

ORIGINAL ARTICLE

Angustifoline inhibits human colon cancer cell growth by inducing autophagy along with mitochondrial-mediated apoptosis, suppression of cell invasion and migration and stimulating G2/M cell cycle arrest

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Summary

Purpose: The prime objective of the present study was to investigate the anticancer properties of angustifoline against COLO-205 human colon cancer cells. Its effects on cell autophagy, apoptosis, cell invasion and cell migration, and cell cycle arrest were also evaluated in the current study.

Methods: WST-1 assay was used to study cytotoxic effects of the compound on the cell viability. Effects on apoptosis and cell cycle arrest were evaluated by flow cytometry. In vitro wound healing assay and matrigel assay were carried out to study the effects of angustifoline on cell migration and cell invasion respectively. To confirm autophagy, we evaluated the expression of several autophagy-associated proteins using Western blot assay along with transmission electron microscopy (TEM).

Results: The findings indicated that angustifoline induced dose- and time-dependent cytotoxicity in COLO-205 human colon cancer cells along with inhibiting cancer cell colony

formation. Angustifoline-treated cells exhibited cell shrinkage along with distortion of the normal cell morphology. Angustifoline-treated cells were also arrested in the G2/M phase of the cell cycle, showing strong dose-dependence. The compound also led to inhibition of cell migration and cell invasion. The results showed that treatment of these cells led to generation of autophagic cell vesicles. Furthermore, it was observed that the expression of Beclin-1 and LC3-II proteins was significantly upregulated in the angustifoline-administered COLO-205 cells.

Conclusions: In brief, the present study hints towards the potent anticancer potential of the natural product angustifoline against COLO-205 human colon cancer cells with in depth mechanistic studies.

Key words: angustifoline, autophagy, cell migration, colon cancer, flow cytometry

Introduction

Researchers directed at screening of molecules for anticancer activities have indicated that natural products could prove promising in the development of safe anticancer agents [1]. Among natural products, plant-derived secondary metabolites have been shown to exhibit potent anticancer activities [2]. Alkaloids of plant origin have been reported to

exhibit a wide array of bioactivities which include but are not limited to antimicrobial and anticancer [3]. In this study the anticancer activity of one of the important plant-derived natural products, angustifoline, was determined against COLO-205 cancer cell line. Angustifoline is of immense pharmacological potential and a wide array of activi-

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ties has been attributed to this natural product [4]. In this study we report for the first time the anticancer activity of angustifoline against colon cancer cells. Colon cancer is one of the very lethal types of cancer and is responsible for alarming morbidity and mortality across the world [5]. Although advancements have been made in the field of cancer research, yet the overall survival of colon cancer still remains unsatisfactory. The main current treatment for colon cancer involves surgery followed by chemotherapy. Radiation therapy and targeted agents are also used. However, chemotherapy is associated with a number of side effects that adversely affect the patient health and quality of life [6]. Hence, there is urgent need to isolate and screen new molecules for their anticancer activity and take the best ones to the clinic. Microbes and plants have served as great drug repositories in the past and will continue to offer new drugs in the future [7].

In this study we observed that angustifoline exhibited significant antiproliferative effects on the COLO-205 cancer cells and its anticancer effects were mainly due to the induction of apoptosis in the COLO-205 cells. Furthermore, it was observed that angustifoline could also induce autophagy in the COLO-205 cells. Analysis of the cell cycle indicated that angustifoline caused the arrest of COLO-205 cancer cells in the G2/M phase of the cell cycle. Angustifoline also inhibited the migration and invasion of the COLO-205 cells, indicative of the potential of angustifoline as anticancer agent.

Methods

Cell lines and culture conditions

Colon cancer COLO-205 cell line was obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China, and was cultured in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), at 37 °C in a humidified incubator.

WST-1 assay

The cancer cell's viability was assessed by WST-1 colorimetric assay. Briefly, the COLO-205 colon cancer cells were seeded in 96-well plates at the density of 2×10^5 cells/well and treated with different concentrations of angustifoline. The cells were then incubated with WST-1 at 37°C for 4 hrs. The absorbance at 450 nm was then taken by a microplate reader to determine the viability of colon cancer cells.

Transmission electron microscopy (TEM)

For TEM, the untreated and angustifoline-treated COLO-205 cells were fixed in glutaraldehyde (2.5%) in

phosphate buffer for 35 min and post-fixed in 1% osmium tetroxide in the same buffer for 35 min. This was followed by dehydration of the cells in molecular graded ethanol and subsequent washing with propylene oxide and then embedded in Epon. This was followed by sectioning on a Reichert-Jung ultramicrotome at 90-nm thickness. The sections were then stained with 5% uranyl acetate and 5% lead citrate and observed under a Hitachi H7100 transmission electron microscope at 75 kV.

Detection of apoptosis

The nuclear morphology of the COLO-205 cancer cells was assessed by fluorescence microscopy after subjecting the cells to DAPI. Ten fields with 100 cells/field were selected randomly for estimation of the cells with condensed nuclei. Annexin V/propidium iodide (PI) double staining was used for the determination of apoptotic colon cancer cells as described previously [8].

Cell cycle analysis

To perform the cell cycle analysis, the angustifoline-treated colon cancer cells were cultured in medium containing FBS (10%) for 24 hrs. For the preparation of the cell samples for flow-cytometry, the cells were subjected to treatment with trypsinization followed by fixation with 70% ethanol. The DNA of the cells was stained by incubation with PBS containing PI (100 µg/ml) and RNase (40 U/ml) for 35 min at 37°C. The cell samples were finally analyzed with the help of fluorescence-activated cell sorter (FACS).

Cell migration assay

The migration of COLO-205 cells was determined with wound healing assay. The cells were grown till 80% confluence and a scratch was made with a scratching device. The cells were then incubated for 48 hrs again, and the wound healing capacity was evaluated by comparing the widths of the wounds.

Matrigel invasion assay

Invasion was evaluated with the help of Matrigel®-coated invasion chambers. The angustifoline-treated and untreated COLO-205 cells that reached the lower surface of the membrane were subjected to staining with crystal violet (CV), and images of CV-stained cells were taken. The crystal violet complexes formed were dissolved in 10% acetic acid and the cell invasion was determined by measuring the absorbances of the resultant solutions at 600 nm in a spectrophotometer.

Western blotting analysis

The COLO-205 colon cancer cells were collected and treated with lysis buffer. The extracts were boiled for 10 min in the presence of loading buffer, followed by separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and subjected to blocking with skim milk (5%). Membrane incubation with primary antibodies was performed overnight at 4°C and subsequently in-

cubated with horseradish peroxidase-linked biotinylated secondary antibodies at 1:1,000 dilution for 2 hrs. The membranes were then subjected to washing with PBS and the immune-reactive bands were visualized using ECL-PLUS kit. Development of the immune complexes was carried out using an ECL detection kit according to the manufacturer's protocol. The bands were analyzed with GelDoc 2000 imaging system.

Statistics

Data are shown as mean \pm SD and were statistically analyzed using Student's Newman t-test. A p value <0.05 was considered to be statistically significant.

Results

Angustifoline inhibits the proliferation of colon cancer cells

The antiproliferative effect of angustifoline against COLO-205 colon cancer cell line (Figure 1) was assessed by WST-1 assay. The results of the WST-1 assay revealed that angustifoline exerted concentration-dependent antiproliferative activity on this colon cancer cell line (Figure 2). The IC_{50} of angustifoline against COLO-205 cells was found to be 10 μ M.

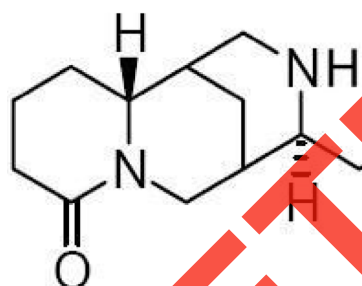


Figure 1. Molecular structure of angustifoline.

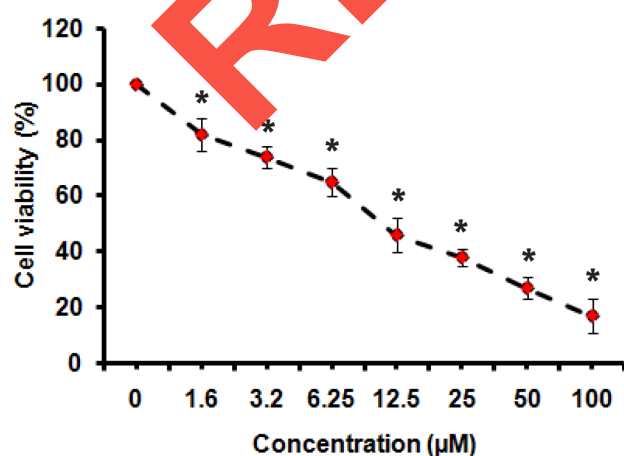


Figure 2. The effect of angustifoline on the cell viability of COLO-205 human colon cancer cells evaluated by MTT cell viability assay. The experiments were done in triplicate. The values are mean \pm SD (n=3, *p<0.05).

Angustifoline induces apoptosis in colon cancer cells

Previous studies have indicated that several plant-derived compounds induce apoptosis in cancer cells and therefore we investigated whether angustifoline could trigger apoptotic cell death in COLO-205 cancer cells. Therefore, COLO-205 cancer cells were treated with varying concentrations

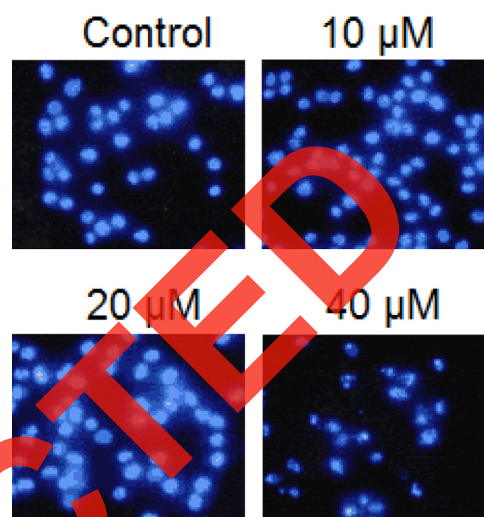


Figure 3. The effect of angustifoline on apoptosis of COLO-205 human colon cancer cells, evaluated by fluorescence microscopy using DAPI staining. The experiments were performed in triplicate. The Figure shows that apoptosis of COLO-205 cells increases with increase in the concentration of angustifoline.

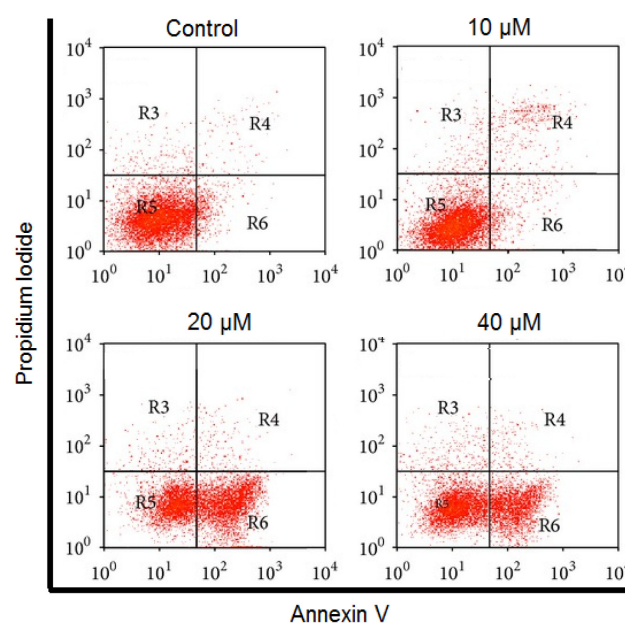


Figure 4. Percentage of apoptotic COLO-205 human colon cancer cells as shown by annexin V/PI staining using flow cytometry. The experiments were performed in triplicate. The Figure depicts that the percentage of apoptotic cells increases with the increase in the concentration of angustifoline.

of angustifoline and subjected to DAPI staining. Interestingly, it was observed that angustifoline induced apoptosis in these cells in dose-dependent manner (Figure 3). Furthermore, the results of annexin V/PI staining revealed that the apoptotic cell populations increased from 2.6% in control to 38.8% at 20 μ M (Figure 4). In addition, angustifoline upregulated the expression of Bax which was

also associated with downregulation of Bcl-2, further confirming the apoptotic cell death of COLO-205 cancer cells (Figure 5).

Angustifoline induced autophagy in COLO-205 colon cancer cells

To seek further information about the underlying mechanism of the angustifoline-induced anti-cancer effects we carried out TEM of the untreated and angustifoline-treated COLO-205 cells. The results showed that treatment of these cells led to generation of autophagic vesicles in these cells (Figure 6). To further validate the autophagic potential of angustifoline we determined the expression of autophagy-associated proteins in the untreated and angustifoline-treated cells and observed that the expression of Beclin-1 and LC3-II proteins was significantly upregulated in the angustifoline-administered COLO-205 cells. Moreover, the expression of p62 was found to be significantly decreased in the angustifoline-treated COLO-205 cells. However, no visible alterations were observed on the expression of LC3-I and Vps34 (Figure 7).

Angustifoline induces G2/M cell cycle arrest of colon cancer cells

To assess whether angustifoline causes arrest of cancer cells, the COLO-205 cells were subjected

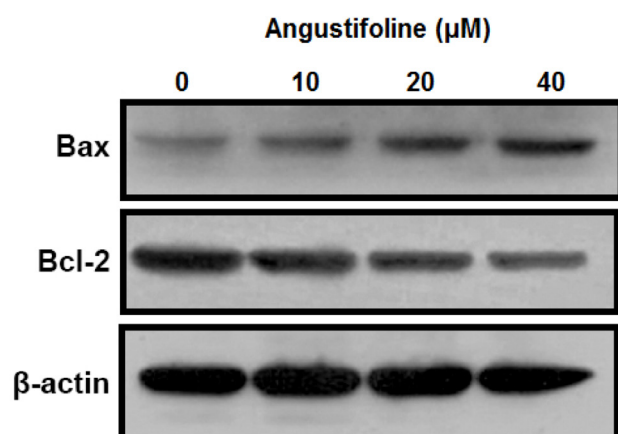


Figure 5. The effect of angustifoline on apoptosis-related proteins (Bcl-2 and Bax) in COLO-205 human colon cancer cells as depicted by western blotting. The experiments were performed in triplicate. The Figure shows that the expression of Bax increases and of Bcl-2 decreases with increase in the concentration of angustifoline.

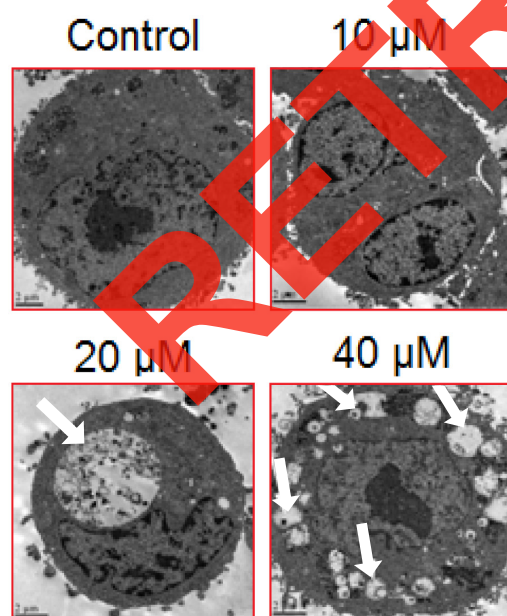


Figure 6. Induction of autophagy in COLO-205 human colon cancer cells as indicated by Transmission electron microscopy (TEM) at different concentrations of angustifoline drug. The experiments were performed in triplicate. Autophagosomes, which are shown by arrows indicate the onset of autophagy. The Figure shows that the number of autophagosomes increases with increase in the concentration of angustifoline.

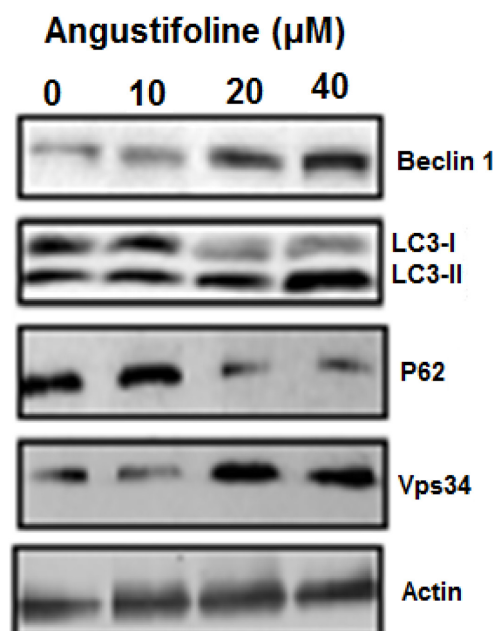


Figure 7. The effect of increasing concentrations of angustifoline on the protein expression of autophagy-related proteins as measured by western blotting method. The experiments were performed in triplicate. The Figure depicts that angustifoline increased the expression of Beclin-1, LC3-II and Vps34 while it decreases the expression of p62 and LC3-I in a concentration-dependent manner.

to treatment with angustifoline followed by flow cytometry. The results revealed that angustifoline caused remarkable increase in the percentage of the COLO-205 cells in the G2 phase of the cell cycle indicating that angustifoline induces G2/M cell cycle arrest of the colon cancer cells (Figure 8).

Angustifoline suppresses the migration and invasion of colon cancer cells

The effect of angustifoline was also assessed on the cell migration and invasion ability of the colon cancer cells at IC_{50} of angustifoline ($10 \mu M$). The results revealed that angustifoline could inhibit the migration of the COLO-205 colon cancer cells (Figure 9). Similar effects were also observed on the cell invasion of the COLO-205 colon cancer cells (Figure 10).

Discussion

Colon cancer causes alarming morbidity and mortality across the globe and is one of the prime causes of cancer-related mortality in humans [9]. Despite the recent advancements in the field of cancer research, overall survival rate of colon cancer is far from satisfactory. The currently used chemotherapeutic agents have a number of adverse effects that compromise the overall health of the patients. Hence, there is need to look for novel, potent and safer molecules for the management

of this malignancy in particular and other types of cancer in general. The molecules isolated from plants are mostly considered safe and a number of currently used anticancer drugs originate from plants [10]. Plants produce a vast diversity of secondary metabolites to defend themselves against pathogens and other stresses. Human beings have utilized these plant secondary metabolites for their own benefit [11]. Among the plant-derived products, alkaloids constitute an important group and a number of bioactivities have been attributed to this group of metabolites [12]. In this study the anticancer activity of a plant-derived alkaloid, angustifoline, was determined against the COLO-205 cancer cells and it was observed that angustifoline inhibits the growth of the COLO-205 cancer cells dose-dependently. This is also supported by previous investigations wherein a number of plant-derived alkaloids have been reported to exert anticancer effects on colon cancer cells [13]. The anticancer

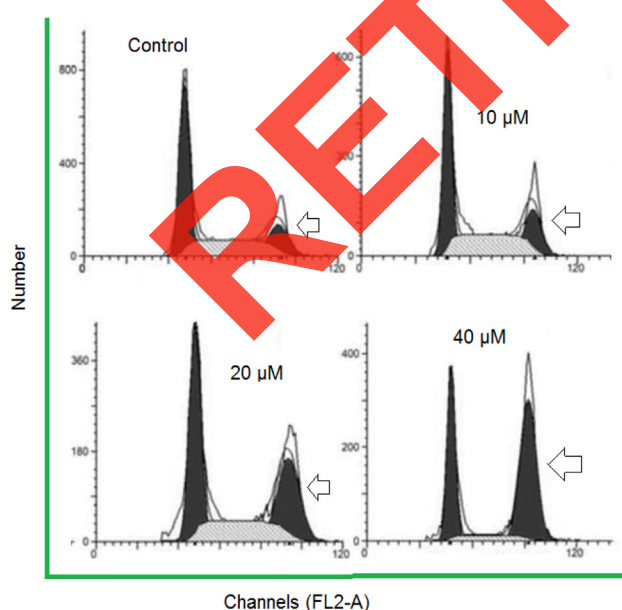


Figure 8. The effect of angustifoline on the migration of COLO-205 human colon cancer cells. The experiments were repeated thrice. The Figure depicts that angustifoline causes arrest of the COLO-205 cells at G2/M checkpoint in a concentration-dependent manner. Arrows indicate the G2/M phase cells.

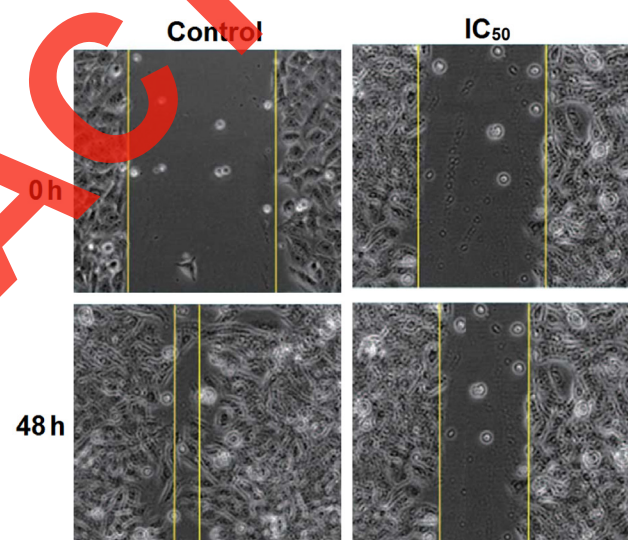


Figure 9. The effect of angustifoline on cell invasion of COLO-205 human colon cancer cells. The experiments were done in triplicate. The Figure shows that angustifoline inhibits the migration of the COLO-205 cells at IC_{50} .

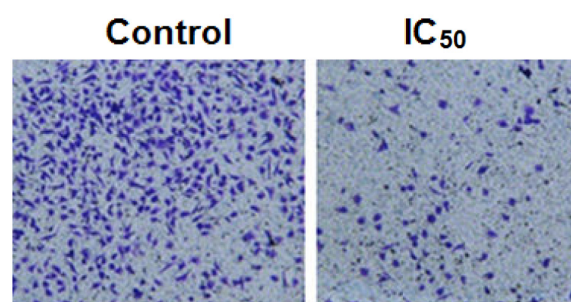


Figure 10. The effect of angustifoline on cell invasion of COLO-205 cells. The experiments were done in triplicate. The Figure reveals that angustifoline inhibits the invasion of COLO-205 at IC_{50} .

agents employ a number of mechanisms to curb the growth of cancer cells which autophagy, apoptosis and cell cycle arrest [14]. The results of this study showed that angustifoline exerted autophagic cell death which was also associated with alteration in the expression of autophagy-related proteins. Secondly, angustifoline also triggered apoptotic cell death of the COLO-205 cancer cells which was accompanied with alteration in the expression of Bax and Bcl-2. Most importantly, angustifoline also triggered G2/M cell cycle arrest of the COLO-205 cells. These results suggest that angustifoline exerts its anticancer effects via different mechanisms and may therefore prove a potent candidate for the treatment of colon cancer. Cell migration and invasion are two important phenomena that determine the metastasis of cancer cells [15] and in this study

we observed that angustifoline could also inhibit the migration and invasion of cancer cells.

Conclusion

Taken together, it is concluded that angustifoline exerts anticancer effects on the colon cancer cells by triggering autophagy, apoptosis and cell cycle arrest. Furthermore, angustifoline inhibited the migration and invasion of colon cancer cells. Hence, angustifoline may prove to be an important lead molecule for the treatment of colon cancer and merits further research.

Conflict of interests

The authors declare no conflict of interests.

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