Original Paper

Anti-Cancer Effects of Radix Angelica Sinensis (Danggui) and N-Butylidenephthalide on Gastric Cancer: Implications for REDD1 Activation and mTOR Inhibition

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Key Words

N-butylidenephthalide • Gastric cancer • MTOR • REDD1 • Metastasis

Abstract

Background/Aims: Radix *Angelica Sinensis* (*danggui* in Chinese) is widely used in traditional Chinese medicine (TCM). N-butylidenephthalide (BP), a bioactive compound in *danggui*, is a potential antitumor agent for various cancer types. However, its clinical effect and mechanism in the treatment of gastric cancer remain undetermined. **Methods:** The *in vivo* protective effect of *danggui* in patients with gastric cancer were validated using data from Taiwan's National Health Insurance Research Database (NHIRD). The genes induced by BP-treatment were analyzed by whole transcriptome RNA sequencing (RNA-seq) and validated by real-time

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PCR, western blot and siRNA transfection. The effect of BP on AGS cell migration and invasion was evaluated in transwell assays. The antitumor effects of BP were evaluated *in vivo* in an AGS xenograft animal model. **Results:** *Danggui* users were found to have an increased survival rate when compared with *danggui* nonusers (log-rank test $p = 0.002$). The use of *danggui* highly associated with decreased mortality (the adjusted hazard ratio (HR) of *danggui* user was 0.72 [95 % CI, 0.57-0.92] ($p = 0.009$)). The *in vitro* results showed that BP inhibited gastric cancer cell proliferation, and triggered cellular apoptosis depending on the activation of mitochondrial apoptotic pathway. Using RNA-seq analysis we found that REDD1 was the highest transcript induced by BP in gastric cancer cells. BP induce an increase of REDD1 expression that inhibits mTOR signaling, thus inhibiting gastric cancer growth. We used RNA interference to demonstrate that the knock-down of REDD1 attenuated the BP-induced mTORC1 activation and growth inhibition. BP suppressed the growth of AGS xenografts tumor *in vivo*. **Conclusion:** *Danggui* can prolong the survival rate of gastric cancer patients in Taiwan. BP caused gastric cancer cell death through the activation of mitochondria-intrinsic pathway and induced the REDD1 expression leading to mTOR signal pathway inhibition in gastric cancer cells. BP inhibited the *in vivo* growth of AGS xenograft tumors. These results may provide the basis for a new therapeutic approach toward the treatment of gastric cancer progression.

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Introduction

Gastric cancer (GC) is the second leading cause of cancer-related death in the world, and the most prevalent cancer in East Asia. In the United States, approximately 21,000 new cases of gastric cancer were diagnosed in 2010, causing an estimated 10,570 deaths [1]. Currently radical resectioning of cancerous lesions is the only available cure for gastric cancer, but multi-disciplinary therapy can palliate the disease and even help to prolong life. The treatment options for gastric cancer patients include adjuvant therapy, perioperative chemotherapy, preoperative chemotherapy and combinations of these treatments [2].

Angelica sinensis (*Danggui*) is a type of traditional Chinese medicine (TCM) widely-used in Taiwan and the prescription of which is covered by Taiwan's National Health Insurance scheme. Taiwan's National Health Insurance Research Database (NHIRD) records the medical history of nearly Taiwan's entire population, and thus provides a means of investigating the incidence of gastric cancer with or without *Angelica sinensis* exposure. Several studies have used the NHIRD to investigate associations between traditional Chinese medicine and different diseases with high accuracy and validity for population-based research [3, 4]. Recently, complementary medicine including TCM, have become more widely accepted as potential alternative medicines for cancer treatment. *N*-butylidenephthalide (BP; $C_{12}H_{12}O_2$) is a naturally occurring compound isolated from the extract of *danggui*, and has been reported to exhibit antitumor activity on various types of cancer cell, including glioblastoma multiforme [5, 6], prostate cancer [7, 8], lung cancer [9] and hepatoma [10]. For instance, BP down-regulates the expression of AP-2 leading to repressing transcriptional activity of hTERT and inhibits the telomerase activity in lung cancer [9]. BP down-regulates the expression of S-phase kinase-associated protein (Skp2) leading to an increase in p16 and p21 expression which cause tumor senescence and cell cycle G0/G1 arrest [5]. BP also modulates the mitochondrial-dependent and ER stress pathway, resulting in cell apoptosis [7, 8]. These findings indicate that BP is a potential anticancer agent for clinical applications. However, the effect of BP on gastric cancer remains unclear and requires further clarification.

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) pathway is an important regulation signaling pathway for cell growth, metabolism, survival, metastasis, and resistance to chemotherapy [11, 12]. Genetic alterations in the PI3K/Akt/mTOR pathway play essential roles in the development and prognosis of gastric cancer [13, 14]. Various upstream repressors of mTOR signaling have been identified including a 25-kD protein REDD1. REDD1 (regulated in development and DNA damage responses 1), also known as DDIT4, is an mTORC1-dependent signaling pathway inhibitor

[15, 16]. It is known to inhibit mTORC1 activity through activation of the TSC1/2 complex [17], and reduction of the phosphorylation of the mTOR substrate: 70-kD ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). The identification of PI3K/Akt/mTOR pathway inhibitors may have the potential to improve the efficacy of gastric cancer treatments.

In this study, we examined the protective effects of *danggui* and its compound against gastric cancer. To investigate these effects *in vivo*, we analyzed the survival rate of gastric cancer patients by using data obtained from the NHIRD. To clarify the possible molecular mechanism of BP-induced growth inhibition in gastric cancer, we performed whole transcriptome RNA-seq analysis of BP-treated gastric cancer cells to identify potential transcriptional targets for BP. We found that REDD1 was expressed over 23 fold at 3 h and 16 fold at 24 h after BP treatment. We further confirmed the marked induction of REDD1 transcripts and proteins in gastric cancer cell lines. We then demonstrated that REDD1 expression leads to the inhibition of mTORC1 by decreasing the phosphorylation of mTORC1 targets S6K and 4EBP1. The role of REDD1 in BP mediated cell growth and mTOR inhibition was confirmed by REDD1 RNA interference. We then demonstrated that BP suppressed tumor growth with a marked increase in REDD1 and apoptosis-related proteins in tumors by immunohistochemistry in an AGS-xenograft model. Our findings suggest that BP might be a novel anticancer agent for gastric cancer therapy.

Materials and Methods

Patients and Study Design

This study used NHIRD records between 1 January, 2003 and 31 December, 2013. In this case-control study, we initially identified patients who had been newly diagnosed with gastric cancer (International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) code 151.XX) from inpatient files. This method has been used in other studies to identify gastric cancer patients. The control cohort included one age-matched non-gastric cancer patient for each gastric cancer patient, randomly selected from the same dataset to reduce selection bias by bundling many confounding covariates that may be present in an observational study with this number of variables. Patients who received Chinese herbal products (CHPs) containing *danggui* between 2003 and 2013 were then identified. The date of initiation of *danggui* exposure was used as the patient's index date. A total of 32, 279 (11, 203 women and 21, 076 men) patients were included in the study cohort. The *danggui* non-exposure group included all of other patients not receiving treatment with Chinese herbal products.

Charlson Comorbidity Index Score

The Charlson Comorbidity Index Score (CCIS) is a widely accepted measure for risk adjustment in administrative claims data sets [18-20]. The CCIS were calculated for each patient by assigning 1 point each for myocardial infarct, congestive heart failure, peripheral vascular disease, dementia, cerebrovascular disease, chronic lung disease, connective tissue disease, ulcer, chronic liver disease or diabetes; assigning 2 points each for hemiplegia, moderate or severe kidney disease, diabetes with end organ damage, tumor, leukemia or lymphoma; 3 points for moderate or severe liver disease; and 6 points each for malignant tumor, metastasis or acquired immune deficiency syndrome.

Other Variables

The study subjects were classified into three groups by socio-economic status using monthly income as a proxy: (1) low SES, <US\$583, (2) moderate SES, US\$583-833, and (3) high SES, ≥ US\$833 [21]. Low income was set at US\$583 because the NTD equivalent (NTD17500) is the government-stipulated minimum wage for full-time employees in Taiwan in 2009. Geographic region of residence was recorded as northern, central, southern, and eastern Taiwan.

Cell lines and Cell cultures

The human gastric cancer cell line AGS, NCI-N87 and TSGH-9201 were purchased from BCRC (Bioresource Collection and Research Center, Hsinchu, Taiwan). Each cell line was cultured in its standard medium as recommended by the BCRC. Culture medium, fetal bovine serum, penicillin and streptomycin were all purchased from Invitrogen, Carlsbad, CA, USA. Cell lines were authenticated annually by short-tandem repeat analysis and routinely tested for mycoplasma contamination (BCRC).

Chemicals and reagents

BP ($C_{12}H_{12}O_2$, 95 %) was purchased from Lancaster Synthesis Ltd. (Newgate Morecambe, UK). Cisplatin, Dimethyl sulfoxide (DMSO), [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT), crystal violet, Tween-20, methanol and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibodies were all purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Polyvinylidene fluoride (PVDF) membranes, BSA protein assay kit and chemiluminescence reagents were purchased from Amersham Biosciences (Arlington Heights, IL, USA).

RNA isolation, Sequencing and Bioinformatics

Total RNA was extracted from cell lines using an-RNeasy Mini Kit® (Qiagen, Valencia, CA, USA) and reverse transcribed at 37 °C for 60 min with an Omniscript RT Kit® (Qiagen) according to the manufacturer's instructions. After RNA extraction, the RNA integrity (RIN Score > 9.0) and quantity was determined using Nanodrop (Thermoscientific, Rochester, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions. Total RNA samples (2 µg) were processed by Beijing Genomics Institute (BGI) for mRNA enrichment by using oligo (dT) magnetic beads. Indexed dsDNA libraries constructed using the Illumina TruSeq RNA sample preparation kit (Illumina, San Diego, CA) were run on a HiSeq2000 for SR50 according to the BGI experiment pipeline. Genes with an adjusted P of less than 0.05 were selected as differentially expressed genes (DEGs) for further gene ontology and pathway enrichment analyses using DAVID [22, 23] and X2K Expression2Kinases software [24].

Western Blot Analysis

5×10^5 cells per 6-cm petri dish were lysed with 200 µl M-PER mammalian protein extraction reagent containing protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and centrifuged at 13,000 x g at 4 °C for 10 min. The protein concentration in the supernatants was quantified using a BSA Protein Assay Kit. Electrophoresis was performed on a 12 % SDS-PAGE gel in a Mini-PROTEAN Tetra cell electrophoresis System (BioRad, Hercules, USA) using 20 µg of protein extract for each lane. Resolved proteins were transferred to PVDF membranes, blocked with 5 % skim milk for 1 h at room temperature, and finally probed with the specific primary antibodies at 4 °C overnight. The PVDF membrane was washed three times with TBS/0.2 % Tween-20 at room temperature and then incubated with the appropriate secondary antibody labeled with horseradish peroxidase (goat anti-mouse or anti-rabbit, 1:10000, Sigma Chemical, St. Louis, MO, USA) for 1 h at room temperature. All resolved proteins bands were detected using Western Lightning Chemiluminescence Reagent Plus (Amersham Biosciences, Arlington Heights, IL, USA).

Cell Proliferation Assay

The viability of the cells following treatment with various BP dosages was evaluated using MTT assay performed in triplicate. Briefly, cells (4×10^4 /well) were incubated in 24-well plates containing 0.5 ml of serum-containing medium. Cells were allowed to adhere for 18-24 h and were washed with phosphate-buffered saline (PBS). Solutions were always prepared fresh by dissolving 0.1 % DMSO (control) or BP in the culture medium before being added to cells. The BP-containing medium was removed after treatment for 24 or 48 h as indicated. The cells were then washed with PBS, replenished with culture medium containing 300 µg/ml MTT, and incubated for 1 h at 37 °C. After the MTT medium was removed, 0.5 ml of DMSO was added to each well. Absorbance at 570 nm was detected by a multi well plate reader Infinite 200 Pro Tecan™ (Tecan, Mannedorf, Switzerland). The absorbance for DMSO-treated cells was considered as 100 %.

Cell Cycle Analysis

The cell cycle was determined by flow cytometry following DNA staining to reveal the total amount of DNA. Approximately 5×10^5 of gastric cancer cells were incubated with 50 (AGS) or 75 (NCI-N87 and TSGH-9201) $\mu\text{g/ml}$ BP for the indicated time. Cells were harvested with trypsin/EDTA, collected, washed with PBS, fixed with cold 100 % ethanol overnight, and then stained with a solution containing 20 $\mu\text{g/ml}$ PI, 0.2 mg/ml RNase A, and 0.1 % Triton X-100 for 30 min in the dark. The cells were then passed through an Accuri C6 flow cytometer to measure the DNA content. The data were obtained and analyzed with CFlow® software.

Cell Migration and Invasion Assay

Cell migration was determined by the trans-well assays performed using Hanging inserts (Millipore Co., MA, USA) in a 24-well plate. Cells were treated without or with 50 (AGS) or 75 (NCI-N87) $\mu\text{g/ml}$ BP for 24h, then 5×10^4 cells were seeded in the hanging inserts with serum-free culture medium. Culture medium supplemented with 10 % FBS was added to the bottom chamber. Incubation was carried out at 37 °C for the indicated time points (migration: 24 h, invasion: 48 h). The hanging inserts were washed with PBS, and cells on the upper filter surface were wiped away with a cotton swab. The inserts were subsequently fixed with 10 % formalin for 10 min at room temperature, stained with 0.2 % w/v crystal violet, and washed with PBS; the remaining cells were counted on the opposite side of the filter under a light microscope (Olympus CKX41 fluorescence microscope, Melville, NY, USA) at 200 X magnification. The migration cell numbers of the control group were considered as 100 %. For the invasion assay, a Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) was coated to the upper side of the hanging inserts at a concentration of 2 mg/ml. Cells were seeded onto the coated hanging inserts and followed by migration assay protocol.

Real-time RT-PCR Analysis

Real-time RT-PCR analysis was performed in triplicate in a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with Power SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 20 μl /reaction. Threshold cycle (Ct) value of each tested gene was normalized to the Ct value of the GAPDH control from the same RNA preparation. The ratio of transcription of each gene was calculated as $2^{-\Delta\text{Ct}}$, where ΔCt is the difference $\text{Ct}(\text{test gene}) - \text{Ct}(\text{GAPDH})$. Real-time RT-PCR primer sequences used in this study were: N-cadherin F-5'-ACAGTGGCCACCTACAAAGG-3', R-5'-CCGAGATGGGGTTGATAATG-3', E-cadherin F-5'-ACGTCGTAATCACCACACTGA-3', R-5'-TTCGTCACGTCTACGTGTAGAA-3', CDK1 F-5'-CACATGAGGTAGTAACACTCTG-3', R-5'-ATGGTGCCTATACTCCA-3', CyclinB1 F-5'-CGAAGATCAACATGGCAGG-3', R-5'-CTTGAGAGAGGCAGTATCAACC-3', Cyclin E F-5'-TTTGCAGGATCCAGATCAAGA-3', R-5'-CACAGACTGCATTATTGTCCCAAG-3', CDC25A F-5'-TAAGACCTGTATCTCGTGGCTG-3', R-5'-CCCTGGTTCACGTCTATCTCT-3', GAPDH F-5'-CCATGGAGAAGGCTGGG -3', R-5'-CAAAGTTGTCATGGATGACC -3'.

Animal Studies

Ethics Statement: The animal use protocol listed below has been reviewed and approved by Institutional Animal Care and Use Committee (IACUC), Hualien Tzu Chi Hospital, approval No: 104-02. All procedures were performed in compliance with the standard operating procedures and relevant guidelines of the Tzu Chi University Laboratory Animal Center (Hualien, Taiwan). Tumors were generated as described previously [25].

Statistical Analysis

All data are shown as mean $\pm$ S.D. Statistical differences were analyzed using Student's t-test for normally distributed values and by nonparametric Mann-Whitney U-test for values with a non-normal distribution. Significant differences between groups were evaluated using analysis of variance (ANOVA) with Games-Howell test as post-hoc test.

Results

Protective effect of danggui in gastric cancer patients from Taiwan

A total of 32, 279 gastric cancer patients were selected from NHIRD records from January 1, 2003 to December 31, 2013. Table S1 shows the number of patients, age, gender, and distribution of various comorbidities. After 11 years follow-up, the survival rate analyses demonstrated a strong association between the use of *danggui* and survival (log-rank test $p = 0.002$, Fig. S1). For all supplemental material see www.karger.com/doi/10.1159/000492641. Using the multivariate Cox model controlling for age, gender, income, urbanization, Charlson comorbidity index (CCIS) and region, the use of *danggui* remained highly associated with decreased mortality (1:4 matched cohort, the adjusted HR of *danggui* user was 0.72; 95 % CI, 0.57-0.92 [$p = 0.009$]) (Table 1).

Table 1. Multivariable cox proportional hazard model hazard ratios of mortality in Danggui status in gastric cancer patients

Characteristics	HR (95 % CI)	p-value
Danggui status		
No exposure	1	
Exposure	0.72 (0.57-0.92)	0.009
Age	1.00 (0.99-1.00)	0.056
Gender		
Female	1	
Male	1.34 (1.12-1.60)	0.002
CCIS	1.14 (1.10-1.17)	<0.001
Socioeconomic status		<0.001
Low SES	1	
Moderate and high SES	0.66 (0.56-0.78)	
Urbanization		<0.001
Urban	1	
Un-urban	0.52 (0.44-0.62)	
Geographic region		0.229
Northern/Central	1	
Southern/Eastern	0.89 (0.73-1.08)	

BP inhibited survival rates of human gastric cancer cells

Table 1 shows the protect effect of *danggui* for the gastric cancer patients in Taiwan. N-butylenephthalide (BP), one of the major components found in the chloroform extract fraction of *danggui*, shown to possess anticancer activity against various human cancer cells. To determine the cytotoxicity effect of BP on gastric cancer cells, 3 human gastric cancer cell lines were treated with increasing concentrations of BP for 24 and 48 h, and subsequently evaluated for cell viability using the MTT assay. As shown in Fig. 1A, all 3 gastric cancer cell lines showed obvious cell rounding up and shrinkage at 48 h after BP treatment as compared to the untreated cells. The MTT assay further demonstrated that BP significantly decreased the viability of various gastric cancer cell lines in a dose- and time-dependent manner (Fig. 1B). Treatment of AGS cells with 50 or 75 $\mu\text{g/ml}$ BP for 48 h resulted in respective cell survival rates of 49 % and 24.1 %. Treatment of NCI-N87 cells with 50 or 75 $\mu\text{g/ml}$ BP for 48 h resulted in respective cell survival rates of 65.2 % and 44.1 %. Treatment of TSGH-9201 cells with 50 or 75 $\mu\text{g/ml}$ BP for 48 h resulted in respective cell survival rates of 84.3 % and 64.6 %. The IC_{50} at 48h of BP treatment in gastric cancer cells were as follows: AGS, 51.9 $\mu\text{g/ml}$; NCI-N87, 69.6 $\mu\text{g/ml}$; and TSGH-9201, 84.1 $\mu\text{g/ml}$. Accordingly, we used 50 (AGS) and 75 (NCI-N87 and TSGH-9201) $\mu\text{g/ml}$ BP in the subsequent experiments.

Transcriptome analysis of BP-treated human gastric cancer cells

To understand how BP inhibits proliferation and promotes cell death in human gastric cancer cells, we treated AGS cells with 50 $\mu\text{g/ml}$ BP for 3 or 24 h and performed global profiling of the transcriptome of BP-treated cells with RNA-seq to identify the candidate genes induced by BP treatment. In RNA-seq analysis, we found 630 significantly up-regulated and 536 significantly down-regulated differentially-expressed genes (DEGs) in BP-treated cells compared with control cells. These DEGs were further investigated using Gene Ontology (GO) enrichment analysis, with biological processes pulled out as being significantly enriched shown in Fig. S1. We found that BP-treated cells had 15 enriched biological processes from the up-regulated genes (Fig. S2A) and 44 enriched biological processes from the down-regulated genes (Fig. S2B). Intriguingly, enriched up-regulated biological processes in BP-

treated cells are mostly involved in metabolism (e.g., carboxylic acid and organic acid transport) and cell death (e.g., cell morphogenesis, proliferation, induction of apoptosis and programmed cell death) while the enriched down-regulated biological processes in BP-treated cells are mostly related to cell cycle and nucleic acid processing. To further pull out potential biological pathways regulated by BP treatment, we used DAVID software to analyze the DEGs from the comparison of BP-treated cells to control cells by KEGG pathway analysis. We found 12 enriched pathways (with at least 3 DEGs in each pathway) in the up-regulated DEGs (Fig. 1C) and 16 enriched pathways in the down-regulated DEGs from BP-treated cells (Fig. 1D). Among these 16 pathways, the down regulation of the cell cycle pathway is consistent with the rest of our experimental results (Fig. 1E). Real time PCR verifications of some representative genes of the cell cycle including Cyclin B1, CDC25A and Cyclin E were down-regulated after BP treatment for 24h in gastric cancer cell lines. These data demonstrated that cell cycle inhibition following BP treatment might involve in BP-mediated anti-proliferation effects in gastric cancer cells.

Transcriptome analysis revealed that BP up-regulates REDD1 expression that contributes to inhibit mTOR signaling in human gastric cancer cells

Among these 12 up-regulated KEGG pathways (Fig. 1C), we found the mTOR signaling pathway is of great interest. Notably, since REDD1 is the upstream regulator of the mTOR signaling pathway and among the significant differential expressed genes (DEGs), REDD1 was nearly the top transcript at 3 and 24 h following BP treatment (respectively 23.8 and 16.1-fold of up-regulated expression). We therefore confirmed the expression of REDD1 in human gastric cancer cell lines by using quantitative RT-PCR (qRT-PCR) and western blotting analysis. We founded that BP can increase REDD1 expression in a dose-dependent fashion as high as 13.8-fold in AGS cells, 9.5-fold in NCI-N87 cells and 2.8-fold in TSGH-9201 cells (Fig. 2A). Western blotting showed that BP induced REDD1 protein expression as early as

Fig. 1. Effects of BP on human gastric cancer cells. [A] Human gastric cancer cells (AGS, NCI-N87 and TSGH-9201) were treated with 0.2 % DMSO as control or 75 µg/ml BP for 48 h; Scale bar: 50 µm. [B] Human gastric cancer cells were treated with increasing concentration of BP (12.5 to 100 µg/ml) for 24 (□) and 48 h (■), respectively, and the survival rate was evaluated with MTT assay. [C] AGS cells were treated with 50 µg/ml BP followed by RNA-seq analysis. KEGG pathway analysis of BP up-regulated and down-regulated [D] DEGs. [E] Validation (from qRT-PCR) of the KEGG cell cycle pathway that were significantly inhibited in the down-regulated genes of the comparison of BP-treatment cells to control cells. Data are presented as means ± S.D. obtained from three different experiments. ** p < 0.01 versus vehicle.

2 h after treatment in all human gastric cancer cell lines (Fig. 2B). REDD1 has been shown to inhibit mTORC1 activity through activation of the TSC1/2 complex, and the inhibition of mTORC1 will abrogate the activation of downstream targets by alleviating the phosphorylation of mTOR, p70S6K and 4EBP1. We found that BP also inhibited mTORC1 signaling by decreasing p70S6K and 4EBP1 phosphorylation as early as 2 h after BP treatment (Fig. 2B). These results demonstrate that BP-induced REDD1 expression and repressed the mTOR signaling pathway in human gastric cancer cell lines.

REDD1 involves in the BP-mediated inhibitory effect of gastric cancer cell proliferation

We further used a siRNA approach to determine the role of REDD1 in the anti-proliferation effect of BP in gastric cancer. AGS cells were transfected with siRNAs for REDD1, and then treated with or without 50 µg/ml BP for 48 h. In western blot analysis, transfection of si-REDD1 suppressed REDD1 expression induced by BP in AGS cells, as compared with cells transfected with control scrambled siRNA. The expression inhibition of REDD1 restored the phosphorylation of downstream targets including mTOR, p70S6K and 4EBP1 (Fig. 2C). Cell death induced by BP treatment was respectively rescued by 22.4 and 17.3 % in cells transfected with 20 or 40 nM REDD1 siRNA, as compared to scrambled siRNA-transfected cells (Fig. 2D). Taken together, these results indicated that BP-induced cell death might be at least partly due to the induction of REDD1 expression that down-regulated the mTOR signaling pathway.

BP induced apoptotic cell death in human gastric cancer cells

To explore the underlying mechanism in BP-induced gastric cancer cell death, human gastric cancer cells were treated with BP for 24 or 48 h and analyzed by flow cytometry (Fig. S3). The appearance of the sub-G1 cell population can be considered to indicate the extent of early apoptotic cell death. As shown in Fig. S3, the sub-G1 population increased to 9.7

Fig. 2. BP induced REDD1 expression and suppression of the mTOR signaling pathway in human gastric cancer cells. [A] qRT-PCR analysis of REDD1 in human gastric cancer cells treated with increasing concentration of BP (25 to 75 µg/ml) for 24 h. [B] Human gastric cancer cells were treated with 0.2 % DMSO (control) or BP for indicated time points, and western blot analysis was performed for REDD1 and mTOR signaling pathway-related proteins. β-actin was used as an internal control. [C] AGS cells were transfected with scramble siRNA (#), 20, or 40 nM REDD1 siRNA for 48 h using the RNAifect transfection reagent. After 48 h treatment with 50 µg/ml BP, western blot analysis was performed for REDD1 and mTOR signaling pathway-related proteins. [D] BP-induced anti-proliferative activity was measured using MTT assay in AGS cells transfected with scramble siRNA (#), 20 nM or 40 nM REDD1 siRNA for 48 h and then treated with 50 µg/ml BP for 24 h. Data are expressed as means ± S.D. from three independent experiments, ** p < 0.01 versus vehicle.

Fig. 3. BP induced mitochondrial-mediated apoptosis in human gastric cancer cells. [A] Human gastric cancer cells were analyzed by Annexin V-FITC staining in the vehicle group (0.2 % DMSO) for 3 h or at presence of 50 (AGS) or 75 (NCI-N87 and TSGH-9201) $\mu\text{g/ml}$ BP for 3, 18 and 24 h. The percentage of annexin V-FITC positive and PI negative population in gastric cancer cells is shown. [B] Human gastric cancer cells were treated with 25, 50 and 75 $\mu\text{g/ml}$ BP for 48 h, and western blot analysis was performed for cleaved PARP, caspase 9 and caspase 3. [C] Human gastric cancer cells were treated with BP for 0, 24, 48, or 72 h, and western blot analysis were performed for cleaved PARP, caspase 9 and caspase 3. [D] Human gastric cancer cells pretreated with caspase 3 inhibitor Z-DEVD-fmk (10 or 20 μM) were subjected to MTT assay for 1 h and then treated in the presence or absence of BP for 48 h. The BP-induced cell death was partly blocked after caspase 3 inhibitor pretreatment as compared to the BP alone group. The values are the mean $\pm$ S.D. from three independent experiments, * $p < 0.05$ versus vehicle, ** $p < 0.01$ versus vehicle.

% (AGS), 5 % (NCI-N87), and 6.3 % (TSGH-9201) after BP treatment for 48 h. For further demonstrate the involvement of apoptosis, human gastric cancer cells were treated with BP for indicated time points, and apoptotic cells were accessed by annexin V-FITC staining (Fig. 3A). Apoptosis was noted as early as 3 h after BP treatment; the PI-negative, annexin V-FITC positive populations denote as early apoptotic cells and increased after BP treatment as compared to the vehicle group (For BP treatment 24 h: AGS: 7.6 %; NCI-N87: 7.9 % and TSGH-9201: 4.5 %). These results suggested that BP induced apoptotic cell death in human gastric cancer cells.

BP induced mitochondrial-mediated apoptosis in human gastric cancer cells

Caspase activation plays a central role in the induction of apoptosis. To evaluate whether the growth inhibition induced by BP is due to apoptosis, we determined the crucial apoptotic signal caspase -9/3 activation in gastric cancer cell lines. Cleavages of caspase -9 and -3 increased dose- and time-dependently in gastric cancer cells after BP treatment (Fig. 3B and C). However, the activation of caspase 8 was not noted (data not shown). These results suggested that gastric cancer cells might undergo mitochondria-mediated apoptosis after exposure to BP. To further demonstrated the role of caspase 3 in BP-induced apoptosis, cells were pretreated with caspase 3 inhibitor Z-DEVD-fmk (10 or 20 μ M) for 1 h and then treated in the present or absent of BP for 48 h (Fig. 3D). The BP-induced cell death was partly blocked after caspase 3 inhibitor pretreatment (20 μ M group: 15.6 % in AGS, 14.1 % in NCI-N87, 16.1 % in TSGH-9201) as compared to the BP alone group. These data suggest that BP induced gastric cancer cell apoptosis via the mitochondria-dependent pathway. To a lesser extent, these results further confirm that the caspase-dependent pathway is involved in BP-induced human gastric cancer cell death.

Effect of BP on the migration and invasion of human gastric cancer cells

Tumor cell malignancy usually correlates to their degree of proliferation and invasion. Trans-well assays were performed to elucidate the effect of BP on the motility of gastric cancer cells. The migration and invasion of BP-treated human gastric cancer cells decreased significantly as compared to the control group in gastric cancer cells (Fig. 4A). The migrated cells accounted for 21.9 % (AGS) and 11.6 % (NCI-N87), as compared to the non-treated cells. The invasive cells accounted for 0.6 % (AGS) and 59.2 % (NCI-N87), as compared to the non-treated cells.

The modulation of epithelial-mesenchymal transition (EMT) is seen as an important target in metastatic regulation. To evaluate whether BP could affect the metastatic potential of gastric cancer cell lines, the expression levels of EMT-related genes were examined using real-time RT-PCR. BP treatment for 24 h significantly up-regulated the mRNA expressions of E-cadherin (2.1 fold in AGS and 2.2 fold in NCI-N87), whereas down-regulated the expressions of N-cadherin (0.8 fold in AGS and 0.5 fold in NCI-N87) (Fig. 4B). The EMT-related genes expression was regulated after BP treatment. The up-regulation of epithelial marker E-cadherin is only visible in AGS cells. The down-regulation of mesenchymal markers N-cadherin and Vimentin, transcription factor Slug at the protein level is evidenced by western blot (Fig. 4C). These results suggest that BP is a potential inhibitor of EMT in human gastric cancer cells.

BP suppresses tumor growth of AGS cancer cells in NOD-SCID mice

To evaluate the antitumor activity of BP *in vivo*, human gastric cancer xenografts were established by injection of 5×10^5 AGS cells into the dorsal subcutaneous tissue of NOD-SCID mice. As shown in Fig. 5A, the relative tumor volumes in mice treated with 300, 500 and 700 mg/kg BP were smaller than the vehicle-treated control mice on Day 31 (48.6 %, $P=0.02$; 41.9 %, $P=0.01$; 34.8 %, $P=0.002$, respectively). Tumors in the control and therapeutic groups were removed and photographed (Fig. 5B). Tumor weight was significantly decreased by 36.2 % (700 mg/kg, $P=0.007$) in the BP-treated groups as compared with the control group. Hematoxylin/eosin (H&E) staining revealed that BP-treated tumor specimen had dark and

Fig. 4. Effect of BP on the migration and invasion of human gastric cancer cells. [A] Human gastric cancer cells (AGS and NCI-N87) were pretreated with 0.2 % DMSO as control or 50 and 75 $\mu\text{g/ml}$ BP for 24 h and then seeded onto the trans-well hanging insert coating with (invasion) or without (migration) matrigel for different time points (migration: 24 h, invasion: 48 h). Images were captured using an inverted microscope with 200-x magnification; Scale bar: 50 μm . The migration and invasion of human gastric cancer cells were quantified by enumerating the stained cells that migrated into the underside of the hanging insert membrane. [B] Human gastric cancer cells were treated with 50 or 75 $\mu\text{g/ml}$ BP for 24 h, and the mRNA expression levels of E-cadherin and N-cadherin were determined by qRT-PCR analysis. c Human gastric cancer cells were treated with 25, 50 and 75 $\mu\text{g/ml}$ BP for 48 h, and western blot analysis was performed for EMT-related genes. Data are presented as means $\pm$ S.D. from three different experiments. * $p < 0.05$ versus vehicle; ** $p < 0.01$ versus vehicle.

dense nuclei as compared to the control group. In addition, control group tumor specimens had large and irregular nuclei and a large number of signet ring cells which are associated with a poor prognosis (Fig. 5C). Furthermore, immunohistochemistry for the proliferation marker Ki67 showed more markedly increased Ki67 expression in the control group than the BP-treated group (Fig. 5C). Upregulation of REDD1 in the BP-treatment tumor was observed by immunohistochemistry staining (Fig. 5C). Caspase-3 activation was also observed in BP treatment tumors (Fig. 5D). No significant body weight differences were found between the control and BP-treated groups. These results suggest that BP-induced gastric cancer cell death might be correlated with REDD1 expression *in vivo*. Further tumor formation experiments using the REDD1 knock-down cells are needed to establish a cause-effect relationship.

Fig. 5. BP inhibits xenographic growth of AGS cells in NOD-SCID mice. [A] Approximately 5×10^5 AGS cells were injected into the dorsal subcutaneous tissue of NOD-SCID mice. When the tumor reached 100–250 mm³, AGS tumor-bearing mice were administrated s.c. with vehicle control or 300 to 700 mg/kg BP on days 0–4 for 5 days. The relative tumor volumes of the control and the BP-treated groups are shown as means $\pm$ S.D. of tumor volume at each time point. [B] Tumors from the control and therapeutic groups were removed and weighed on Day 31. Average tumor weight from the BP-treated group (700 mg/kg) was 36.2 % smaller than that of the control group. [C] Tumor tissue sections with HE staining. Ki67 and REDD1 expressions were immuno-histochemically identified in the control and the BP-treated groups. The REDD1-positive cells were stained brown. Expression of active form caspase-3 in the AGS xenograft tumor tissue were upregulated after BP administration as compared to control group by western blotting analysis [D].

Discussion

The popularity of *danggui* in Taiwan provides an opportunity to investigate the incidence of gastric cancer with or without *danggui* exposure through the NHIRD. Given the time and cost required for large-scale clinical trials, the NHIRD presents a reasonable and viable approach for research on herbal and complementary and alternative medicine (CAM). The NHIRD suffers from several limitations including lack of information about patient lifestyle, the severity of the cancer and family history of cancer. However, previous studies have shown that the NHIRD database provides approximately 95 % consistency between patient records and claims data, indicating a high degree of validity and accuracy. A total of 32, 279 patients (both with and without gastric cancer) were selected from NHIRD records from January 1, 2003 to December 31, 2013. Table S1 shows the number of patients, ages, genders, and distribution of various comorbidities. Overall, patients with exposure to *danggui* displayed a significant decrease in mortality (adjusted HR of *danggui* user was 0.72; 95 % CI, 0.57-0.92 [$p = 0.009$]). Although NHIRD data indicates *danggui* exhibits a protective effect on gastric cancer patients in Taiwan, serious randomized, double-blind, and placebo-controlled trials are necessary to confirm this finding. In addition, to clarify the relationship between *danggui* and gastric cancer, we carried out *in vitro* and *in vivo* experiments to provide supportive evidence for the therapeutic effects of BP in gastric cancer therapy.

BP has been reported to prolong the survival of ALS mice by down-regulating autophagy and upregulating mTOR expression [26]. Combining bone marrow-derived mesenchymal stem/stromal cells (BM-MSCs) with two main components of *Radix Angelica Sinensis*, BP and sodium ferulate (SF), contribute to angiogenesis after cerebral ischemia which is associated with AKT/mTOR signaling activation [27]. Interestingly, BP induced different degrees of mTOR regulation which might explain cell or tissue specificity. Our study demonstrated that BP causes a marked increase in REDD1 expression and inhibits the mTOR signaling pathway. REDD1 plays a major role in the inhibition of mTOR activity but may also play a partial role in growth inhibition induced by BP in gastric cancer cells. The inhibition of REDD1 expression by RNA interference did not completely block the growth inhibitory effect of BP in AGS cells (Fig. 2D). BP also induced cell cycle arrest- and mitochondrial-dependent apoptosis gene

expression in gastric cancer cells. We have further demonstrated the tumor suppression potential in xenograft animal models. Taken together, these results suggest the therapeutic potential of BP in clinical applications.

REDD1 positively controls caspase-2-dependent cell death by inhibiting mTOR in ovarian cancer cells [28]. The blockade of fatty acid synthase (FASN) induced an ATF4-dependent transcriptional induction of REDD1, facilitating caspase-2 dimerization/activation and leading to ovarian cancer cell death. Interestingly, our RNA-seq data showed that the FASN gene was downregulated 1.4-fold, but ATF4 and caspase-2 were respectively upregulated 2.4 and 1.17-fold after 24 h of BP treatment in AGS cells as compared to the control cells. These data suggest that the novel REDD1-mTOR-caspase-2 pathway may be involved in BP-mediated gastric cancer cell death. In addition, REDD1 was reported to mediate mTOR inhibition and cell cycle arrest through the induction of metformin in cancer cells [29]. Similar cell cycle arrest effects were also observed in BP-treated gastric cancer cells demonstrated by real-time PCR. Taken together, REDD1 and mTOR inhibition may be one of several mechanisms involved in BP-mediated gastric cancer growth inhibition.

Previous report identified that REDD1 encodes a shared transcriptional target for p53-dependent stress response, as well as in p63-mediated regulation of epithelial differentiation [30]. In addition, suppression of mTORC1 signaling to 4E-BP1 requires the coordinated activity of two tumor suppressors, p53 and p63 [31]. BP was also shown to induce p53 expression and phosphorylation in brain tumor cells [6], which suggests that BP induced REDD1 expression via the p53 and p63 activation, which merits further investigation.

REDD1 was identified in screening for genes that are upregulated by hypoxia [32]. The hypoxia-induced inhibition of mTOR signaling requires REDD1 and TSC1/TSC2 complex [16, 33, 34]. However, this requirement is cell-type specific, for example REDD1 is not required for hypoxia-induced mTORC1 inhibition in hepatocytes [35]. Moreover, REDD1 ablation had no effect on glucocorticoid-induced mTORC1 inhibition and apoptosis in thymocytes *ex vivo* [36]. Our results showed that BP increases REDD1 expression and decreases mTOR and p70S6K phosphorylation nearly simultaneously (Fig. 2B). Furthermore, the knock-down of REDD1 only partially attenuated the BP-induced mTORC1 activation and growth inhibition (Fig. 2C). Thus, we cannot rule out the involvement of the REDD1-independent mechanism in BP-induced mTOR inhibition and apoptosis induction.

Gastric cancer genesis and metastasis is closely related to EMT. Several studies have shown that the initiation of gastric cancer and its malignancy are related to E-cadherin mutations [37, 38]. In addition, N-cadherin expression has been associated with the invasive phenotype of gastric cancer and may override the function of E-cadherin [39]. The levels of N-cadherin, Snail (Snail1), Slug (Snail2), and Vimentin, are all up-regulated in patients with dysplasia or early Gastric cancer, while the level of E-cadherin is decreased in these patients [40]. In the present study, transwell assay results showed BP suppressed the migration and invasion of gastric cancer cells *in vitro*. Since BP inhibits proliferation and induces apoptosis in gastric cancer cells, we could not exclude the possibility that the results obtained were partly affected by the impact of BP on cell proliferation/apoptosis. BP clearly up-regulated the expression of E-cadherin in AGS cells but not in NCI-N87 cells. BP down-regulated the expression of mesenchymal markers N-cadherin, Slug, and Vimentin, suggesting that BP is a potential agent to control and inhibit EMT in gastric cancer cells.

Conclusion

The present study demonstrated that *danggui* has protective effects for gastric cancer patients in Taiwan. BP inhibits proliferation and induces mitochondrial-dependent apoptosis in gastric cancer cells. BP suppresses mTOR signaling through up-regulated REDD1 expression. BP also inhibits the migration and invasion of gastric cancer cells through modulated the EMT. BP suppresses the growth of AGS xenografts tumor *in vivo*. Together, these results suggest that BP might be a potential candidate for gastric cancer therapy.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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