

ORIGINAL ARTICLE

## Amarogentin secoiridoid inhibits *in vivo* cancer cell growth in xenograft mice model and induces apoptosis in human gastric cancer cells (SNU-16) through G2/M cell cycle arrest and PI3K/Akt signalling pathway

Jian-guo Zhao<sup>1\*</sup>, Ling Zhang<sup>2\*</sup>, Xiao-jun Xiang<sup>2</sup>, Feng Yu<sup>2</sup>, Wan-li Ye<sup>1</sup>, Dong-ping Wu<sup>1</sup>, Jian-fang Wang<sup>1</sup>, Jian-ping Xiong<sup>2</sup>

<sup>1</sup>Department of Oncology, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, Shaoxing 312000, China;

<sup>2</sup>Department of Oncology, The First Affiliated Hospital of Nanchang University, Jiangxi, Nanchang, 330006, China

\*These authors contributed equally to this work

### Summary

**Purpose:** To investigate the *in vitro* and *in vivo* antitumor effects of amarogentin in SNU-16 human gastric cancer cells as well as in nude mice xenograft model. The effects of this compound on cell apoptosis, cell cycle phase distribution and PI3K/Akt and m-TOR signalling pathways were also studied in detail.

**Methods:** MTT assay was used to study the effect of amarogentin on SNU-16 cell viability while clonogenic assay indicated the effect of the compound on colony formation tendency of these cells. Phase contrast microscopy revealed the effect on cellular morphology while flow cytometry was engaged to study the effects on cell apoptosis and cell cycle arrest. SNU-16 cancer cells were subcutaneously inoculated into nude mice to investigate the *in vivo* antitumor effects of amarogentin.

**Results:** Amarogentin induced potent, dose-dependent as well as time-dependent cytotoxic effects on the growth of SNU-16 human gastric cancer cells. Amarogentin also inhibited the colony forming capability of these tumor cells and its treatment led to morphological alterations in these cells in which

the cells became withered and rounded, detached from one another and adopted irregular shapes while floating freely in the culture medium. In comparison to untreated control cells, the amarogentin treated cells with 10, 50 and 75  $\mu$ M exhibited 32.5, 45.2 and 57.1 % apoptotic cells, respectively. Amarogentin induced potent and dose-dependent G2/M cell cycle arrest in these cells and led to downregulation of m-TOR, p-PI3K, PI3K, p-Akt and Akt and upregulation of cyclin D1 and cyclin E protein expressions. The tumor tissues obtained from the amarogentin-treated mice were much smaller than the tumor tissues derived from the control group.

**Conclusion:** Amarogentin exerts potent *in vitro* and *in vivo* antitumor effects in SNU-16 cell model as well as in nude mice xenograft model. These antitumor effects were found to be mediated through apoptosis induction, G2/M cell cycle arrest and downregulation of PI3K/Akt/m-TOR signalling pathways.

**Key words:** amarogentin, anticancer activity, cell cycle arrest, flow cytometry, gastric cancer

### Introduction

Gastric cancer is a deadly disease, being the second most common cause of cancer-related deaths globally and the fourth most common malignancy worldwide. It remains one of the major health problems worldwide because of its

high mortality-to-incidence ratio and poor prognosis. Risk factors include infection by *Helicobacter pylori*, high alcohol consumption, low intake of fruits and vegetables, excess dietary salt and smoking. Gastric cancer is one of the few cancers

that is directly associated with an infectious organism [1-3]. There is a significant difference in the global distribution of gastric cancer cases with Asian countries including most of the global gastric cancer cases while North America and European countries account for less number of these cases. This indicates that there is a wide-spectrum of factors which are linked with the prevalence, survival and mortality of the disease [4]. Therapeutic strategies rely on the site and volume of the primary cancer tissue. Surgical resection is the preferred therapeutic method with total or partial gastrectomy. Chemotherapy is used in combination with surgery or radiotherapy and this has resulted in much improved survival rates. However, survival rates in case of metastatic gastric cancer or its advanced stages is very poor and median overall survival remains less than 1 year [5]. Chemotherapy involves the use of drugs like 5-fluorouracil plus leucovorin, epirubicin, cisplatin etc. However, these chemotherapeutic drugs are associated with numerous severe side effects because of their non-selective activity against cancer cells, killing both normal as well as tumor cells. Therefore, there is an urgent need to discover, design and develop novel anticancer agents from natural sources which can be used against this deadly disease.

The main objective of the present study was to investigate the *in vitro* and *in vivo* anticancer and apoptotic effects of amarogentin in SNU-16 human gastric cancer cells and to evaluate its mechanism of action by examining its effect on cell cycle and PI3K/Akt signalling pathway.

## Methods

### *Chemicals and other reagents*

Amarogentin (purity  $\geq 95\%$ , obtained from high performance liquid chromatography [HPLC] analysis) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amarogentin was dissolved in dimethylsulfoxide (DMSO) to make a stock solution at a concentration of 100 mM, which was further diluted to the desired concentration with culture medium before each experiment. Control experiments contained DMSO alone. The antibodies against  $\beta$ -actin, AKT, mTOR, PI3K were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### *Cell line and culture conditions*

SNU-16 cells were procured from the Institute of Biochemistry and Cell Biology, Shanghai Institute

for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

### *Cell proliferation assay*

SNU-16 cells were seeded at a density of  $2 \times 10^6$  cells per well in a 96-well culture plate. After 24 hrs, amarogentin was dissolved in DMSO at various concentrations (0, 2.5, 5, 10, 25, 50, and 75  $\mu\text{M}$ ) before treating the cells with it. Then before adding MTT solution into each well, the cells were incubated for 12, 24 and 48 hrs time intervals. The supernatant was discarded and supplemented with 100  $\mu\text{l}$  DMSO. The number of viable cells was proportional to the amount of formazan crystals which were dissolved in ethanol and the optical density (OD) was measured on a microplate reader (Bio-Rad; Hercules, CA, USA) at a wavelength of 570 nm.

### *Clonogenic assay*

SNU-16 cells at the exponential growth phase were harvested and counted with the help of a hemocytometer. Afterwards, the cells were plated at about 500 cells per well, incubated for 12 hrs and then treated with different doses (0, 10, 50 and 75  $\mu\text{M}$ ) of amarogentin. The cells were incubated for another 6-8 days, then washed with PBS twice and stained with 0.5% gentian violet for 20 min and the colonies formed were counted under light microscope.

### *Phase contrast microscopic study of cell morphology*

SNU-16 cells were seeded into 6-well plates at a density of  $2 \times 10^6$  cells/well. The cells were treated without or with different concentrations (0, 10, 25 and 75  $\mu\text{M}$ ) of amarogentin for 48 hrs. The morphological changes were observed and the images were captured under a phase contrast microscope (Olympus, Olympus Optical Co., LTD, Tokyo, Japan) after 48 hrs. The same spot of cells was marked and captured. The images were captured at a magnification of 400x.

### *Annexin V-FITC assay of cell apoptosis*

SNU-16 cells were seeded at a density of  $2 \times 10^6$  cells per ml into 12-well plates and incubated overnight. After treating cells with different doses (0, 10, 50 and 75  $\mu\text{M}$ ) of amarogentin, the cells were washed with PBS and then resuspended in binding buffer containing Annexin V-fluorescein isothiocyanate and propidium iodide for 20 min. Cells grown in media containing an equivalent amount of 0.11 % DMSO without any drug served as control. Fluorescence intensity was measured using flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

### Cell cycle analysis

Briefly, SNU-16 cells were seeded at a density of  $2 \times 10^6$  cells/ml into a 6-well plate and incubated for 48 hrs for complete cell attachment. The cells were then exposed to different doses of amarogentin (0, 10, 50 and 75  $\mu\text{M}$ ) for 48 hrs. Then, the cells were harvested by trypsinization, centrifuged at 15,000 rpm for 10 min, washed with PBS twice and fixed with ice-cold 70% ethanol at  $-20^\circ\text{C}$  for 2 hrs. After the cells were resuspended in 400  $\mu\text{L}$  of PBS, with 50  $\mu\text{L}$  propidium iodide (PI) and 50  $\mu\text{L}$  RNase A, they were finally analyzed on a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Data on 10,000 cells were acquired and processed with Cell Quest software.

### Western blot analysis

Next, we performed Western blot assay to demonstrate the main proteins involved in the biofunctions of these cancer cells. SNU-16 cells were harvested and lysed with RIPA buffer, and the collected protein samples were quantified by using bichinonic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). About 100  $\mu\text{g}$  of cellular protein from each sample were applied to 8-10% SDS-polyacrylamide gels and probed with specific antibodies followed by exposure to horseradish peroxidase-conjugated goat anti-mouse antibodies. Blots were then developed using the West Pico Chemiluminescent substrate (Pierce; Woburn, MA, USA).

### Tumor xenograft mouse model of SNU-16 cells

Six to eight weeks old female BALB/c nude mice (National Rodent Laboratory Animal Resources, Shanghai, China) weighing about 20-25 g were used for *in vivo* studies. All the mice were maintained in a laminar airflow cabinet under pathogen-free conditions and a 12-h light-dark cycle, and fed with a standard diet *ad libitum*. All the animal procedures were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of Shaoxing Hospital of Zhejiang University. The mice were subcutaneously injected with either vehicle or amarogentin (10 mg/kg, 25 mg/kg and 50 mg/kg). Suspensions of  $2 \times 10^6$  SNU-16 cells in 200  $\mu\text{l}$  PBS were subcutaneously inoculated into the right flank of the nude mice. The mice were then divided into four groups (N= 5/group): Vehicle control (Group-1), amarogentin (10 mg/kg/) (Group-2), amarogentin (25 mg/kg/) (Group-3) and amarogentin (50 mg/kg/) (Group-4).

### Statistics

Each experiment was performed in triplicate. The data were expressed as the mean value  $\pm$  standard deviation (SD). The results of the different groups were compared using one-way analysis of variance (ANO-

VA). Differences were considered statistically significant and highly significant at  $p < 0.05$ ,  $p < 0.01$ .

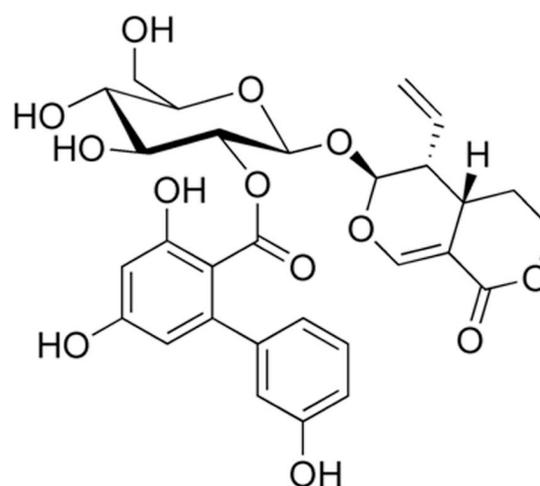
## Results

### *In vitro* antiproliferative activity of amarogentin in SNU-16 cells

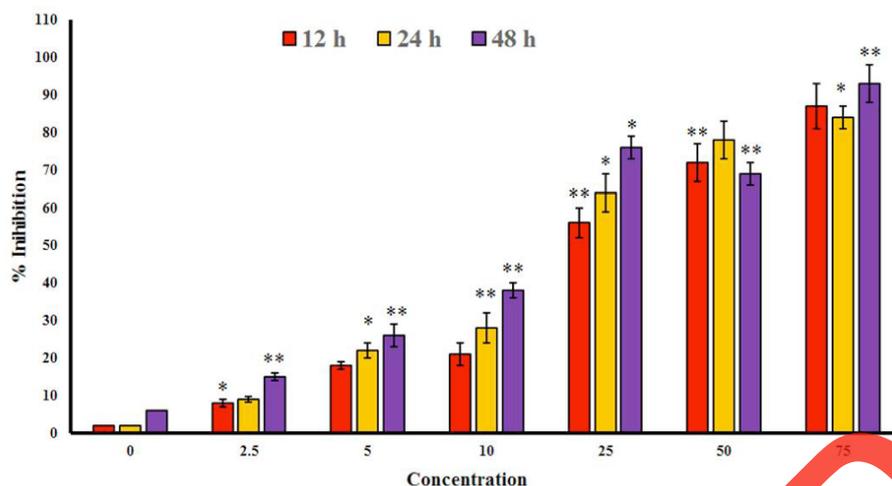
The chemical structure and antitumor effects of amarogentin are shown in Figures 1 and 2, respectively. MTT cell viability assay was used to examine the antitumor effects of amarogentin at various doses as well as at various incubation times. The results showed that amarogentin induced dose-dependent as well as time-dependent cytotoxic effects in SNU-16 cells. The activity of the compound was determined in terms of  $\text{IC}_{50}$  values which is a measure of the effectiveness of a drug. It gives an idea how much quantity of a compound is required to inhibit cell growth by 50%. The  $\text{IC}_{50}$  values at 12, 24 and 48 hrs time intervals for the compound were 23.6, 17.8 and 12.4  $\mu\text{M}$ , respectively.

### Amarogentin inhibited the colony forming tendency of SNU-16 cells

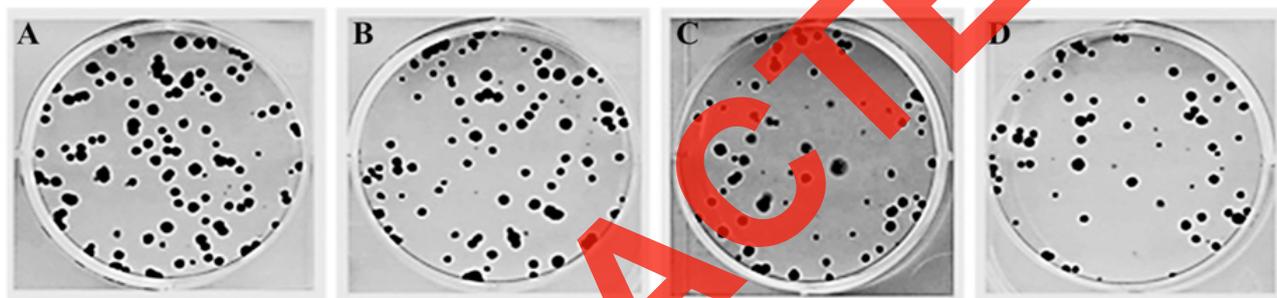
The effect of amarogentin on the colony forming ability in SNU-16 cells is depicted in Figure 3, A and B, respectively. Amarogentin inhibited the clonogenic activity of the SNU-16 cells by decreasing the number of tumor forming cell colonies which also showed dose-dependence. Thus MTT and clonogenic assay indicated that amarogentin has both antiproliferative and anticlonogenic properties.



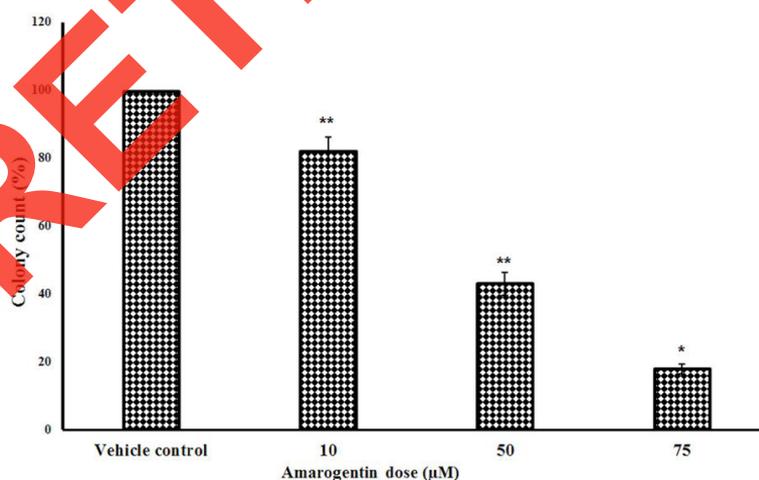
**Figure 1.** Chemical structure of amarogentin.



**Figure 2.** Dose and time-dependent cytotoxic effects of amarogentin in human gastric cancer cells (SNU-16). Data are means  $\pm$  SD of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , vs 0  $\mu\text{M}$  (control).



**Figure 3.** The inhibitory effect of amarogentin on the colony formation tendency of SNU-16 human gastric cancer cells. Representative images of colony-forming assay using light microscope. **A, B, C** and **D** represent the effect of 0, 10, 50 and 75  $\mu\text{M}$  of amarogentin on the colony formation in SNU-16 cells.



**Figure 4.** Graphical representation of the inhibitory effect of amarogentin on the colony-forming tendency in SNU-16 cancer cells. Data are means  $\pm$  SD of three independent experiments. \* $p < 0.05$  \*\* $p < 0.01$  vs control group.

*Amarogentin induced significant morphological changes and led to decreased cell viability in SNU-16 cells*

In this experiment, the amarogentin-induced morphological changes in SNU-16 cells were examined under a phase contrast microscope. As can

be seen in Figure 4, A-D, as compared with the control untreated cells, the amarogentin-treated cells at 10, 50 and 75  $\mu\text{M}$  dose exhibited visible signs of morphological changes including rounding and withering of cells with unorganized cell layers. The cells became disconnected from one another and freely floated in the medium.

#### *Amarogentin induced both early and late apoptosis in SNU-16 cells*

The fact that amarogentin induced early and late apoptosis in SNU-16 cells was evaluated by annexin V-FITC assay using flow cytometry. The results of this assay are depicted in Figure 5, A-D, and indicated that in comparison to untreated control cells (Figure 5 A), which showed that only 12.4% of cells underwent apoptosis, the amarogentin-treated cells displayed apoptosis in 32.5, 45.2 and 57.1% of the cells with 10, 50 and 75  $\mu\text{M}$  of amarogentin, respectively. The different quadrants R1, R2, R3 and R4 represent necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population, respectively.

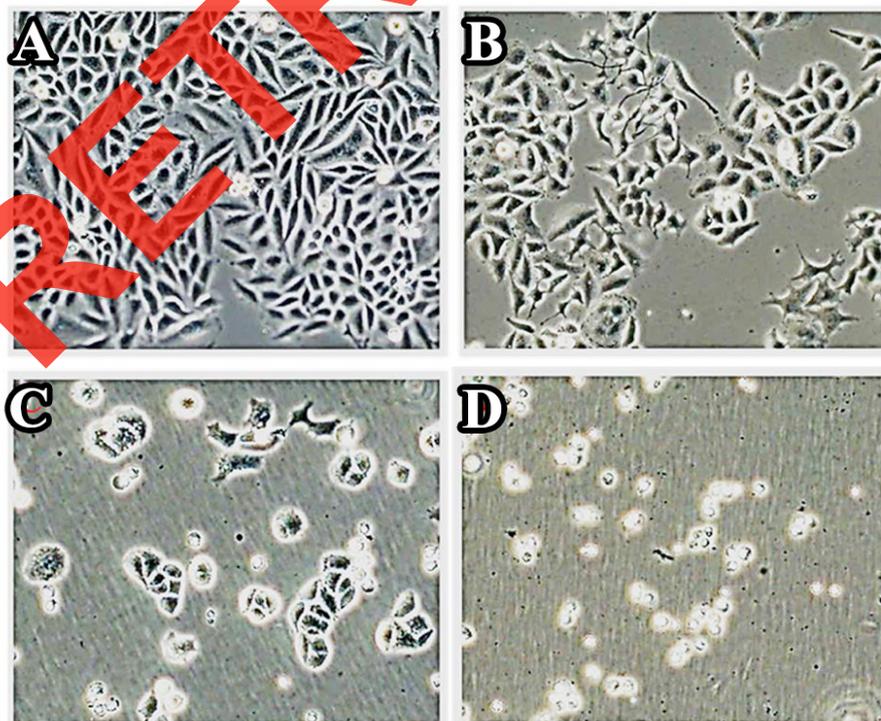
#### *Amarogentin induced G2/M cell cycle arrest in SNU-16 cells*

In this experiment, the effect of amarogentin

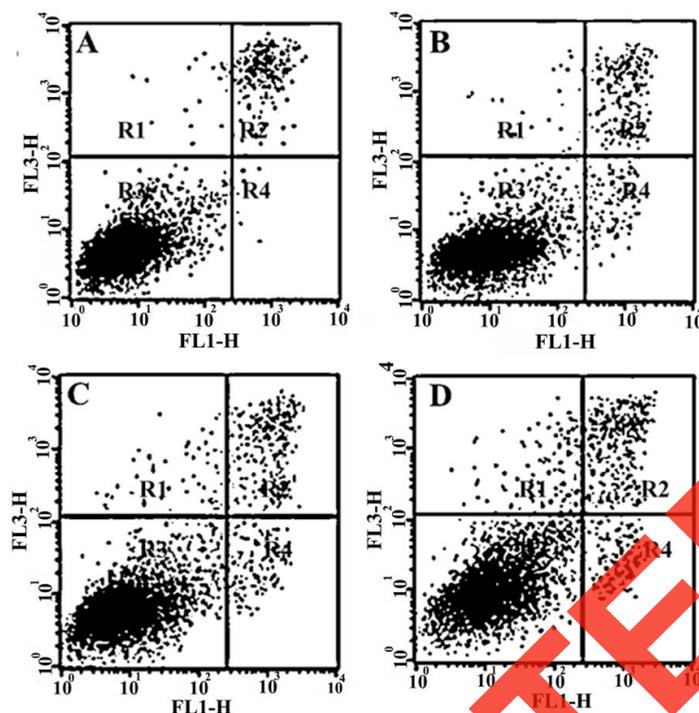
on cell cycle phase distribution was demonstrated using PI as probe and flow cytometer for analysis. The results are shown in Figure 6, A-D and reveal that as compared to the control untreated cells which showed only 12.1% of cells in G2/M phase, the percentage of G2/M cells in 10, 50 and 75  $\mu\text{M}$ -amarogentin treated cells increased to 18.7, 45.3 and 75.8% respectively. This was accompanied by a corresponding decrease in the percentage of cells in the S-phase of the cell cycle. Figure 6-B shows the graphical representation of the amarogentin-induced G2/M cell cycle arrest in SNU-16 cells.

#### *Effect of amarogentin on the cyclin D1, cyclin E, PI3K/Akt and m-TOR signalling pathway in SNU-16 cells*

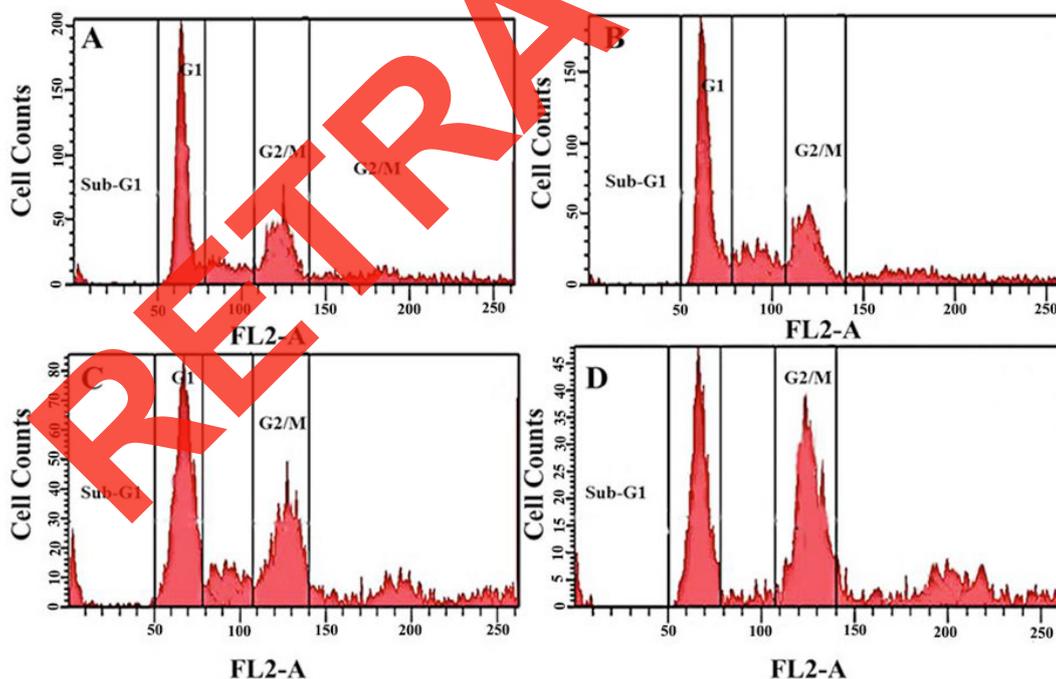
Western blot assay was used to study the effect of amarogentin on various crucial cell signalling pathways including cyclin D1, cyclin E, PI3K/Akt and m-TOR signalling pathways. The results which are shown in Figure 7 indicate that expressions of m-TOR, p-PI3K, PI3K, p-Akt and Akt proteins were significantly lower than that in the untreated control group, indicating a downregulation of these proteins on amarogentin treatment. However, the expressions of cyclin D1 and cyclin E were significantly much higher than that in the untreated control group, indicating an upregulation



**Figure 5.** Effect of amarogentin on the cell viability of SNU-16 human gastric cancer cells. Phase contrast microscopic images of the amarogentin-treated cells at different doses: 0 (A), 10 (B), 50 (C) and 75 (D)  $\mu\text{M}$  for 48 hrs.



**Figure 6.** Annexin-V-FITC assay of apoptosis quantification in SNU-16 human gastric cancer cells. The cells were treated without (A) and with 10 (B), 50 (C) and 75 (D)  $\mu$ M of amarogentin for 48 hrs and then analyzed by flow cytometer. The quadrants R1, R2, R3 and R4 represent necrotic cells, late apoptotic cells, live cells and early apoptotic cells respectively. As the dose of amarogentin increased, the percentage of early and late apoptotic cells also increased.



**Figure 7.** Amarogentin induced potent G2/M cell cycle arrest in SNU-16 human gastric cancer cells. The cells were treated without (A) and with 10 (B), 50 (C) and 75 (D)  $\mu$ M of amarogentin for 48 hrs and then analyzed by FACSCalibur flow cytometer. As compared to the control untreated cells which showed only 12.1% of cells in G2/M phase, the percentage of G2/M cells in 10, 50 and 75  $\mu$ M amarogentin-treated cells increased to 18.7, 45.3 and 75.8%, respectively.

tion of these proteins. Downregulation of m-TOR, p-PI3K, PI3K, p-Akt and Akt and upregulation of cyclin D1 and cyclin E were found to be related to amarogentin-dose.

*Amarogentin suppressed tumor growth in nude mice xenograft model*

Finally, we evaluated the *in vivo* antitumor effects of amarogentin in a nude mice xenograft

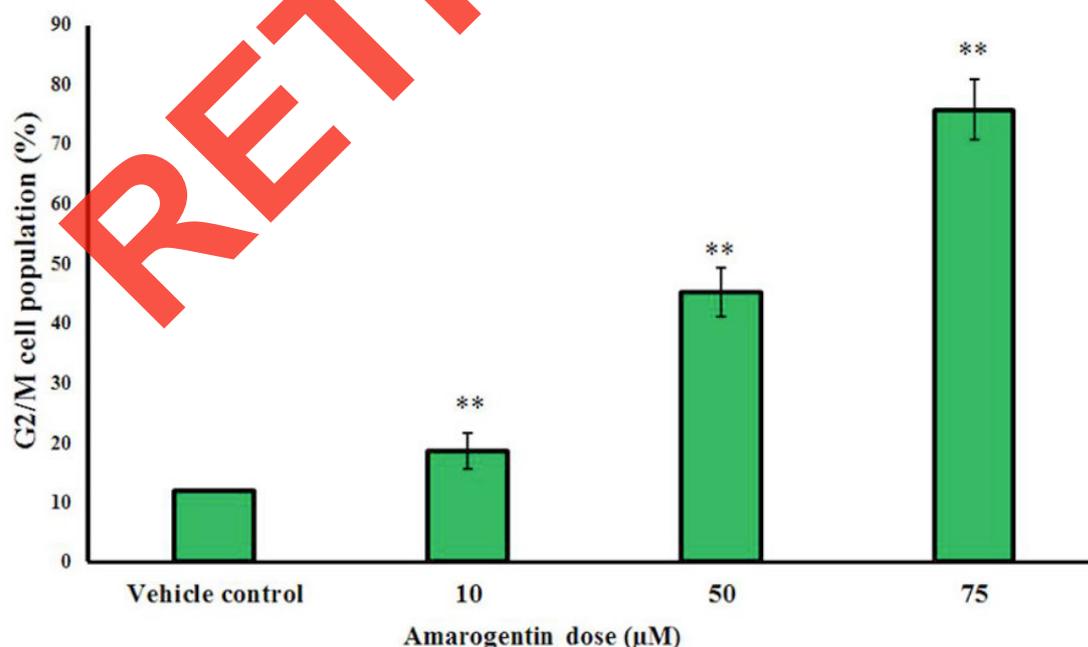
model. The tumors were initiated in these mice by transplanting SNU-16 gastric cancer cells subcutaneously into the right flank of each mice. The results showed that the tumor tissues developed in the amarogentin-treated mice were much smaller than the tumor tissues in the control group. Among amarogentin-treated mice, the group with 50 mg/kg dose exhibited the smallest tumor size followed by 25 mg/kg dose and 10 mg/kg dose. There was a statistically significant difference between the amarogentin-treated and the control group (Figure 8). Five mice were used in each group. The differences in the tumor sizes of the four groups was significant ( $p < 0.05$ ).

## Discussion

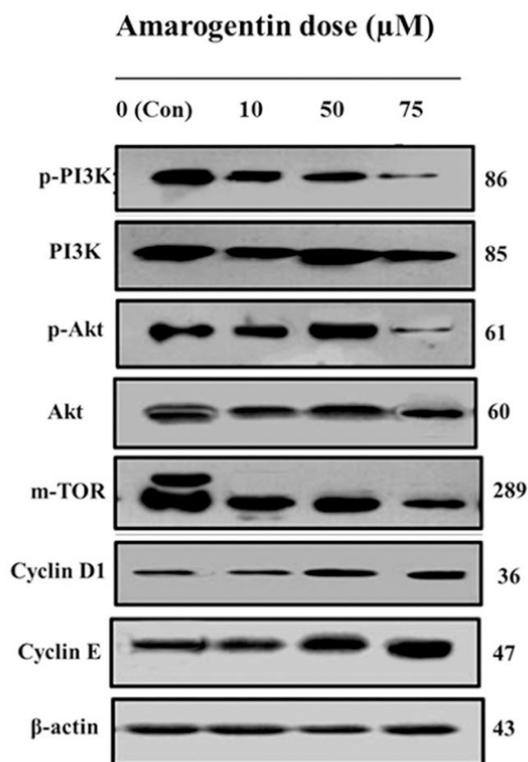
Programmed cell death or apoptosis is a highly specialized biochemical mechanism with the help of which multicellular organisms get rid of abnormal or damaged cells. The apoptotic cells can be easily distinguished from normal cells from their characteristic morphological features including membrane blebbing, cell shrinkage, as well as from their molecular expressions. In case of cancer cells, the induction of cell apoptosis plays a key role as it helps in the prevention, management, as well as treatment of different tumors. Apoptotic dysfunction is associated with numerous diseases, in particular cancer. There

exists a wide-spectrum of natural products which have the tendency to induce apoptosis in different cancer cells [6-8]. Sixty percent of drugs approved by FDA up to date were isolated from natural sources, particularly plants. Among 121 drugs prescribed for cancer treatment, 90 are derived from herbal medicine [9]. Our results indicate that amarogentin also induced early and late apoptosis in SNU-16 human gastric cancer cells in a dose-dependent manner. Annexin V-FITC assay using flow cytometry revealed that in comparison to untreated control cells, which showed only 12.4% cells had undergone apoptosis, the amarogentin-treated cells with 10, 50 and 75  $\mu\text{M}$  dose showed apoptotic rates of 32.5, 45.2 and 57.1 %, respectively. Phase-contrast microscopy revealed that amarogentin induced morphological changes in SNU-16 cells, characteristic of apoptosis.

The PI3K/Akt signalling pathway plays a crucial role in cell apoptosis, cell proliferation, cell survival and cell differentiation. It has been reported that phosphorylated Akt results in facilitation of the cell viability and cell proliferation. There is an intimate relationship between the excessive expression and activation of Akt kinase and the malignant biological behavior of various cancers. The PI3K/Akt signaling pathway has been reported to play key roles in the occurrence of various malignant tumors including non-small cell lung cancer and endometrial cancer [10-13].

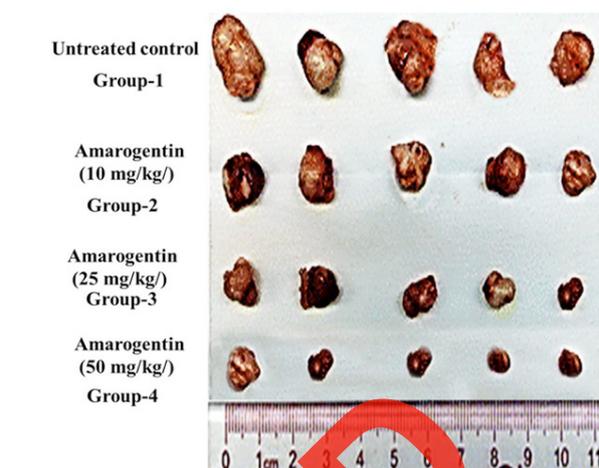


**Figure 8.** Graphical representation of the amarogentin-induced G2/M cell cycle arrest in SNU-16 human gastric cancer cells. As compared to the control, amarogentin-treated cells showed a significant proportion of cells in the G2/M phase and this percentage increased with increasing dose of the compound. Data are means  $\pm$  SD of three independent experiments. \*\*  $p < 0.05$  vs control group.



**Figure 9.** Effect of amarogentin on the m-TOR, cyclin D1, cyclin E, PI3K/Akt pathways in human gastric (SNU-16) cancer cells. The expressions of these proteins were evaluated by Western blot analysis. On amarogentin treatment, it was observed that there was upregulation of cyclin D1 and cyclin E, while there was downregulation of m-TOR, PI3K/Akt proteins.  $\beta$ -actin served as control.

The results of the present study revealed that amarogentin leads to downregulation of the protein expressions of m-TOR, p-PI3K, PI3K, p-Akt and Akt in comparison to the untreated cells. However, the expressions of cyclin D1 and cyclin E were significantly much higher than that in the untreated control group, indicating an upregulation of these proteins. Amarogentin also led to G2/M phase cell cycle arrest in these cells. The tumor tissues developed in the amarogentin-treated



**Figure 10.** *In vivo* antitumor effect of amarogentin on nude mice bearing SNU-18 gastric cancer cells. Dissected tumor tissues from the untreated and treated mice. As can be seen from the figure, the tumor tissues obtained from the amarogentin-treated mice were much smaller than the tumor tissues derived from the control group. There was a statistically significant difference between the amarogentin-treated and the control group. Five mice were used in each group (N=5).

mice were much smaller compared with the control group. Among amarogentin-treated mice, the group treated with 50 mg/kg dose exhibited the smallest tumor size, followed by 25 mg/kg and 10 mg/kg dose.

In conclusion, amarogentin induced significant, *in vitro* as well as *in vivo* antitumor effects in SNU-16 gastric cancer cells and nude mice model and these antitumor effects were mediated via apoptosis induction, G2/M cell cycle arrest and downregulation of m-TOR, p-PI3K, PI3K, p-Akt and Akt protein expressions.

### Conflict of interests

The authors declare no conflict of interests.

### References

1. Ferro A, Peleteiro B, Malvezzi M et al. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. *Eur J Cancer* 2014; 50:1330-1344.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69-90.
3. Shen L, Shan YS, Hu HM et al. Management of gastric cancer in Asia: resource-stratified Guidelines. *Lancet Oncol* 2013;14:e535-e547.
4. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893-2917.

5. Cervantes A, Roda D, Tarazona N, Roselló S, Pérez-Fidalgo JA. Current questions for the treatment of advanced gastric cancer. *Cancer Treat Rev* 2013;39:60-67.
6. Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-257.
7. Daniel PT, Koert U, Schuppan J. Apoptolidin: induction of apoptosis by a natural product. *Angew Chem Int Ed Engl* 2006;45:872-893.
8. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251-306.
9. Naveen Kumar DR, Cijo George V, Suresh PK, Ashok Kumar R. Cytotoxicity, apoptosis induction and anti-metastatic potential of *Oroxylum indicum* in human breast cancer cells. *Asian Pac J Cancer Prev* 2012;13:2729-2734.
10. Osaki M, Oshimura M. PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis* 2004;9:667-676.
11. Franke TF. PI3K/Akt: getting it right matters. *Oncogene* 2008;27:6473-6488.
12. Geering B, Cutillas PR. Regulation of class IA PI3Ks: is there a role for monomeric PI3K subunits? *Biochem Soc Trans* 2007;35:199-203.
13. Tang JM, He QY. Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis. *Lung Cancer* 2006;51:181-191.

**RETRACTED**