

Anti-tumor effects of bakuchiol on human gastric carcinoma cell lines are mediated through PI3K/AKT and MAPK signaling pathways

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Abstract. Bakuchiol is extracted from *Psoralea corylifolia*, a member of the Leguminosae family, has been used in Indian Ayurvedic and Chinese traditional medicine, and it possesses an anticancer effect. The primary aim of the present study was to identify the molecular mechanisms underlying the anticancer effect of bakuchiol monoterpenes. Bakuchiol treatment significantly inhibited NUGC3 human gastric cancer cell viability in a concentration dependent manner. In addition, bakuchiol significantly increased the apoptotic cell population in the sub-G1 phase, and Annexin-V-fluorescein isothiocyanate/propidium iodide double staining confirmed the increase in apoptosis. Nuclear fragmentation and the formation of apoptotic organelles were promoted in bakuchiol-treated NUGC3 cells. Western blotting results indicated that bakuchiol treatment significantly decreased procaspase-3,6,8,9 and poly (ADP-ribose) polymerase (PARP) expression levels, increased cleaved caspase-3 and cleaved PARP expression levels, and increased the B cell lymphoma-2 associated X, apoptosis regulator:B cell lymphoma-extra large ratio. Bakuchiol-treated NUGC3 cells demonstrated significantly reduced phosphorylated (p-) protein kinase B (AKT) protein expression levels and elevated p-extracellular signal related kinase 1/2 (ERK1/2), p-c-Jun N-terminal kinase (JNK) and p-p38. Bakuchiol-induced cell death was mitochondrial dependent, through modulation of phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase signaling pathways. These findings demonstrated that bakuchiol possesses potential for treating human gastric cancer.

Introduction

Gastric cancer is one of the most common malignant cancers globally. It is estimated that gastric cancer alone causes almost 10% of all cancer-associated mortality with a higher (10-12%) incidence rate in Asia and Europe (1). Despite global reductions in prevalence and mortality over the past 20 years, gastric cancer is still ranked as the second major cause of cancer-associated mortality and considered as the foremost gastrointestinal infectious disease in eastern Asia (2). Understanding of the molecular mechanisms underlying gastric cancer have improved, but there remains a distinctive lack of targeted treatments in the clinical development of the disease (3). Therefore, there is a necessity to establish novel therapeutic agents that diminish the mortality rate of patients with gastric cancer, with minor side effects.

Plant-derived natural products have been used as a source of medicinal agents useful to treat humans, and the hunt for novel, effective therapeutic compounds continues. Bakuchiol is a typical prenylated monoterpene phenolic compound separated from *Psoralea corylifolia*, a member of the Leguminosae family. Previous reports have demonstrated that molecular structures containing styryl moieties in conjugation with other structural features, including chromones, quinazolines and pyrenes, have multiple biological uses including the treatment of cancer and HIV (4-7). Bakuchiol has previously been demonstrated to exhibit cytotoxicity in certain human cancer cell lines (8). Based on these prior examinations, the present study investigated the anticancer effects of bakuchiol in gastric cancer, aiming to produce novel insights to enhance understanding of the underlying molecular mechanism.

Materials and methods

Materials and reagents. Bakuchiol (Fig. 1), MTT reagent and dimethyl sulfoxide (DMSO) were ordered from Sigma Aldrich; Merck KGaA (Darmstadt, Germany). Cell culturing RPMI-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin antibiotics were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-rabbit monoclonal B cell lymphoma-2 associated X, apoptosis regulator (Bax; cat. no. ab32503; 1:1,000), anti-rabbit monoclonal B cell lymphoma-extra large (Bcl-xL; cat. no. ab32370; 1:1,000),

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anti-rabbit monoclonal procaspase-3 (cat. no. ab32150; 1:500), anti-rabbit monoclonal procaspase-6 (cat. no. ab3263, 1:800), anti-mouse monoclonal procaspase-8 (cat. no. ab38271; 1:1,000) and anti-rabbit polyclonal procaspase-9 (cat. no. ab135544; 1:800), anti-rabbit monoclonal poly (ADP-ribose) polymerase (PARP; cat. no. ab191217; 1:1,000), anti-rabbit monoclonal cleaved PARP (cat. no. ab32064; 1:1,200), anti-rabbit monoclonal protein kinase B (AKT; cat. no. ab183758; 1:1,000), anti-rabbit monoclonal phosphorylated (p-AKT; cat. no. ab81283; 1:800), anti-rabbit monoclonal, p-c-Jun N-terminal kinase (JNK; cat. no. ab179461; 1:1,200), anti-rabbit monoclonal extracellular signal-regulated kinase 1/2 (ERK1/2; cat. no. ab184699; 1:1,000), anti-rabbit polyclonal cleaved caspase-3 (cat. no. ab2302; 1:1,200), anti-rabbit monoclonal β -actin (cat. no. ab32575; 1:1,500) and anti-mouse monoclonal β -actin (cat. no. ab8226; 1:1,500) antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; cat. no. ab97023; 1:10,000) and anti-rabbit IgG (cat. no. ab6721; 1:10,000) were also obtained from Abcam (Cambridge, UK). The Muse Annexin V and Dead Cell Assay kit (cat. no. MCH100105) and the Muse Cell Cycle Assay kit (cat. no. MCH100106) were ordered from EMD Millipore (Billerica, MA, USA). DAPI was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). All other chemicals and materials utilized for electrophoresis were acquired from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Cell culture. NUGC3 human gastric cancer cell lines were purchased from the Japanese Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in RPMI-1640 medium and supplemented with heat-inactivated 10% FBS (v/v) and 1% penicillin and streptomycin in humidified conditions of 5% CO₂ at 37°C.

MTT assay. To test the effect of bakuchiol on human gastric cancer cell viability, NUGC3 cells were seeded in a 96 well plate at a density of 6x10⁴ cells/ml, then treated with 0, 20, 40, 60, 80, 100 or 120 μ g/ml bakuchiol and incubated for 24 h at 37°C. The negative control cells were treated with DMSO. Following incubation, 0.5 g/ml MTT was added to each well and the plates were further incubated for 3 h at 37°C. The medium was then removed and DMSO was added to dissolve the formazan crystals formed. The absorbance was recorded at a 540 nm wavelength using an iMark (model 550) ELISA microplate absorbance reader (Bio-Rad Laboratories, Inc.).

Cell cycle distribution and cell apoptosis analysis. NUGC3 cells were treated with 0, 50 and 100 μ g/ml bakuchiol and incubated for 24 h at 37°C. The cell lines were collected and cleaned using cold PBS and centrifuged at 1,000 x g for 10 min at 37°C. Cold ethanol 70% (v/v) was used to fix the pellet at -20°C for 3 h, and the cells were then washed using PBS and 200 μ l was transferred to a fresh tube. Muse Cell Cycle Assay kit solution (100 μ l) was added to each well and incubated for 30 min in the dark at room temperature. The pellet was resuspended in 1 ml RPMI-1640 medium and 100 μ l was transferred to a fresh tube for the analysis of apoptosis. Again, 100 μ l of Muse Annexin V and Dead Cell Assay kit solution was added and incubated at room temperature for 30 min in the dark at 37°C and analyzed using the Muse cell analyzer

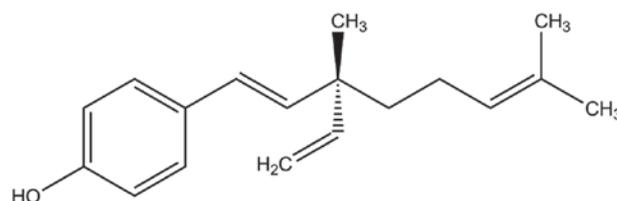


Figure 1. The structure of bakuchiol.

(0500-3115) using Muse count and viable software (version 1.05.0) from EMD Millipore (Billerica, MA, USA).

Morphological changes and DAPI staining. NUGC3 cells were treated with 0, 50 and 100 μ g/ml bakuchiol and incubated for 24 h at 37°C. A total of 1x10⁴ cells were then washed with cold PBS at room temperature. Then the cells were fixed with 3.7% paraformaldehyde (1 ml) and 95% ethanol at room temperature for 10 min and followed by washing with PBS. The fixed cells were stained using DAPI (DAPI staining kit solution). The morphological nuclear changes were examined using fluorescence microscopy at x400 magnification (Model-DMLS) from Leica Microsystems (GmbH, Wetzlar, Germany).

Western blot analysis. NUGC3 cells were treated with 0, 50 and 100 μ g/ml bakuchiol at 37°C for 72 h and then lysed with NP-40 (1% w/w) ice cold radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), sodium deoxycholate (1% w/v), SDS (0.1% w/v), NaCl (0.15 M), EDTA (2 mM) sodium phosphate buffer (0.01 M, pH 7.2) and sodium fluoride (50 mM). The resultant cell lysates were centrifuged at 3,000 x g for 10 min at 37°C and total cellular protein concentration was determined by Bio-Rad Bradford protein assay method (Bio-Rad Laboratories, Inc.) (9). Equal quantities of protein (50 μ g/lane) was separated by SDS PAGE on 12% gel and then transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with Tris-buffered saline containing Tween-20 and 5% skimmed milk for 4 h at 37°C and probed with the primary antibodies overnight at 37°C, followed by incubation with secondary antibodies conjugated to horseradish peroxidase for 2 h at 37°C. The blots were examined using an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Chalfont, UK) and quantification was performed using Image J software from the National Institutes of Health (version 2.8; Bethesda, MD, USA). All assays were performed in triplicate.

Statistical analysis. The statistical analyses were performed using SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA). The results were expressed as the mean \pm standard deviation of three independent experiments. Differences between the experimental groups were determined using one-way analysis of variance followed by Dunnett's multiple comparison post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Bakuchiol inhibits NUGC3 cell viability. To determine the concentration of bakuchiol that inhibits cell viability, NUGC3

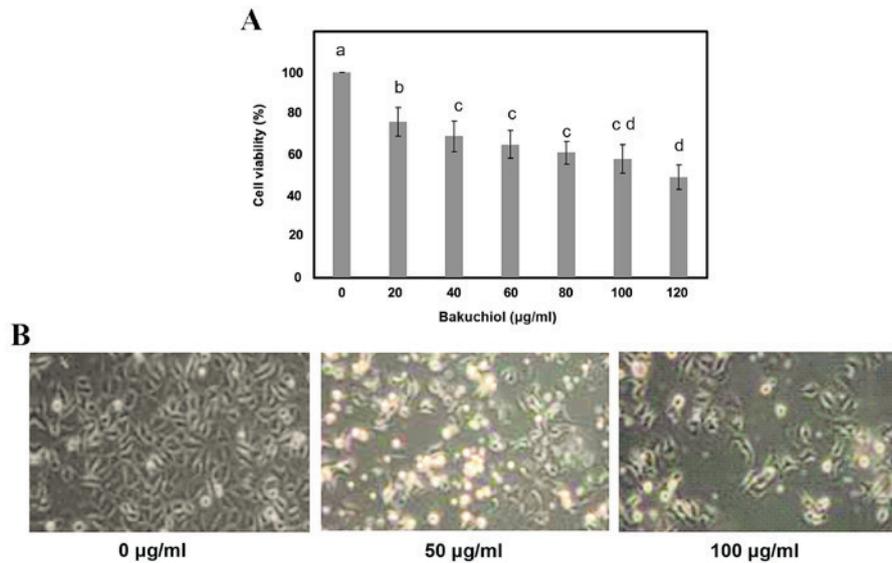


Figure 2. Inhibitory effect of bakuchiol on NUGC3 cell viability. (A) NUGC3 cell viability following treatment with bakuchiol, as determined by MTT assay. (B) Morphological changes in NUGC3 cells following treatment with 0, 50 or 100 µg/ml bakuchiol, examined under light microscopy (magnification, x400). Data bearing different letters (a-d) were significantly different from each other (all $P < 0.05$).

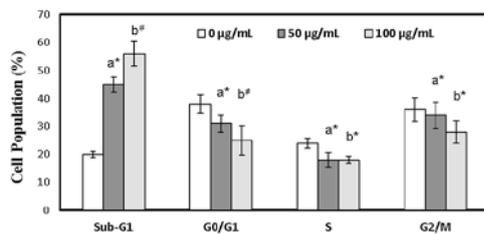


Figure 3. Cell cycle progression of NUGC3 cells treated with 0, 50 or 100 µg/ml bakuchiol for 24 h. The data represents the mean ± standard deviation of three independent experiments. * $P < 0.05$; # $P < 0.01$. vs. 0 µg/ml.

cells were treated with 0, 20, 40, 60, 80, 100 or 120 µg/ml bakuchiol for ~24 h, and cell viability was then measured by MTT assay. Following bakuchiol treatment and 24 h incubation, cell viability was inhibited in a concentration-dependent manner when compared with the control, without bakuchiol treatment ($P < 0.05$; Fig. 2A). The concentration that produced 50% inhibition (IC_{50} value) was revealed to be ~120 µg/ml. Therefore, bakuchiol concentrations of 0, 50 and 100 µg/ml were used for further examination. Microscopic study indicated changes in the cell structure, including visible shrinkage of cells and reduced cell counts, in bakuchiol treated cells compared with controls (Fig. 2B).

Bakuchiol induces apoptosis in NUGC3 cells. Cell cycle distribution and the apoptotic cell population in bakuchiol-treated NUGC3 cells was determined using flow cytometry analysis. Bakuchiol treatment elevated the percentage of sub-G1 cells from 13% to 28 and 39% at 50 ($P < 0.05$) and 100 µg/ml ($P < 0.01$), respectively (Fig. 3). Meanwhile, bakuchiol treatment significantly reduced the cell population of G₀/G₁, S and G₂/M compared with the control ($P < 0.05$; Fig. 3). In addition, the effect of bakuchiol on apoptosis induction in NUGC3 cells was examined using Annexin V-fluorescein isothiocyanate/propidium iodide double staining and flow cytometry.

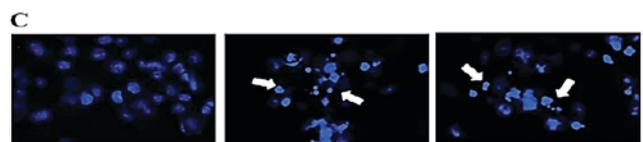
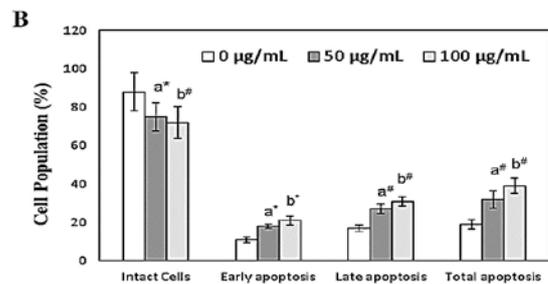
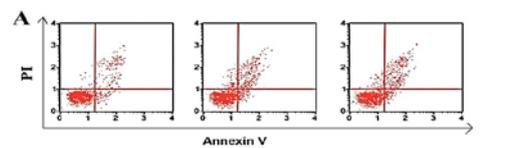


Figure 4. (A) Effect of 0, 50 or 100 µg/ml bakuchiol treatment on apoptosis of NUGC3 cells. (B) The data are expressed as the mean ± standard deviation of three independent experiments. (C) DAPI staining of NUGC3 cells with nuclear fragmentations indicated by white arrows, analyzed using a fluorescent microscope (magnification, x400). * $P < 0.05$; # $P < 0.01$. vs. 0 µg/ml.

The mild elevation of sub-G1 cells in the untreated group was attributed to the effect of DMSO on NUGC3 cells.

Treatment with bakuchiol (50 µg/ml; $P < 0.05$ and 100 µg/ml; $P < 0.01$) significantly increased the percentage of early apoptosis, late apoptosis and total apoptosis in NUGC3 cells in a concentration dependent manner compared with the control (Fig. 4A and B). Nuclear fragmentation and apoptotic organelles were observed following 50 and 100 µg/ml bakuchiol treatment, using DAPI staining (Fig. 4C). These data suggested that bakuchiol treatment induced cell death in NUGC3 cells.

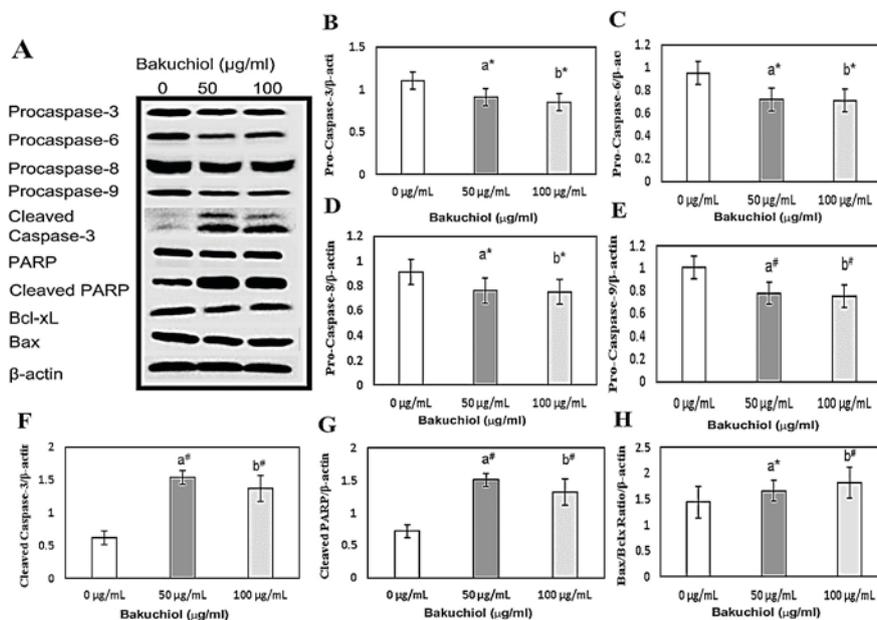


Figure 5. (A) Western blot analysis of caspase activation and PARP cleavage in NUGC3 cells treated with 0, 50 or 100 μ g/ml bakuchiol for 24 h. Densitometric analyses of (B) procaspase-3, (C) procaspase-6, (D) procaspase-8, (E) procaspase-9, (F) cleaved caspase-3, (G) PARP and (H) Bax:Bcl-xL. *P<0.05; #P<0.01. vs. 0 μ g/ml. PARP, poly (ADP-ribose) polymerase; Bax, B cell lymphoma-2 associated X, apoptosis regulator; Bcl-xL, B cell lymphoma-extra large.

Caspase activation and PARP cleavage was induced by bakuchiol. To confirm whether bakuchiol-induced apoptosis was caspase dependent, western blotting analysis was performed (Fig. 5A). Furthermore, the levels of apoptosis-associated proteins, Bax and Bcl-xL, were measured in bakuchiol-treated NUGC3 cells. Levels of procaspase-3,6,8 and 9 were significantly decreased following treatment with 50 and 100 μ g/ml bakuchiol treatment compared with the control (P<0.05; Fig. 5B-E, respectively), while cleaved caspase-3 and cleaved PARP levels were significantly elevated following 50 and 100 μ g/ml bakuchiol treatment compared with the control (P<0.01; Fig. 5F and G, respectively). The ratio of Bax/Bcl-xL in NUGC3 cells was also significantly upregulated following bakuchiol (50 μ g/ml; P<0.05 and 100 μ g/ml; P<0.01) treatment compared with the control (Fig. 5H). These data suggested that bakuchiol treatment induced caspase-dependent apoptosis in NUGC3 cells.

Bakuchiol-induced apoptosis is regulated by the phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways in NUGC3 cells. Cell proliferation and apoptosis are regulated by the PI3K/AKT and MAPK signaling pathways. As AKT activity is maintained by phosphorylation, the phosphorylation of PI3K/AKT and MAPK was analyzed using western blotting during bakuchiol-induced apoptosis in NUGC3 cells (Fig. 6A). Bakuchiol treatment (50 and 100 μ g/ml) resulted in significant decrease (50 μ g/ml; P<0.01 and 100 μ g/ml; P<0.05) in the levels of phosphorylated AKT (p-Akt) compared with the control (Fig. 6B). Furthermore, phosphorylated ERK1/2, JNK and p38 levels were significantly upregulated upon treatment with bakuchiol compared with the control (P<0.01; Fig. 6C-E, respectively). These experimental findings suggested that bakuchiol induced apoptosis in NUGC3 cells by regulation of the PI3K/AKT and MAPK signaling pathways.

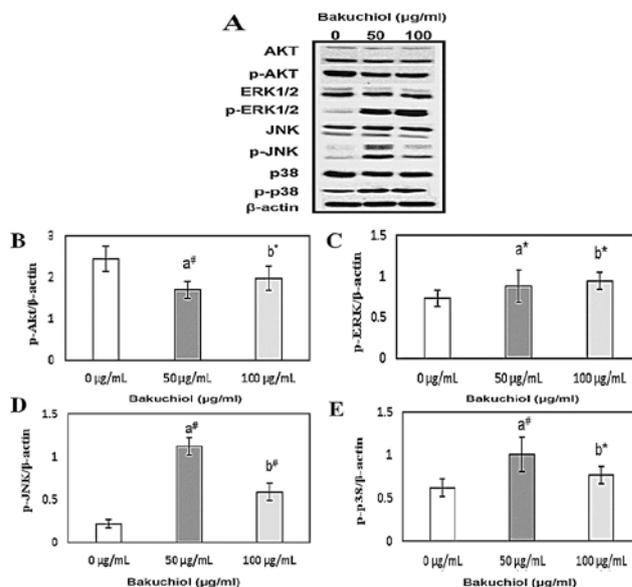


Figure 6. (A) Effect of 0, 50 or 100 μ g/ml bakuchiol treatment for 24 h on PI3K/AKT and MAPK signaling pathways in NUGC3 cells, analyzed by western blotting. Densitometric analyses of (B) p-Akt, (C) p-ERK1/2, (D) p-JNK and (E) p-p38. *P<0.05; #P<0.01. vs. 0 μ g/ml. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; MAPK, mitogen-activated protein kinase; p-, phosphorylated; ERK1/2, extracellular signal related kinase 1/2; JNK, c-Jun N-terminal kinase.

Discussion

The aim of the present study was to investigate whether bakuchiol treatment induced gastric cancer cell death, and to further determine the underlying molecular mechanisms of bakuchiol-induced apoptosis in NUGC3 cells. Successful medicinal treatment using chemotherapeutic agents is primarily dependent on their potential to induce apoptosis

in cancer cells (10). Isolated bakuchiol, which is a major chemical component of *Psoralea corylifolia*, a member of the Leguminosae family (11), is one of the primary medicinal plants in Indian Ayurveda and Chinese traditional medicinal treatments owing to its inhibitory properties on DNA polymerase (12) and topoisomerase II (13). A previous study on bakuchiol observed that its antibacterial activity was significantly enhanced via chemical modification to obtain reduction of MIC up to 8-fold against Gram (+) and (-) bacteria (14). The present study focused on the anticancer effects of bakuchiol against human gastric cancer cell lines.

The results of the MTT assay revealed that bakuchiol significantly reduced NUGC3 cell viability in a concentration dependent manner. Type I programmed cell death, or apoptosis, is the primary mechanism by which different anti-tumor and chemoprotective agents, inclusive of naturally derived products, exert anti-cancer properties (15). Earlier reports demonstrated that abnormalities in the cell cycle may result in apoptosis in various cancer cell lines (16,17). Bakuchiol treatment resulted in sub-G1 phase cell accumulation in NUGC3 cells. Annexin V-FITC/PI double staining confirmed the induction of apoptosis and nuclear fragmentation and apoptotic organelles were observed in bakuchiol-treated NUGC3 cells. These data revealed that bakuchiol suppressed NUGC3 gastric cancer cell viability and was responsible for the induction of apoptosis.

To explore the involved molecular mechanisms, western blotting was performed and the results indicated that procaspase-3,6,8 and 9 expression levels were significantly decreased. The vital caspase is caspase-3, which activates PARP cleavage and results in the induction of the apoptosis process. Elevated cleaved caspase-3 expression concomitantly induced the cleavage of PARP. These data confirmed that bakuchiol induced apoptosis in a caspase-3 dependent manner. Mitochondrial apoptotic functions are regulated by apoptotic regulatory proteins of the Bcl-2 family. Cell viability and death were determined by the levels of Bcl-2 family pro- and anti-apoptotic proteins. The ratio of Bax and Bcl-xL has been demonstrated to be an apoptosis determining factor. In the present work, Bax protein expression levels remained the same while Bcl-xL levels were significantly downregulated, but following bakuchiol treatment the upregulation of the Bax:Bcl-xL ratio was observed in NUGC3 cells. When released from the mitochondria to the cytosol, cytochrome c has previously been observed to interact with apoptotic peptidase activating factor-1 and thereby lead to Bax/Bcl-xL ratio increment, thus activating caspase-3 and resulting in apoptosis (18).

To further evaluate the molecular mechanisms and involved signaling pathways in bakuchiol-induced apoptosis, PI3K/AKT and MAPK pathway phosphorylation levels were analyzed by western blotting. Bakuchiol inhibited levels of p-AKT, the downstream target of PI3K, which is known to control proliferation and cell apoptosis. Similar to the results of the present study, PI3K/AKT signaling pathway inhibition induces apoptosis in different cancer types (18,19). The MAPK signaling pathway also participates in cell viability, proliferation and apoptosis, and classified into 3 major divisions: JNK, p38 and ERK MAPKs (20). Although ERK1/2 pathway activation is associated with cell growth and proliferation, it has also been reported to induce apoptosis in T cells via the Fas expression

ligand (21). ERK1/2 promotes apoptosis through preventing inactivation of the pro-apoptotic Bcl-2 family member, BCL2 associated agonist of cell death (22). JNK is a representative MAPK downstream kinase family member, which has been reported to maintain Fas and Fas ligand receptor expression in apoptosis (23). JNK is also involved in the apoptosis intrinsic pathway, where activated JNK controls the pro-apoptotic proteins BH3 interacting domain death agonist and Bax, and induces cytochrome c release into the cytosol from mitochondria (24). JNK activation results in anti-apoptotic Bcl-2 protein downregulation (25). It has previously been noted that activated p38 induces apoptosis in different cell line (26,27). The results of the present study exhibited similar patterns in which p-ERK, p-JNK and p-p38 levels were increased following bakuchiol treatment in er NUGC3 gastric cancer cells (Fig. 6), indicating the involvement of PI3 K/AKT and MAPKs in bakuchiol-induced apoptosis in NUGC3 cells.

In conclusion, bakuchiol was demonstrated to decrease cell viability and induce caspase-dependent apoptosis in NUGC3 gastric cancer cells. PI3K/AKT and MAPK signaling pathways triggered apoptosis following bakuchiol treatment in the NUGC3 gastric cancer cell line. To the best of our knowledge, the present study is the first to demonstrate the anticancer effect of bakuchiol treatment in NUGC3 cells. Therefore, bakuchiol may be an effective novel chemotherapeutic agent for treating human gastric cancer.

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