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/p38, 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 monoterpane 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. ab814699; 1:1,000), anti-rabbit polyclonal cleaved caspase-3 (cat. no. ab2302; 1:1,200), anti-rabbit monoclonal β-actin (cat. no. ab32575; 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. MCH100106) and the Muse Cell Cycle Assay kit (cat. no. MCH100105) were ordered from EMD Millipore (Billerica, MA, USA). The results were expressed as the mean ± 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
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 (IC50 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. 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.
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 µg/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 µg/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 µg/ml; P<0.05 and 100 µg/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 µg/ml) resulted in significant decrease (50 µg/ml; P<0.01 and 100 µg/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.

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 NUGC 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 proteins 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 peptide activating factor-I 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, p83 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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