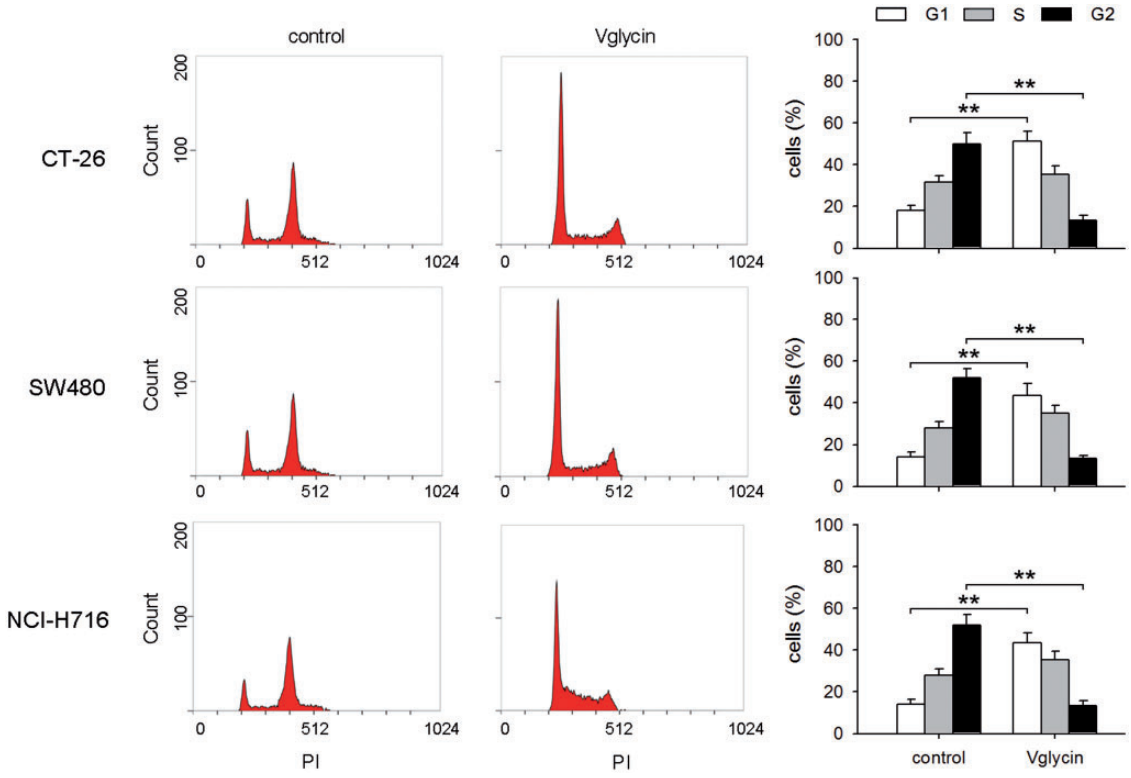

Figure 3 Vglycin caused a G1 cell cycle arrest of colon cancer cells. (a) CT-26, SW480, and NCI-H716 cells were treated with 10 μmol/L Vglycin for 24 h as described in ''Material and methods'' section, and the cell cycle distribution was examined. Data are representative of three independent experiments (left) or pooled from three independent experiments with a total of six samples in each group (right). $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

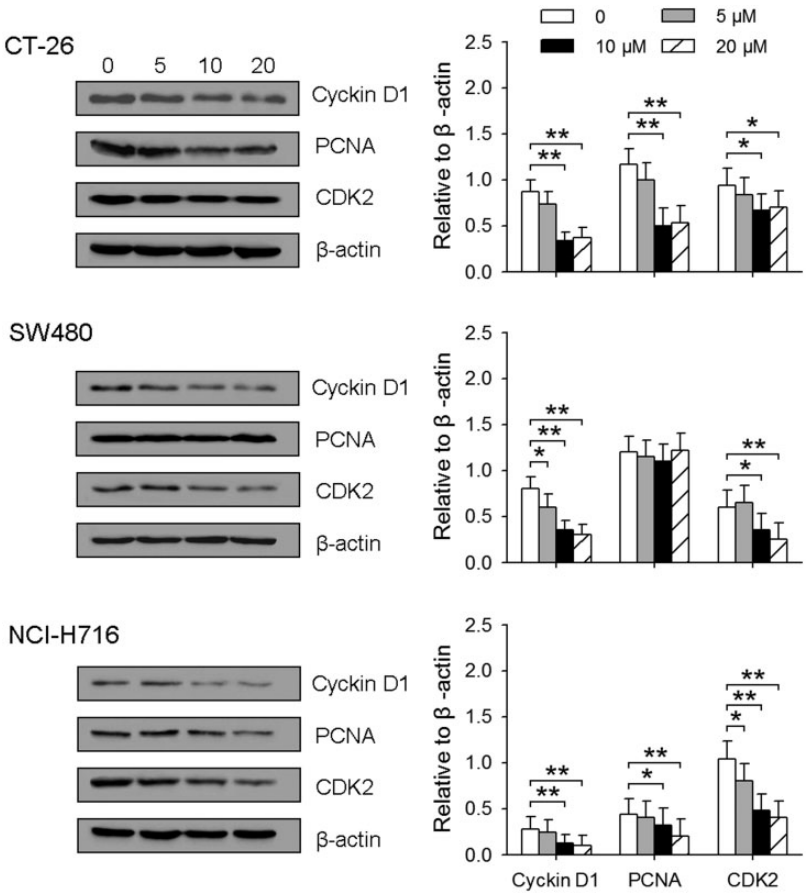

Figure 4 Vglycin down-/up-regulates protein expression involving cell cycle progression of cancer cells. CT-26, SW480, and NCI-H716 cells were incubated with 5, 10, or 20 μmol/L Vglycin for 24 h, and the expression of Cyclin D1, PCNA, and CDK2 was detected by Western blotting (left). The ratios of Cyclin D1, PCNA, and CDK2 to β-actin were calculated after densitometric analysis (right). Data are representative of three independent experiments (left), or pooled from three independent experiments with a total of six samples in each group (right). $^{*}P < 0.05$, $^{**}P < 0.01$

NCI-H716 cells when they were treated with 5, 10, and 20 μmol/L Vglycin (Figure 4).

Furthermore, we validated the enhanced inhibition of tumor growth by checking the status of the cell cycle regulatory protein CDK2. The CDK2 levels were significantly lower in the tumor lysates of Vglycin-treated cells compared to the control. These results indicated that Vglycin inhibited the growth of CT-26, SW480, and NCI-H716 cells by modulating cell cycle regulatory proteins (Figure 4).

Vglycin promotes apoptosis in CCCs

To determine whether the reduction in cell proliferation of CT-26, SW480, and NCI-H716 cells was due to apoptosis, CT-26, SW480, and NCI-H716 cells were treated with Vglycin at different time duration. Vglycin treatment for 12 h did not result in a significant increase in apoptosis (data not shown). A 10 μmol/L Vglycin treatment for 24 h caused a significant increase in apoptotic CT-26 cells. The percentage of apoptotic cells increased from 1.2% to 35.2% (Figure 5(a)).

For SW480 and NCI-H716 cells, the apoptotic cells rate increased from 0.6% and 5.5% to 24.7% and 59%, respectively (Figure 5(a)). Moreover, in line with the flow cytometry results, the increased caspase-3 activity of the Vglycin-treated group also indicated that Vglycin promoted apoptosis in CCCs (Figure 5(b)).

Vglycin mediated the apoptosis of CCC by modifying the expression of antiapoptotic proteins Bcl-2, Mcl-1 and pro-apoptotics protein Bax

To further clarify the mechanism involved in Vglycin-induced apoptosis, the expression of Bcl-2 family was measured. Vglycin could upregulate the Bax expression, a pro-apoptotic protein. In CT-26 and SW480 cells, it increased in a dose-dependent manner. In NCI-H716 cells, a similar increase in the Bax expression was observed after Vglycin treatment (Figure 6(a)). Besides, the expression of the Bcl-2 and Mcl-1 was reduced by the treatment with Vglycin. In CT-26, SW480, and NCI-H716 cells, significant inhibition of Bcl-2 and Mcl-1 expression was observed after Vglycin treatment (Figure 6(a)).

The Bcl-2/Bax ratio was significantly decreased after Vglycin treatment (Figure 6(b)). These results suggested that the down-regulation of Bcl-2 and Mcl-1, and the up-regulation of Bax may be involved in the Vglycin-induced apoptosis in CT-26, SW480, and NCI-H716 cells.

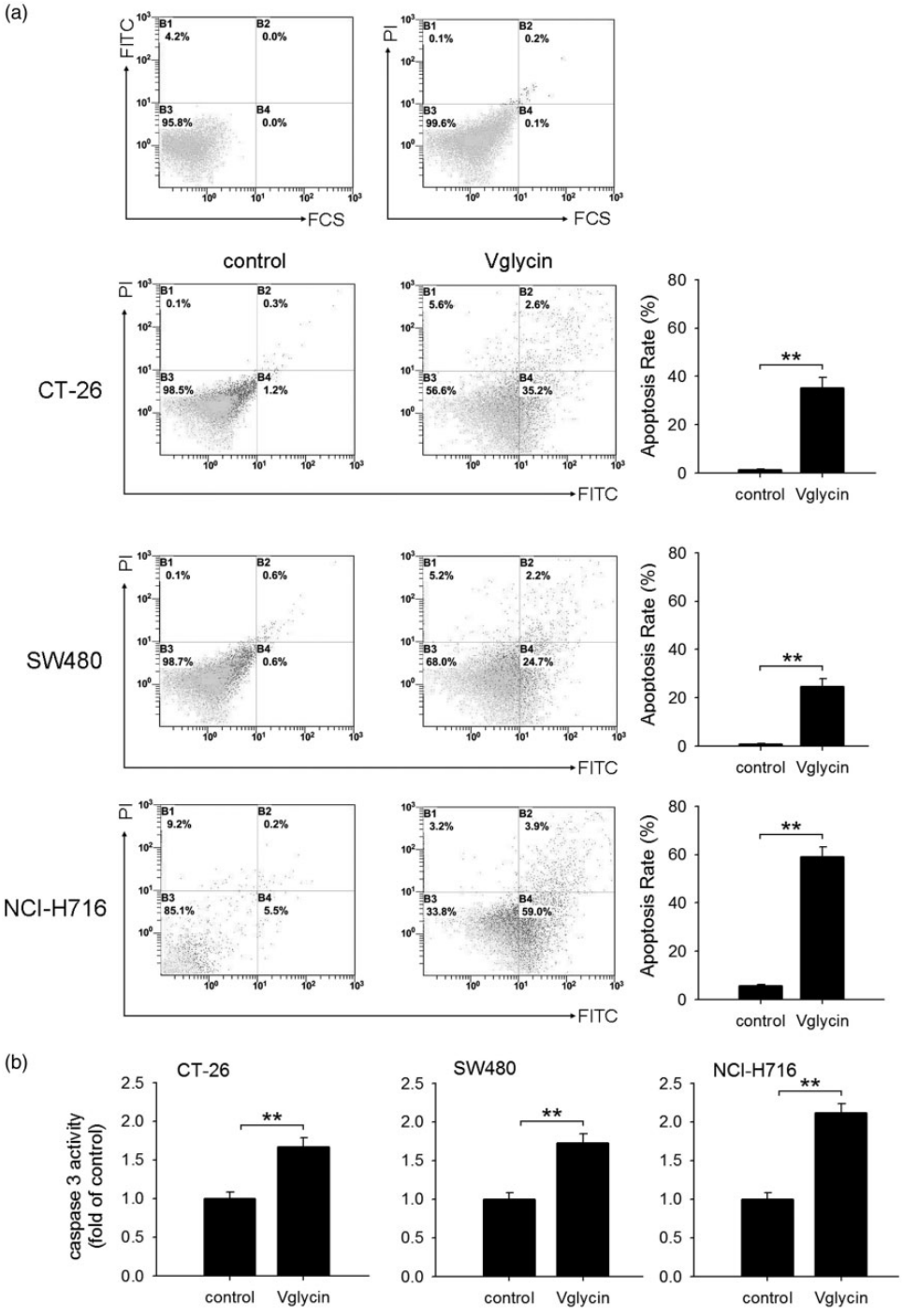

Figure 5 Vglycin promoted apoptosis in colon cancer cells. (a, b) CT-26, SW480, and NCI-H716 cells were treated with 10 μmol/L Vglycin for 24 h, and the percentage of apoptotic cells was detected as described in ''Materials and methods'' section (a), analysis of caspase-3 activity (b). Data are representative of three independent experiments (a, left) or pooled from three independent experiments with a total of eight samples in each group (a, right; b). $^{*}P < 0.05$, $^{**}P < 0.01$

Discussion

Our preliminary results has shown that Vglycin induces differentiation of the poorly differentiated human colon adenocarcinoma cell line NCI-H716.[7] In our study, the capability of Vglycin to inhibit proliferation in the CCCs and the underlying mechanism for apoptosis induction were demonstrated for the first time.

In vitro functional studies showed that Vglycin treatment caused significant cytotoxicity against CT-26, SW480, and NCI-H716 cells with IC_{50} values of 4.21, 3.68, and 3.62 μmol/L, respectively, suggesting a strong ability of Vglycin in decreasing the growth and viability of CCCs. Moreover, Vglycin exhibited remarkable inhibitory effects on colony formation in CT-26, SW480, and NCI-H716 cells, whereas normal colon epithelial cell lines (NCM460) were unaffected, indicating that Vglycin is likely to selectively inhibit the growth of cancerous cells. Consistent with this *in vitro* result, we did not observe any toxic effects of

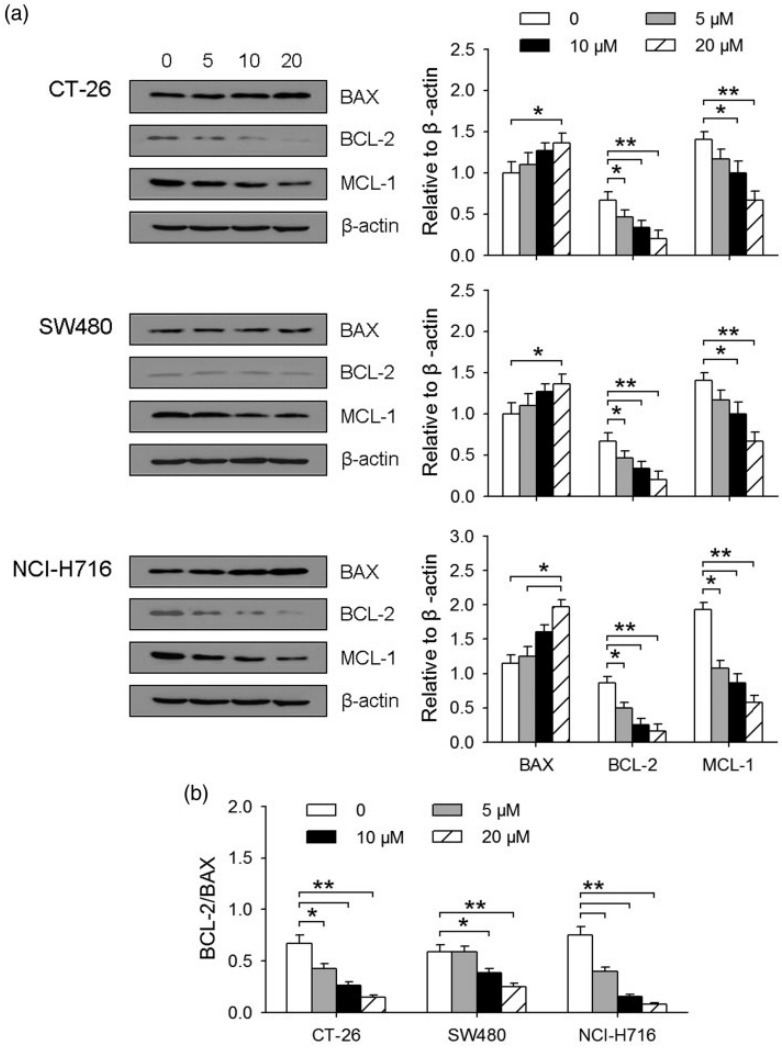

Figure 6 Vglycin regulates the expression of antiapoptotic proteins Bcl-2 and Mcl-1, and pro-apoptotic protein Bax in colon cancer cells. (a, b) CT-26, SW480, and NCI-H716 cells were incubated with 5, 10, or 20 μmol/L of Vglycin for 24 h, and the expression of Bax, Bcl-2, and Mcl-1 was detected by Western blotting (a, left). The ratios of Bax, Bcl-2, Mcl-1 to β-actin were calculated after densitometric analysis (a, right). Ratios of Bcl-2 to Bax were calculated. Data are representative of three independent experiments (a, left) or pooled from three independent experiments with a total of six samples in each group (a, right). $^{*}P < 0.05$, $^{**}P < 0.01$

Vglycin on major organ functions of normal BALB/c mice in our previous studies.[7] Even more important, differences in the growth and viability of Vglycin-treated CT-26, SW480, and NCI-H716 cells were detected. Above results indicate that the cytotoxic effects of Vglycin on CCCs is cell-type dependent, which is possibly due to the various biological differences between the three CCCs.

PCNA, a DNA polymerase delta auxiliary protein, is a marker of cell proliferation, as it represents cell replication capability,[11] and PCNA expression levels in colorectal adenocarcinomas are related to survival times.[12] We analyzed the effects of Vglycin on PCNA expression in CT-26, SW480, and NCI-H716 cells by Western blot analysis. The results revealed a down-regulation of PCNA expression levels both in the Vglycin treated CT-26 and NCI-H716 cells, further providing the evidence that Vglycin effectively reduces the proliferation of CCCs.

An important finding in our studies of the animal model was that Vglycin has the ability to inhibit colon cancer growth *in vivo*. By using a CT-26 xenograft mouse model, we observed an inhibitory rate of 38% in the Vglycin-treated group at 20 mg/kg every day for 21 days compared with the control. The results indicate that Vglycin has an inherently profound effect on tumor growth and progression. Our *in vivo* result is similar to another observation of a breast cancer xenograft model that lunasin, another soybean bioactive peptide, attenuates tumor growth *in vivo*.[13] This supports our finding that Vglycin possesses anticancer potential, and may act as a cancer therapeutic agent.

The ultrastructural analysis of Vglycin-treated CCCs revealed notable and progressive morphological changes in CT-26, SW480, and NCI-H716 cells, including vacuolization, mitochondrial swelling, nuclear chromatin condensation, and margination. These results suggest that Vglycin affects the cellular structures of colon cancer cellular and promotes cell apoptosis. Similar apoptotic effects of Vglycin on CT-26, SW480, and NCI-H716 cells were also detected by Annexin V/PI. Apoptosis of 35.2%, 24.7%, and 59.0% of the

total cell number was induced in CT-26, SW480, and NCI-H716 cells, respectively, by treatment of 10 μmol/L Vglycin. These results demonstrated that Vglycin treatment lead to a significant increase in the percentage of CT-26, SW480, and NCI-H716 cells undergoing apoptosis.

Mechanistic studies demonstrated that the antiproliferative effect of Vglycin on CT-26, SW480, and NCI-H716 cells *in vitro* is caused by cell cycle arrest at the G1/S phase. These results proved the capability of Vglycin in regulating the cell cycle distribution of CCCs and inhibiting cells entry into the mitosis.[14] Considering the roles of Bax, Bcl-2, and Mcl-1 in inducing of cell apoptosis, we further detected the expression of these proteins and the activity of caspase-3. We observed that Vglycin treatment resulted in a dose-dependent down-regulation of the expression of Bcl-2 and Mcl-1 proteins in CT-26, SW480, and NCI-H716 cells. This was concomitant with the up-regulation of the expression of Bax, which is responsible for antagonizing Bcl-2 and inducing apoptosis via the mitochondrial-dependent pathway.[15] Moreover, Vglycin treatment increased caspase-3 activity in CCCs by inducing apoptosis, mainly through regulation of Bax, Bcl-2, and Mcl-1 expression. Therefore, caspase-3 has a central role in Vglycin-induced apoptosis in CCCs.

Numerous studies have shown that the Bcl-2 protein family, including the pro-apoptotic proteins Bax, the anti-apoptotic proteins Bcl-2, and Mcl-1, plays a crucial role in regulating apoptosis. Furthermore, Bcl-2 family members are important treatment targets as a result of their over-expression in a variety of tumors.[16,17] It is reported that Bax expression is positively correlated with better survival in advanced colorectal cancer (CRC).[18] Moreover, Bax/Bcl-2 ratio was regarded as critical to cell apoptosis in the mitochondria-mediated apoptosis pathway,[9] as the change in the Bax/Bcl-2 ratio initiates caspase-cascade signaling.[19] Several studies have shown that caspases are enzymes that are a member of the cysteine family, and are critical to the changes of morphological and biochemistry during apoptosis.[20,21] Caspase-3 is a main executioner caspases in cell and activated by pro-apoptotic signals, and has a key role in the terminal phase of apoptosis induced by diverse stimuli.[21,22] Our data demonstrated that Vglycin-induced apoptosis occurred by modification of Bax and Bcl-2 involved in the apoptotic mitochondrial pathway, and resulted in caspase-3 activation, which promotes apoptosis in CCCs. Specifically, Vglycin alters the Bax/Bcl-2 ratio and may trigger the apoptotic pathway in CCCs.

Additionally, Mcl-1 amplification is a common genetic abnormality occurring in cancers,[23] indicating Mcl-1 also plays a key role in tumorigenesis. In our study, we observed that the Mcl-1protein level was reduced by Vglycin, implying that Vglycin regulates not only the expression levels of Bcl-2 and Bax, but also the Mcl-1 expression level. These results suggested that Vglycin-induced apoptosis of CCCs is mediated by the down-regulation of Bcl-2 and Mcl-1, the up-regulation of Bax as well as the disturbance of the Bax/Bcl-2 ratio, thereby initiating caspase-cascade signaling. Among these proteins, Mcl-1 may contribute to oncogenesis by promoting apoptotic resistance and proliferation of cancer cells, which makes it a critical molecule in cancer initiation and progression.[24]

It is well known that cyclins and cyclin-dependent kinases (CDKs) are also related to mechanisms of cell cycle regulation in cancer. Transition from one to another cell cycle phase (G1–S–G2–M) is regulated by a series of checkpoints where members of the CDK family bind with their respective regulatory subunits (Cyclins), which in turn trigger subsequent downstream processes of the cell cycle by phosphorylating matched target proteins.[25] When CDKs are abnormally up-regulated, cells undergo cycles and generate resistance to apoptosis, which result in uncontrolled proliferation.[26] CDK2, a member of the CDK family, is a serine/threonine protein kinase. It regulates cell cycle conversion of G1- and S-phase. Therefore, it is important for controlling cell proliferation.[27] Cyclins D1 is responsible for integrating extracellular signals in the cells.[28] Animal model studies shows that the overexpression of Cyclin D1 directly contributes to tumorigenesis.[28,29] For this reason, the suppression of the Cyclin D1 function is probably one of the important strategies for cancer treatment.[30]

In this study, we observed that CDK2 and Cyclin D1 are overexpressed in CT-26, SW480, and NCI-H716 cells, and the antiproliferative effect of Vglycin on these cells resulted from the inhibition of expression levels of CDK2 and Cyclin D1. Moreover, Vglycin-induced down-regulation of the expression of CDK2 and Cyclin D1 was associated with a lower Bcl-2/Bax ratio. Taken together, these results imply that Vglycin inactivates CDK2 and Cyclin D1 continuously, which in turn decreases Bcl-2/Bax ratio probably by down-regulating Bcl-2 and up-regulating Bax, as well as by activating caspases-3, which finally attenuates apoptosis resistance in CCCs.

In summary, our results demonstrate the ability of Vglycin to inhibit proliferation, and induce apoptosis and G1/S phase arrest of the CCCs, CT-26, SW480, and NCI-H716 cells. We proposed a model by which Vglycin promotes apoptosis in CCCs *in vitro* through activation of mitochondrial pathways, as evidenced by down-regulation of Bcl-2 expression, up-regulation of Bax expression, alteration of the Bcl-2/Bax rate, and activation of the caspase-3 activity. The CDK2/CyclinD1 signaling pathway has pivotal effect in the initiation and progression of CRC. Our findings may facilitate the understanding of the health effects of pea protein, and, furthermore, imply that Vglycin is a potential candidate for the prevention and treatment of colon cancer in humans.

Authors' contributions: Experiments were conceived and designed by JPF; experiments performed by CG. data analysis performed by JPF, RS, and CG. Manuscript writing done by JPF, RS, and CG. All authors reviewed the manuscript.

ACKNOWLEDGMENT

The authors extend their thanks to Hua Jiang PhD at University of Science and Technology of China for technical assistance. This work was financially supported by the Natural Science Foundation of Hubei province (2012FFB056010); Wuhan

Yellow Crane medical talent program under Grant; Wuhan Science and Technology Bureau (201260523197, 201271130460, 2015061701011626 and 2013060602010259); National Natural Science Foundation of P.R. China (81502461).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Dia VP, Mejia EG. Lunasin promotes apoptosis in human colon cancer cells by mitochondrial pathway activation and induction of nuclear clusterin expression. *Cancer Lett* 2010;**295**:44–53
2. Ortiz-Martinez M, Winkler R, Garcia-Lara S. Preventive and therapeutic potential of peptides from cereals against cancer. *J Proteomics* 2014;**111**:165–83
3. Hernandez-Ledesma B, Hsieh CC, de Lumen BO. Lunasin, a novel seed peptide for cancer prevention. *Peptides* 2009;**30**:426–30
4. de Mejia EG, Bradford T, Hasler C. The anticarcinogenic potential of soybean lectin and lunasin. *Nutr Rev* 2003;**61**:239–46
5. McConnell EJ, Devapatla B, Yaddanapudi K, Davis KR. The soybean-derived peptide lunasin inhibits non-small cell lung cancer cell proliferation by suppressing phosphorylation of the retinoblastoma protein. *Oncotarget* 2015;**6**:4649–62
6. Jiang Q, Pan Y, Cheng Y, Li H, Liu D, Li H. Lunasin suppresses the migration and invasion of breast cancer cells by inhibiting matrix metalloproteinase-2/-9 via the FAK/Akt/ERK and NF-kappaB signaling pathways. *Oncol Rep* 2016;**36**:253–62
7. Jiang H, Feng J, Du Z, Zhen H, Lin M, Jia S, Li T, Huang X, Ostenson CG, Chen Z. Oral administration of soybean peptide Vglycin normalizes fasting glucose and restores impaired pancreatic function in type 2 diabetic Wistar rats. *J Nutr Biochem* 2014;**25**:954–63
8. Ho CM, Hu FW, Lee SS, Shieh TM, Yu CH, Lin SS, Yu CC. ZEB1 as an indicator of tumor recurrence for areca quid chewing-associated oral squamous cell carcinomas. *J Oral Pathol Med* 2015;**44**:693–8
9. Zou Y, Chang SK. Effect of black soybean extract on the suppression of the proliferation of human AGS gastric cancer cells via the induction of apoptosis. *J Agric Food Chem* 2011;**59**:4597–605
10. Broecker-Preuss M, Viehof J, Jastrow H, Becher-Boveleth N, Fuhrer D, Mann K. Cell death induction by the BH3 mimetic GX15-070 in thyroid carcinoma cells. *J Exp Clin Cancer Res* 2015;**34**:69
11. Zorofchian Moghadamtousi S, Rouhollahi E, Karimian H, Fadaeinasab M, Firoozinia M, Ameen Abdulla M, Abdul Kadir H. The chemopotential effect of Annona muricata leaves against azoxymethane-induced colonic aberrant crypt foci in rats and the apoptotic effect of acetogenin annomuricin E in HT-29 cells: a bioassay-guided approach. *PLoS One* 2015;**10**:e0122288
12. Mayer A, Takimoto M, Fritz E, Schellander G, Kofler K, Ludwig H. The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and MDR gene expression in colorectal cancer. *Cancer* 1993;**71**:2454–60
13. Hsieh CC, Hernandez-Ledesma B, Jeong HJ, Park JH, de Lumen BO. Complementary roles in cancer prevention: protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS One* 2010;**5**:e8890
14. Stark GR, Taylor WR. Control of the G2/M transition. *Mol Biotechnol* 2006;**32**:227–48
15. Chan CK, Supriady H, Goh BH, Kadir HA. Elephantopus scaber induces apoptosis through ROS-dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. *J Ethnopharmacol* 2015;**168**:291–304
16. Dutta S, Ryan J, Chen TS, Kougentakis C, Letai A, Keating AE. Potent and specific peptide inhibitors of human pro-survival protein Bcl-xL. *J Mol Biol* 2015;**427**:1241–53
17. Billard C. BH3 mimetics: status of the field and new developments. *Mol Cancer Ther* 2013;**12**:1691–700
18. Nehls O, Okech T, Hsieh CJ, Enzinger T, Sarbia M, Borchard F, Gruenagel HH, Gaco V, Hass HG, Arkenau HT, Hartmann JT, Porschen R, Gregor M, Klump B. Studies on p53, BAX and Bcl-2 protein expression and microsatellite instability in stage III (UICC) colon cancer treated by adjuvant chemotherapy: major prognostic impact of proapoptotic BAX. *Br J Cancer* 2007;**96**:1409–18
19. Danial NN. BCL-2 family proteins: critical checkpoints of apoptotic cell death. *Clin Cancer Res* 2007;**13**:7254–63
20. Galluzzi L, Lopez-Soto A, Kumar S, Kroemer G. Caspases connect cell-death signaling to organismal homeostasis. *Immunity* 2016;**44**:221–31
21. Flanagan L, Meyer M, Fay J, Curry S, Bacon O, Duessmann H, John K, Boland KC, McNamara DA, Kay EW, Bantel H, Schulze-Bergkamen H, Prehn JH. Low levels of Caspase-3 predict favourable response to 5FU-based chemotherapy in advanced colorectal cancer: Caspase-3 inhibition as a therapeutic approach. *Cell Death Dis* 2016;**7**:e2087
22. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998;**281**:1312–6
23. Billard C. BH3 mimetics: status of the field and new developments. *Mol Cancer Ther* 2013;**12**:1691–700
24. Perciavalle RM, Opferman JT. Delving deeper: MCL-1's contributions to normal and cancer biology. *Trends Cell Biol* 2013;**23**:22–9
25. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* 2003;**36**:131–49
26. He X, Xiang H, Zong X, Yan X, Yu Y, Liu G, Zou D, Yang H. CDK2-AP1 inhibits growth of breast cancer cells by regulating cell cycle and increasing docetaxel sensitivity *in vivo* and *in vitro*. *Cancer Cell Int* 2014;**14**:130
27. Shi XN, Li H, Yao H, Liu X, Li L, Leung KS, Kung HF, Lin MC. Adapalene inhibits the activity of cyclin-dependent kinase 2 in colorectal carcinoma. *Mol Med Rep* 2015;**12**:6501–8
28. Benzeno S, Narla G, Allina J, Cheng GZ, Reeves HL, Banck MS, Odin JA, Diehl JA, Germain D, Friedman SL. Cyclin-dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1. *Cancer Res* 2004;**64**:3885–91
29. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 1994;**369**:669–71
30. Weinstein IB. Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. *Carcinogenesis* 2000;**21**:857–64

(Received October 20, 2016, Accepted January 30, 2017)