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ORIGINAL ARTICLE

Bakuchiol inhibits cell proliferation and induces apoptosis and cell cycle arrest in SGC-7901 human gastric cancer cells

Jing Lin[1], Hui-Jing Yao[1], Rui-Yun Li[2]

[1]Department of Gastroenterology and [2]Department of Emergency, Taian City Central Hospital, Shandong 271000, China

Summary

Purpose: *The main purpose of the present study was to evaluate the antiproliferative activity of bakuchiol in human gastric tumor cell line (SGC-7901) along with an effort to demonstrate its mode of action.*

Methods: *he effect of the compound on cell viability was evaluated by MTT assay. Fluorescence and phase contrast microscopic techniques were used to study the effect of the compound on cellular morphology and apoptosis. Flow cytometry was used to assess the effect on cell cycle phase distribution.*

Results: *The results revealed that bakuchiol exerted potent, dose-dependent as well as time-dependent growth inhibitory effects in SGC-7901 cell proliferation with IC_{50} values of 58.4, 42.3 and 32.5 μM at 12, 24 and 48 hrs time intervals, respectively. On treatment with 10, 50 and 100 μM dose of bakuchiol for 48 hrs, phase contrast microscope revealed that the cells got detached from one another making clusters of small number of cells floating in the medium. After the cells were treated with 10, 50 and 100 μM of bakuchiol, cells began to emit orange red fluorescence more heavily at the centre of cells indicating apoptosis. Bakuchiol also induced sub-G1 cell cycle arrest in a dose-dependent manner.*

Conclusion: *The current findings reveal that bakuchiol is a potent cytotoxic agent against gastric cancer cells and its cytotoxicity is mediated through induction of apoptosis and sub-G1 cell cycle arrest.*

Key words: *apoptosis, bakuchiol, cytotoxicity, flow cytometry, gastric cancer*

Introduction

Gastric cancer, which is one of the most common cancers in China, Japan and various East Asian countries, represents the principal cause of cancer-related deaths. Despite the advanced techniques in diagnosis and improved treatment regimens, this cancer remains a serious health threat throughout the world [1,2]. Gastric cancer treatment involves chemotherapy with cisplatin alone or in combination with other chemotherapeutic agents. Combination chemotherapy with cisplatin as first- or second-line treatment for advanced and persistent gastric tumor have yielded decent responses and this treatment modality is well accepted. One of the important criteria for potential anticancer drugs is the ability to selectively kill tumor, without harming normal cells. But most of the anticancer chemotherapeutic drugs kill normal cells in addition to cancer cells [3] resulting in serious, unwanted side-effects. So, there is an urgent need to design and develop new anticancer drugs with minimal side-effects and maximal efficacy. Herbal medicines have been reported to be a potential substitute for cancer therapy because of their low toxicity and cheaper prices.

Apoptosis is a programmed cell suicide characterized by chromatin condensation, DNA fragmentation, cell shrinkage, membrane blebbing and apoptotic body formation. The process of apoptosis plays key role in the development of

Correspondence to: Hui-Jing Yao, MD. Department of Gastroenterology, Taian City Central Hospital, 29 Longtan Road, Taian, Shandong-271000, P. R. China, Tel & Fax: +86 538 6298273, E-mail: yaohuijingdt@hotmail.com
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most of the cancers. Most of the tissues that develop cancer display decreased apoptosis. It has been reported that tumors subjected to radiation and cytotoxic agents showed increased rates of apoptosis, implying that enhanced rate of apoptosis can be used in cancer therapy [4,5]. Bakuchiol is a meroterpene in the class of terpenophenols and can be isolated from various plants including Psoralea corylifolia and Otholobium pubescens.

The objective of the current study was to investigate the anticancer and apoptotic effects of bakuchiol in SGC-7901 human gastric cancer cells along and to evaluate its role in inducing cell cycle arrest in these cells.

Methods

Chemicals and other reagents

Bakuchiol and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO, USA). Bakuchiol was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to get a 100 mM stock solution, which was diluted in the medium to yield the anticipated concentration. An equivalent volume of DMSO in complete culture medium was used as the vehicle control. To exclude the cytotoxicity of DMSO, the ultimate concentration of DMSO for all experiments was kept at less than 0.2%. Minimum Essential Medium (MEM) and RPMI, fetal bovine serum (FBS), penicillin, streptomycin, trypsin, phosphate-buffered saline (PBS) with calcium chloride and magnesium chloride were obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Propidium iodide (PI), acridine orange (AO), and Hoechst 33258 were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China).

Cell line and culture conditions

The SGC-7901 human gastric cancer cell line was purchased from the Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). The cells were cultured in MEM and RPMI supplemented with 10% (v/v) FBS under humidified atmosphere of 5% CO_2 at 37 °C. The medium was replaced every 3 days. Cells were subcultured every 4 days.

MTT cell viability assay

The SGC-7901 human gastric cancer cells were seeded on a 96-well plate at 2×10^5 cells per well. After 24 hrs, the cells were treated with bakuchiol at several doses (0, 10, 30, 50, and 100 μM). After incubation times of 12, 24 and 48 hrs, MTT solution (20 μl) was added. The formazan crystals thus formed were dissolved with DMSO and the absorbance was measured on a microplate reader (FLUOstar Optima, Offenburg, Germany).

Phase contrast microscopy

SGC-7901 human gastric cancer cells were plated in 6-well plates at a density of $2x10^5$ cells/ml and then cultured for 24 hrs. Subsequently, the cells were exposed to treatment with various concentrations of bakuchiol (0, 10, 50 and 100 μM) for 48 hrs. Following drug treatment, culture plates were examined using an inverted light microscope (Nikon Corp., Tokyo, Japan) and images were captured. DMSO was used as a control.

Fluorescence microscopic assay using acridine orange (AO), propidium iodide and Hoechst 33258 staining dyes

SGC-7901 human gastric cancer cells were seeded on a chamber slide (Thermo Scientific Nunc Lab Tek II) at a density of 2×10^5 cells per chamber. The cells were treated with 0, 10, 50 and 100 μM bakuchiol for 48 hrs. Afterwards, 10 μg/mL of AO and 10 μg/mL of PI were added to each chamber and the cells were observed under fluorescence microscope (Olympus IX-70, Tokyo, Japan).

Then, after treating cells with the above mentioned doses, the cells were washed with PBS and fixed with 3.5 % formaldehyde for 20 min. Afterwards the cells were again washed removing the fixing solution and then stained with Hoechst 33258. The cells were again washed before analysis under a fluorescence microscope (Olympus IX 81 Tokyo, Japan).

Cell cycle analysis by flow cytometry

The cell cycle analysis was carried out by flow cytometry (Becton-Dickinson FACS Calibur flow cytometry) equipped with CellQuest 3.3 software. After incubation with bakuchiol for 48 hrs, SGC-7901 human gastric cancer cells were harvested, fixed with 70% ice-cold ethanol for 24 hrs, treated with 20 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA), stained with 20 μg/ml PI, and analyzed by flow cytometer.

Statistics

The data were expressed as means ± SD and came from 3 independent experiments. Differences between the control and treatment groups were examined using the Student's t-test. SPSS 17.0 software was used for data analysis and a p value <0.05 was considered statistically significant.

Results

Bakuchiol induced potent cytotoxic effects in SGC-7901 human gastric cancer cells

The chemical structure and the cytotoxic effects of bakuchiol in SGC-7901 gastric cancer cells

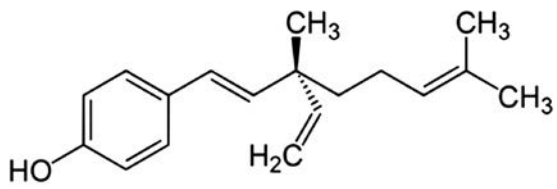

Figure 1. Chemical structure of bakuchiol.

are shown in Figures 1 and 2, respectively. As it is evident from Figure 2, bakuchiol induced potent, concentration-dependent as well as time-dependent cytotoxic effects in SGC-7901 human gastric cancer cells. The IC_{50} values of bakuchiol were found to be 58.4, 42.3 and 32.5 μM at 12, 24 and 48 hrs time intervals, respectively. This indicates that the cytotoxic effect of this compound increases with increase in the incubation time also.

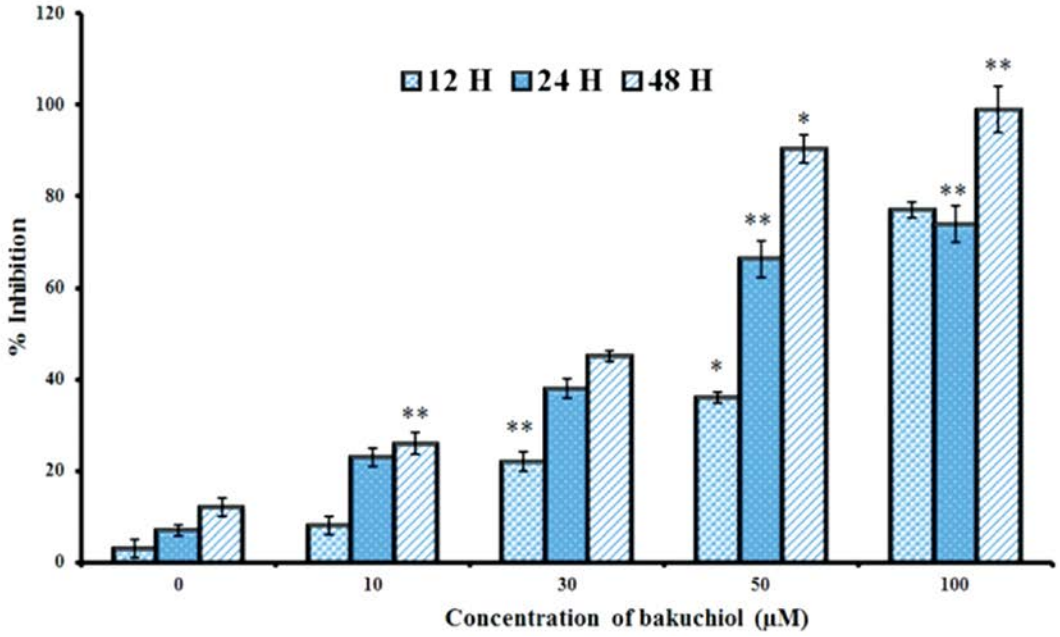

Figure 2. Cytotoxic effect of bakuchiol on the proliferation of SGC-7901 gastric cancer cells. According to dosage (μM) and time of exposure (H). Data are shown as the mean ± SD of three independent experiments. *p<0.05, **p<0.01 vs 0 μM (control).

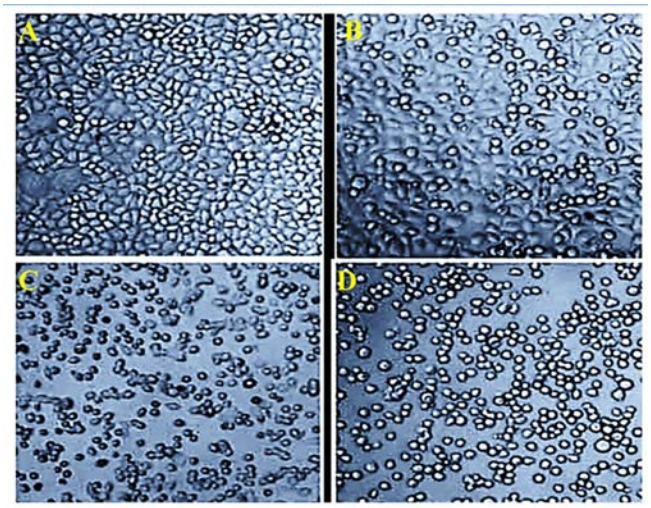

Figure 3. Effects of bakuchiol on the morphology of SGC-7901 gastric cancer cells. Morphological changes were observed under phase-contrast microscopy after treating without **(A,** control**)** and with 10 **(B)**, 50 **(C)** and 100 **(D)** μM of bakuchiol for 48 hrs. Cells got detached from one another making clusters of small numbers of cells floating in the medium (original magnification x200).

Effect of bakuchiol on the cellular morphology of SGC-7901 human gastric cancer cells

In this assay, SGC-7901 gastric cancer cells were exposed to increasing doses of bakuchiol in order to examine any morphological changes induced by this compound. The results of this assay are depicted in Figure 3 A-D, indicating that untreated control cells exhibited normal morphology like round shape and were attached to one another. However, on treatment with 10, 50 and 100 μM of bakuchiol for 48 hrs, phase contrast microscopy revealed that the cells got detached from one another making clusters of small number of cells floating in the medium. These cells also had uneven shape and were incapable to maintain their intact membranes.

Bakuchiol induced characteristic morphological features of apoptosis

Fluorescence microscopy using OA and PI double staining indicated that bakuchiol induced

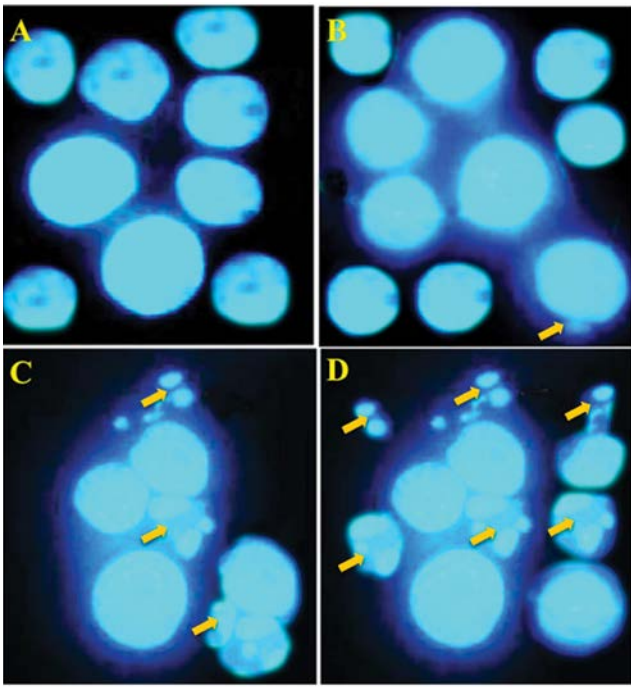

Figure 4. Fluorescence microscopy study of SGC-7901 gastric cancer cells using Hoechst 33258 staining dye. The arrows show the apoptotic cells which are shrunk and condensed with uneven morphology. The cells were treated without bakuchiol (**A**, untreated control), and with 10 **(B)**, 50 **(C)** and 100 **(D)** μM for 48 hrs. Results taken from three independent experiments (original magnification x400).

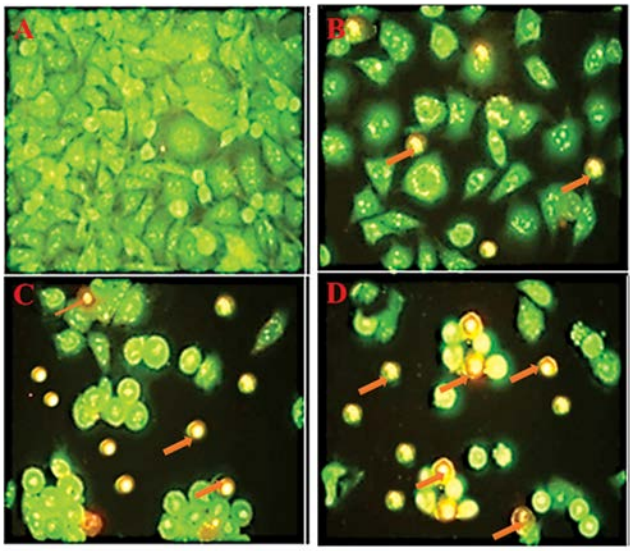

Figure 5. Fluorescence microscopy study of SGC-7901 gastric cancer cells using acridine orange/propidium iodide (AO/PI) staining. The cells were treated without (**A**, untreated control), and with 10 **(B)**, 50 **(C)** and 100 **(D)** μM of bakuchiol for 48 hrs. The arrows show apoptotic cells with morphological features including nuclear fragmentation, chromatin condensation and apoptotic body formation (original magnification x400).

morphological features which were indicative of apoptosis. The results of this assay are shown in Figure 4 A-D, revealing that untreated SGC-7901 human gastric cancer cells showed green fluorescence. However, after the cells were treated with 10, 50 and 100 μM of bakuchiol, these cells began to emit orange red fluorescence more intensively at the centre of cells indicating apoptosis. The number of these apoptotic cells increased with increase in the dosage of bakuchiol.

In case of Hoechst 33258 staining, similar results indicating apoptosis were obtained. The results are shown in Figure 5, indicating that unlike control untreated cells which showed normal morphology and spherical shape (Figure 5 A) the bakuchiol-treated cells showed significant chromatin condensation, chromosomal DNA cleavage, blebbing of the membrane and formation of apoptotic bodies. The appearance of these apoptotic bodies was closely related to the dose of bakuchiol (Figure 5 B-D).

Bakuchiol induced sub-G1 cell cycle arrest in SGC-7901 cells

The fact that bakuchiol induced apoptosis was further confirmed by flow cytometry using PI as a fluorescent probe. After the SGC-7901 cells were treated with 10, 50 and 100 μM of bakuchiol for 48 hrs, it was observed that there occurred an obvious accumulation of cells in the sub-G1 phase of the cell cycle (also called as the apoptotic phase). The results which are shown in Figure 6 A-D reveal that as compared to the untreated control cells which only showed 1.3% of cells in sub-G1 phase, the cells treated with 10, 50 and 100 μM of bakuchiol indicated that the percentage of cells in the sub-G1 phase increased significantly to 6.5, 23.8 and 62.2%, respectively.

Discussion

In the present study, it was observed that bakuchiol induced potent cytotoxic effects in SGC-7901 human gastric cancer cells in a dose- and time-dependent manner. Furthermore, using phase contrast and fluorescence microscopic techniques, it was observed that bakuchiol could induce various morphological features which are characteristic of apoptosis including DNA fragmentation, chromatin condensation, blebbing of the membrane and formation of apoptotic bodies. On treatment with 10, 50 and 100 μM of bakuchiol for 48 hrs, phase contrast microscopy revealed that the cells got detached from one another mak-

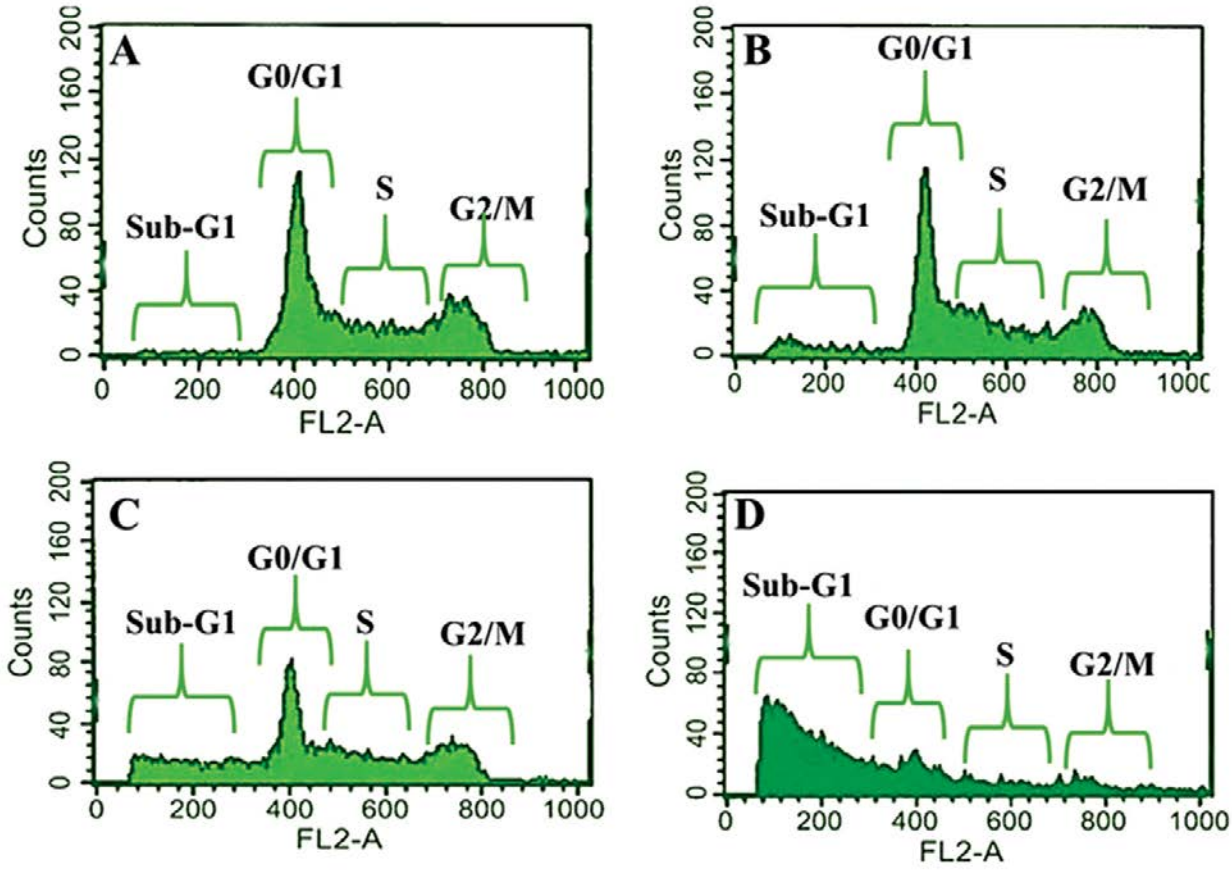

Figure 6. Effect of bakuchiol on the cell cycle phase distribution of SGC-7901 human gastric cancer cells. The cells were treated without (**A**, untreated control), and with 10 **(B)**, 50 **(C)** and 100 **(D)** μM of bakuchiol for 48 hrs and then analyzed by flow cytometry. Bakuchiol induced sub-G1 cell cycle arrest in these cells leading to an increase in cells in the sub-G1 phase (apoptotic phase).

ing clusters of small number of cells floating in the medium. The cells when treated with 10, 50 and 100 μM of bakuchiol began to emit orange red fluorescence more intensively at the centre of cells, indicating apoptosis. Flow cytometry revealed that bakuchiol also induced sub-G1 cell cycle arrest. The cells treated with 10, 50 and 100 μM dose of bakuchiol indicated that the percentage of cells in the sub-G1 phase increased significantly to 6.5, 23.8 and 62.2% respectively.

Natural products have always been used as anticancer agents for many decades. Natural products have played significant role in the design and development of more than 60% of the clinically used anticancer agents. Furthermore, there are numerous natural products or their analogs which are currently in preclinical and clinical stage of evaluation. World Health Organization has estimated that about 75 to 80% of the world population rely on traditional medicines for their main health care [6-8]. Natural products which are highly operative and create less side-effects are a promising substitute for chemotherapy with even deadly side-effects. Non-cytotoxic bioactive molecules have a great prospective for using them against cancer because most of these natural products exhibit pleiotropic properties [9-14].

Bakuchiol is a terpenoid compound mostly isolated from Psoralea corylifolia and Otholobium pubescens [15]. Previous studies have reported that bakuchiol exhibits anticancer, hepatoprotective, antihyperglycemic and antibacterial activities [16-18]. To the best of our knowledge, no such study has been published reporting anticancer activity of bakuchiol in SGC-7901 human gastric cancer cells. Therefore, we undertook this study to evaluate the effect of this compound on this cell line along with studying its mode of action by investigating its effect on cellular apoptosis and cell cycle phase distribution.

Conflict of interests

The authors declare no confict of interests.

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