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Original Article

# Babao Dan, a Traditional Chinese Medicine Formula, Arrests the G1/S Phase Transition in Gastric Cancer Cells by Activating p53 Pathway

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SUMMARY

*Background:* Clinical studies and biological mechanism research showed that Babao Dan (BBD) exerts strong activity against many types of cancer, including gastric cancer (GC). In this article, the effect of BBD on proliferation of human gastric cancer cells (AGS, MGC80-3) was studied.
*Methods:* AGS and MGC80-3 cells were treated with various concentrations of BBD *in vitro*. The cell viability and survival rate were determined by the MTT assay and colony formation assay. The cell cycle analysis was measured by PI staining with flow cytometry. The level of G1/S phase check point related proteins (p53, p-p53, PCNA, Survivin, Cyclin D1, CDK4, p21) were determine by Western Blot.
*Results:* BBD inhibited AGS and MGC80-3 cells viability and decreased survival rate in a dose-dependent manner. The percentage of S-phase of AGS and MGC80-3 were found to be significant decreased in dose-dependent manner after cells were treated by different concentration BBD for 24 h. In addition, increased BBD concentration up-regulated protein p-p53, p21 level and down-regulated the expression of cell survival key protein Survivin, cell proliferation key protein PCNA and cell cycle related protein Cyclin D1, CDK4, which is closely correlated with the G1/S phase checkpoint.
*Conclusion:* BBD is likely to inhibit the proliferation of gastric cancer cells with blocking G1/S cell cycle transition by regulating the p53 pathway. Thus, BBD may become a promising agent used for GC clinical treatment.



## 1. Introduction

As a common malignant tumor of the digestive system, gastric cancer (GC) ranks second in all kinds of tumors in China,[1] especially in Fujian, the incidence ranks first. The prevalence of GC, which is considered a geriatric disease, in the elderly is significantly higher than that in the young,[2] and the median diagnostic age in China is 62.5 years.[3] Because there are not obviously clinical symptoms at the early stage, mostly diagnosed patients of GC have usually been at the middle or late stages. Up to now surgical resection is still the first choice of clinical application for a few early diagnosed GC patients.[4,5] Unfortunately, most patients are diagnosed in middle-advanced stages so that the great majority have missed the best time for surgery opportunity. The chemotherapy is also a kind of standard treatment in perioperative period but the toxic and side effects of chemotherapy drugs largely limit its application.[6–8] Traditional Chinese medicine (TCM) is becoming a kind of increasingly important treatment for GC patients whether they are in early or late stage for its less side-effects, multi-targets and whole regulation effect.

Babao Dan (BBD) is one of classical and famous Chinese patent medicine in China. According to the pharmacology theory of TCM, BBD can clean away heat and dampness, promote blood circulation and detoxicating and remove yellow and pain. In traditional Chinese medical science, the tumor occurrence, development and progression are all closely related to heat, toxic, phlegm and dampness. Encouragingly a large number of clinical studies have confirmed that BBD can not only reduce the side effects of chemotherapy drugs, improve the living standard of patients, but also prolong the life span of patients including GC. Corresponding to clinical study, some biological mechanism research of BBD also showed that it can inhibit tumor cells proliferation and enhance the sensitivity of tumor cells to the chemotherapy drugs for liver cancer, lung cancer and osteosarcoma.[9–10] Thus BBD is generally considered to be one of the potential antitumor drugs with little side-effects. However, at present, there are still unclear about the effects of BBD on the proliferation of GC cells.

Many Chinese herbal extracts have been shown to induce growth arrest or apoptosis of cancer cells via p53 activation and it was never been reported the anti-proliferation effect of BBD on p53 signal pathway in GC cells.[11–15] p53 (a kind of transcription factors) can be used as an inhibitor, which can effectively inhibit the growth

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of cancer cells, and also regulate cell apoptosis, angiogenesis, senescence, DNA repair, and cell-cycle.[16] In all most half of human cancers, the function of p53 is impaired by mutation or deletion.[17] The p53 pathway plays an important role in cell cycle.[18,19] The p53 protein activates the transcription and over-expression of p21 gene, causing cell cycle arrest, DNA replication and mitosis inhibition.[20]

The experimental conditions of cell culture *in vitro* were selected to investigate and analyze the inhibitory effects of BBD on the proliferation of GC cells (AGS and MGC80-3) and its mechanism, providing more evidence for the clinical application of BBD.

## 2. Materials and methods

All materials and methods were performed in compliance with international ethical guidelines and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

### *2.1. Materials*

The author purchased Roswell Park Memorial Institute (RPMI) 1640 Medium, fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin from Gibco (Grand Island, NY, USA). The author purchased Thiazolyl blue tetrazolium bromide (MTT) and crystal violet from Solarbio (Beijing, China). The author purchased Phosphate buffer (PBS) from Hyclone (South Logan, UT, USA). The author got cell culture consumables Bicinchoninic acid (BCA) protein assay kit, RIPA cell lysis buffer from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The author purchased Cycle Test Plus DNA Reagent kit from KeyGEN BioTECH (Nanjing, China). As for other chemical materials, if not specifically mentioned, cell culture consumables were both obtained from Nest (Wuxi, China).

### *2.2. Cell culture*

Two human GC cell lines (AGS, MGC80-3) were gotten from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). In RPMI 1640 Medium containing 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, cells were cultured at 37 °C under 5% $CO_2$.

### *2.3. Preparation of BBD*

BBD was obtained from Xiamen Traditional Chinese Medicine Factory Co., Ltd. (Xiamen, China). Before use, BBD powder should be dissolved into PBS, and the BBD solution with 25 mg/mL concentration should be prepared. After that, BBD solution was diluted in the culture medium or PBS, and then the working concentration of BBD solution was prepared.

### *2.4. MTT assay*

MTT assay can effectively detect the viability of AGS and MGC80-3 cells. Cells were seeded into 96-well plates. Its density is $1 \times 10^5$ cells/well and the volume of total medium is 100 μL. The cells were cultured at 37 °C. After 12 h, the cells were treated with different concentrations of BBD solution (with a final solubility of 0, 0.25, 0.5, 1, 1.5, 2 and 4 mg/mL) for 24 h or 48 h. Then, the medium was discarded and 100 μL MTT (0.5 mg/mL) was added into each well. At 37 °C, it was incubated for 4 h, then MTT solution was removed, and then the purple formaldehyde crystal was dissolved in 100 μL DMSO. The absorbance at 570 nm was examined by using ELISA reader (Thermo, Multiskan FC, MA, USA).

### *2.5. Colony formation assay*

AGS and MGC80-3 cells were inoculated at $2.5 \times 10^5$ cells/well density into 6-well plate. When cell density reached 50%~60%, cells were treated with different concentrations of BBD respectively (with a final solubility of 0, 0.25, 0.5 and 1 mg/mL) for 24 h. And then cells were collected and diluted in a culture medium without BBD. 1000 cells/well were seeded into culture dish in a total volume 2 mL. Replaced new culture medium every 3 days and observed the clone number. After 10 days, the colonies were fixed in 10% formaldehyde solution for 10 min, then stained with 0.01% crystal violet for 20 min, and calculated numbers under inverted microscope (DFC295, Leica, Solms, Germany).

### *2.6. Cell cycle assay*

Flow cytometry and PI staining were used to determine cell cycle. AGS and MGC80-3 cells were treated with various concentrations of BBD (0, 0.25, 0.5 and 1 mg/mL) for 24 h. After incubation, cells were separated from the culture plate with 0.1% trypsin and washed twice with cold PBS solution. Cells were collected at a density of $1 \times 10^6$ cells/mL and fixed in 70% ethanol solution at 4 °C overnight.

The cells were washed twice with cold PBS solution, and then cultured for 30 min with 500 μL PI/RNase solution as the Cycle Test Plus DNA Reagent kit (KeyGEN BioTECH, Jiangsu, Nanjing, CHINA). Then cell cycle was analyzed with flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA, USA) and data was analyzed using Modft LT 3.0 software (Verity Software House, Inc., Topsham, ME, USA).

### *2.7. Western blot*

In 5 mL medium, AGS and MGC80-3 cells ($2.5 \times 10^5$ cells/mL) were inoculated into 25 $cm^2$ flask for 24 h. After that, the cells were treated with BBD (0, 0.25, 0.5 or 1 mg/mL). After 24 h, the cells were treated with RIPA lysis buffer (Beyotime, Shanghai, China) containing protease and cocktails (both Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor for 30 min on ice. After that, the pyrolysate was centrifuged at 10,000 ×g at 4 °C for 20 min, and then the concentration of total protein was determined by the bicinchoninic acid protein assay (Thermo Fisher Scientifc, Inc., Waltham, MA, USA). A 10% SDS-PAGE method was used to separate the same amount of protein cleavage products (40 μg/lane) and then converted them onto polyvinyl dimer membranes. At room temperature, the membrane was blocked by 5% skimmed dry milk for 1 h, the primary antibodies against CDK4 (1:1,000), Cyclin D1 (1:1,000), Survivin (1:500), p21 (1:1,000), p-p53 (1:500), p53 (1:500), PCNA (1:500) and β-actin (1:5,000) were incubated overnight at 4 °C. After that, the membrane was continuly incubated for 1 h with appropriate HRP-conjugated antibodies (1:5000). The last step is to observe the target protein bands with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientifc, Inc., Waltham, MA, USA).

### *2.8. Statistical analysis*

The statistical analysis were performed by SPSS 12.0 software

(SPSS, Inc., Chicago, IL, USA). All quantitative data were represented as mean ± S.D.. Significance of the differences among various groups was statistically analyzed using one-way ANOVA and Student's t-test. p value less than 0.05 was considered with the difference statistically significant.

## 3. Results

### *3.1. BBD inhibited AGS and MGC80-3 cells proliferation in vitro*

MTT assay is used to evaluate the cell viability treated by BBD. Cells treated by BBD (0.25-4 mg/mL) for 24 h or 48 h, AGS and MGC80-3 cells viability were notablely reduced with a dose- and time-dependent manner (Fig. 1, compared with control $p < 0.01$ or $p < 0.05$). The colony formation results showed that different concentrations of BBD decreased the number of colonies at different level (Fig. 2, compared with control group, $p < 0.01$ or $p < 0.05$) which indicated that BBD downregulated the different cell lines survival rate.

### *3.2. BBD blocked AGS and MGC80-3 cell cycle in G1/S phase*

G1/S conversion is one of the two key points to regulate cell proliferation. The cell cycle was examined by flow cytometry after confirmed BBD inhibited cells viability and survival rate by MTT and colony formation assay. The results showed that the percentage of S phase of AGS and MCG80-3 cells is significantly decreased with a dose-dependent manner and the percentage of G0/G1 phase increased gradiently treated by BBD for 24 h (Fig. 3, compared with control group, $p < 0.01$). The results showed that BBD blocked AGS and MGC80-3 cell cycle in G1/S phase.

### *3.3. BBD activated p53 and modulated the G1/S phase check point related protein level in AGS and MGC80-3 cells*

In order to further determine the blocking cell cycle mechanism of BBD, western blot assay was used to detect the G1/S phase check point related protein level. BBD regulated the expression of p53, p-p53, PCNA, Survivin, Cyclin D1, CDK4, p21 in AGS (Fig. 4A) and MGC80-3 (Fig. 4B) after 24 h treatment. The total level of p53 protein was remarkable reduced and the phosphorylated protein level, p-p53, was significant increased compared with the untreated cells in a dose-dependent manner. Survivin and PCNA are key protein of cell proliferation and survival, which were down-regulated by BBD compared with the control group in a dose-dependent manner. BBD also increased p21 protein level in a dose-dependent manner, while the key protein levels of CDK4 and Cyclin D1 at G1/S phase checkpoints decreased significantly.

## 4. Discussion

Although BBD has been used to treat many different types of tumors in clinical, the specific reasons for its anti-cancer effect are not clear. The present study results confirmed that BBD inhibited the growth of GC cells *in vitro*. As we all know the most distinctive feature of cancer cells are unlimited proliferation.[21] This kind of abnormal cell growth will be accumulated and finally invade other organ

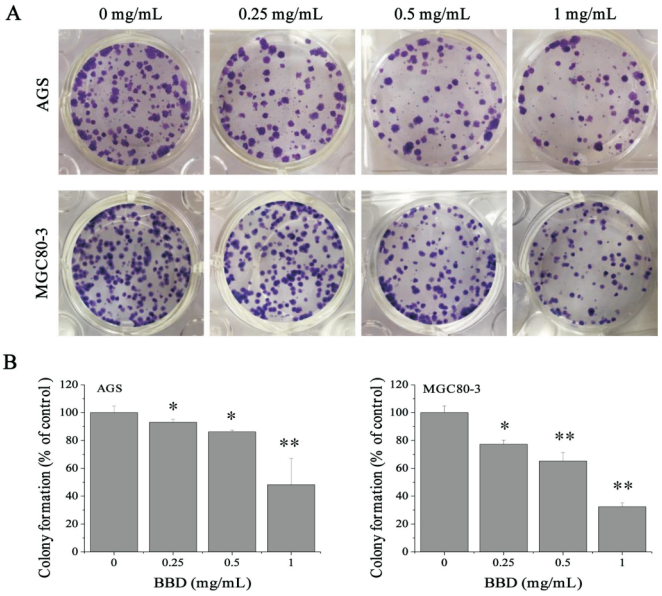


**Fig. 2.** Effect of BBD on AGS and MGC80-3 cell survival rate. AGS and MGC 80-3 cells were treated with 0 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL BBD for 24 h, respectively. Under the microscope, the colony number was calculated after 10 days. A. The photographs were magnified 40 times with a phase contrast microscope. B. Data standardization was used as a control cell colony formation. Data were obtained from means ± SD of three independent experiments. Compared with the control group, * $p < 0.05$ and ** $p < 0.01$.

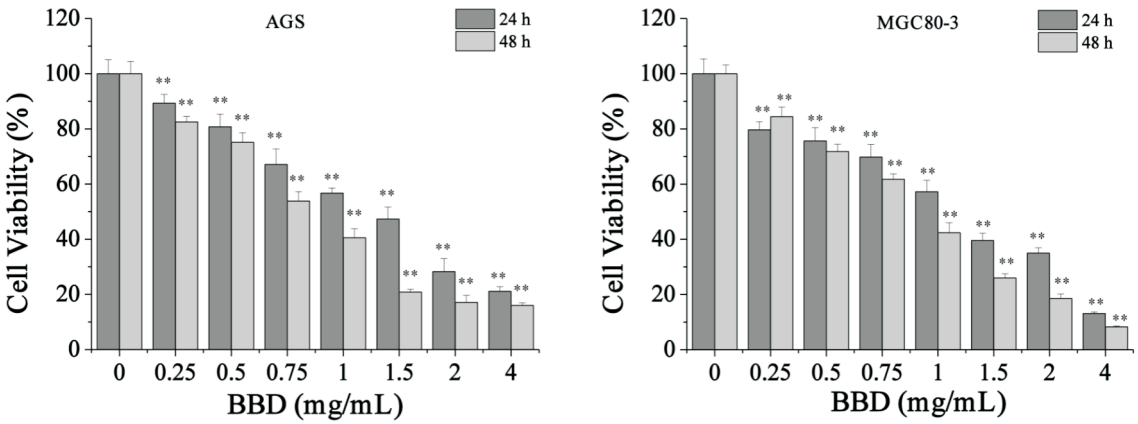


**Fig. 1.** Effect of BBD on the viability of AGS and MGC80-3 cells. MTT assay was used to detect the relative survival rates of AGS and MGC 80-3 cells after 24 h and 48 h of BBD (0, 0.25, 0.5, 1, 1.5, 2 and 4 mg/mL) treatment respectively. Data were standardized as control cells and averaged using SD (error bar) of three independent experiments. Compared with the control group,** $p < 0.01$.

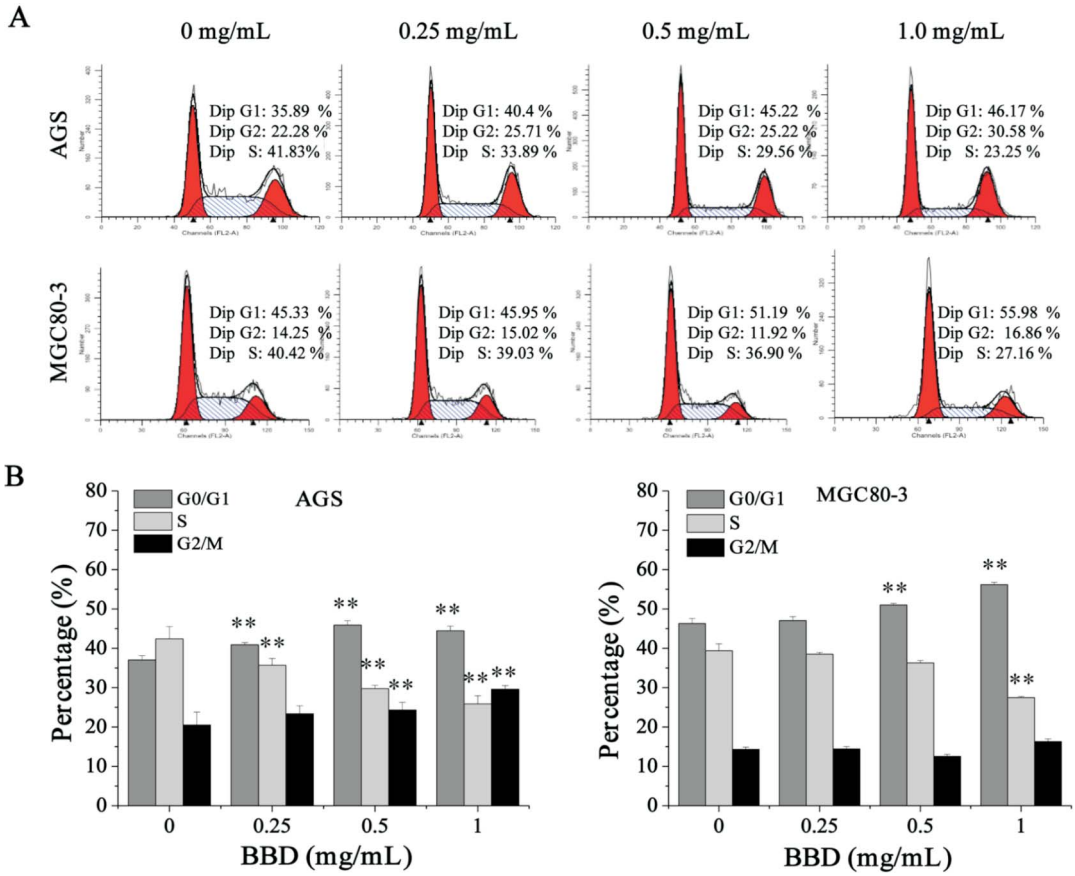


**Fig. 3.** Effect of BBD on the cell cycle blocking of AGS and MGC80-3 cells. The AGS and MGC80-3 cells were treated with different concentration of BBD (0 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL) for 24 h. The cells were stained with PI and detected by FACS. These results were obtained from three independent experiments. B. Modft LT 3.0 software was used to analyze the percentage of G0/G1 cells, S cells and G2/M cells. Three groups of independent experimental data were expressed by means ± SD. Compared with the control group, ** $p < 0.01$.

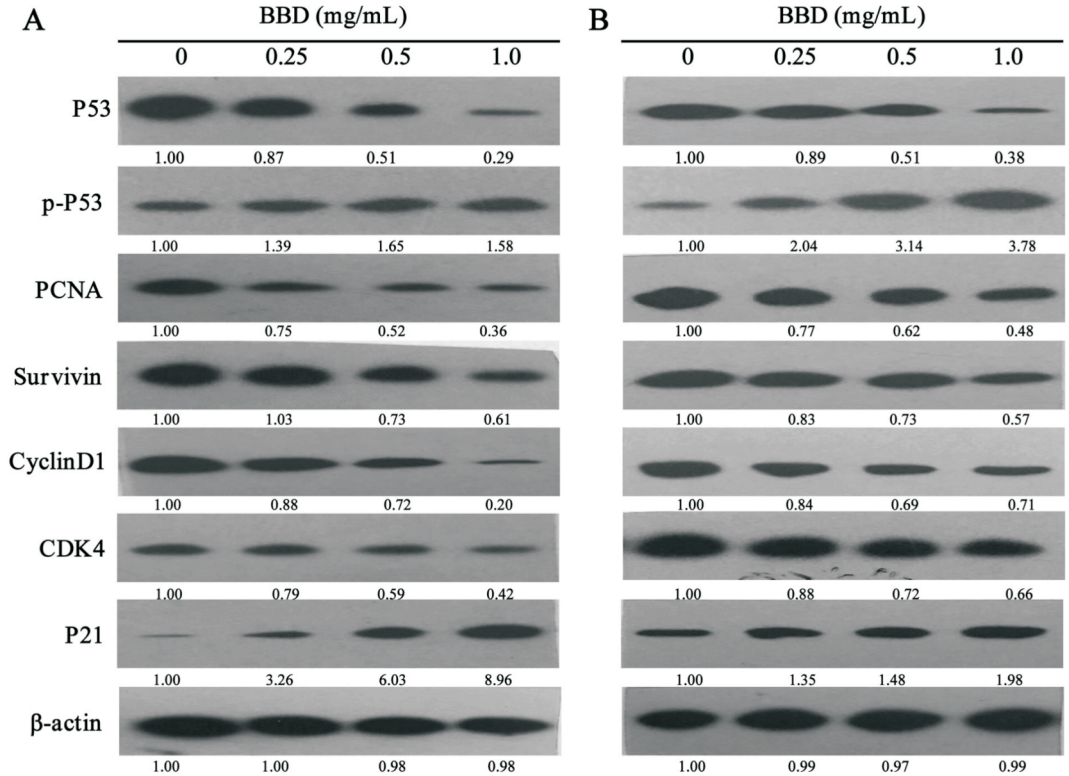


**Fig. 4.** Effect of BBD on the protein level of p53, p-P53, PCNA, Survivin, p21, CDK4, Cyclin D1 in AGS and MGC80-3 cells. The cells were treated with BBD indicated concentration for 24 h, and the protein extracted from AGS (A) and MGC80-3 (B) cells was detected by western blot. Internal control was performed with β-actin. Three individual experiments were performed.

to lead to death. Therefore, finding some new drugs that target the cell proliferation will furtherly provide some favorable helps for GC treatment in clinical. Perhaps, BBD may be a potential possibility of these drugs because BBD gradually induce the decrease of the Survivin and PCNA protein level, a kind of critical protein of cell proliferation in a dose-dependent manner. Survivin is a protein that is able to bind to p21 and block apoptosis to prevent cell death and prolong cell survival.[22] PCNA is a specific marker of cell division and a synthetic product of a short period before S phase in the cell cycle.[23]

It is important to note that BBD furtherly blocked the cell cycle transition from G1 phase to S phase at a dose-dependently manner in AGS and MGC80-3 cells. G1/S conversion is one of the key points in cell cycle, which is mainly related to the beginning and ending stages of DNA replication.[24] By using FACS and PI staining analysis, it can be confirmed that BBD blocked the process of G1/S cell cycle. Some reports indicate that p21 is the main target of p53 activity. Once p53 combined with p21, the complex can block the process of G1/S cell cycle and then inhibit the proliferation of cancer cells. The main mechanism is that p53/p21 complex directly lead to induce apoptosis and inhibit cell proliferation when cells are damaged.[25,26] Our results furtherly clarified that BBD blocked the cell cycle by activating p53/p21 protein for that the level of activated p-p53 and p21 were found evidently up-regulated with the increasing concentrations of BBD. Cyclin D1 combined with CDK4 to synthesize active complex, which can regulates cell proliferation. Its main mechanism is through phosphorylation and inhibition of pocket proteins.[27] It is well known that overexpression of Cyclin D1 and CDK4 is common in different types of cancer.[28–31] It can be concluded that the inhibitory effect of BBD on Cyclin D1 and CDK4 protein levels in AGS cells and MGC80-3 cells is the same as that on G1/S conversion. The function of p21 is to inhibit cyclin-dependent kinase, which can inhibit any Cyclin/CDK complex.[32] Therefore, the increasing p-p53 protein induced by BBD up-regulated p21 and down-regulated Survivin, Cyclin D1-CDK4 complex level and leaded to the cell cycle arrest in GC cells (as shown in Fig. 5).

From the results of this study, it can be concluded that BBD has a certain anti-tumor effect. Its main mechanism is to inhibit the process from G1 phase to S phase, and then hinder the proliferation of GC cells *in vitro*.

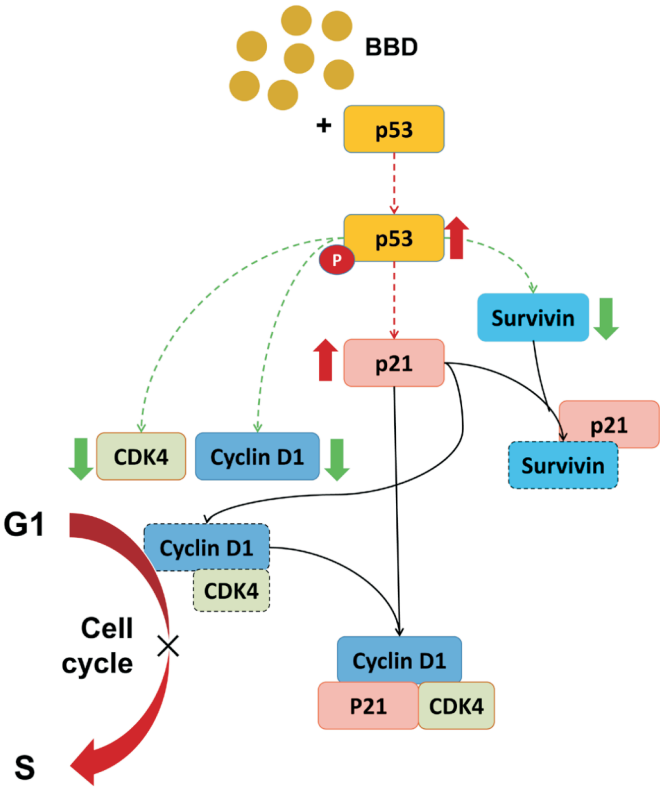


**Fig. 5.** Proposed mechanisms of action for the anticancer activities of BBD. Through the activation of p53 pathway, BBD modulates p53, p21, CDK4, Cyclin D1 and Survivin which are related to cell-cycle regulation.

## Competing interests

The authors declare that they have no competing interests.

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## References

1. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. *CA Cancer J Clin*. 2016;66(2):115–132.
2. Leung WK, Lin SR, Ching JY, et al. Factors predicting progression of gastric intestinal metaplasia: Results of a randomised trial on Helicobacter pylori eradication. *Gut.* 2004;53(9):1244–1249.
3. Wu J, Zhang JF, Dong J. Exploring the distribution of gastric cancer based on SEER database. *Science & Technology Information.* 2018;18:220–222. [In Chinese, English abstract]
4. Bastid J. EMT in carcinoma progression and dissemination: Facts, unanswered questions, and clinical considerations. *Cancer Metastasis Rev.* 2012;31(1–2):227–283.
5. Farran B, Muller S, Montenegro RC. Gastric cancer management: Kinases as a target therapy. *Clin Exp Pharmacol Physiol*. 2017;44(6):613–622.
6. Kaye SB. New antimetabolites in cancer chemotherapy and their clinical impact. *Br J Cancer.* 1998;78(suppl 3):1–7.
7. Meta-Analysis Group In Cancer, Lévy E, Piedbois P, et al. Toxicity of fluorouracil in patients with advanced colorectal cancer: Effect of administration schedule and prognostic factors. *J Clin Oncol*. 1998;16(11):3537–3541.
8. Malet-Martino M, Jolimaitre P, Martino R. The prodrugs of 5-fluorouracil. *Curr Med Chem Anticancer Agents.* 2002;2(2):267–310.
9. Chen Y, Li HL. Effect on sensitivity to cisplatin after Babaodan treatment of human lung adenocarcinoma cells A549 and SPCA-1. *Journal of Binzhou Medical University.* 2016;39(6):414–417. [In Chinese, English abstract]
10. Zhou Z, Lin JH. Proliferation inhibition and apoptosisi induction of Ba-Bao-Dan(BBD) in human osteosarcoma U-2OS cells. *Chinese Journal of Traditional Medical Traumatology & Orthopedics*. 2006;S2:93–95. [In Chinese, English abstract]
11. Zhang Y, Dong H, Li Z, et al. Bing De Ling, a Chinese herbal formula, inhibits cancer cells growth via p53. *Front Biosci (Elite Ed)*. 2010;2:221–230.
12. Vaz JA, Ferreira IC, Tavares C, et al. Suillus collinitus methanolic extract increases p53 expression and causes cell cycle arrest and apoptosis in a breast cancer cell line. *Food Chem*. 2012;135(2):596–602.
13. Liu HR, Meng LY, Lin ZY, et al. Cochinchina momordica seed extract induces apoptosis and cell cycle arrest in human gastric cancer cells via PARP and p53 signal pathways. *Nutr Cancer.* 2012;64(7):1070–1077.
14. Gao J, Morgan WA, Sanchez-Medina A, et al. The ethanol extract of Scutellaria baicalensis and the active compounds induce cell cycle arrest and apoptosis including upregulation of p53 and Bax in human lung cancer cells. *Toxicol Appl Pharmacol.* 2011;254(3):221–228.
15. Cheng YL, Lee SC, Harn HJ, et al. The extract of Hibiscus syriacus inducing apoptosis by activating p53 and AIF in human lung cancer cells. *Am J Chin Med*. 2008;36(1):171–184.
16. Shangary S, Wang S. Targeting the MDM2-p53 interaction for cancer therapy. *Clin Cancer Res.* 2008;14(17):5318–5324.
17. Chène P. Inhibiting the p53-MDM2 interaction: An important target for cancer therapy. *Nat Rev Cancer.* 2003;3(2):102–109.
18. Mazzatti DJ, Lee YJ, Helt CE, et al. p53 modulates radiation sensitivity independent of p21 transcriptional activation. *Am J Clin Oncol*. 2005;

28(1):43-50.
19. Dolan DW, Zupanic A, Nelson G, et al. Integrated stochastic model of DNA damage repair by non-homologous end joining and p53/p21-mediated early senescence signalling. *PLoS Comput Biol*. 2015;11(5):e1004246.
20. Abbas T, Dutta A. p21 in cancer: Intricate networks and multiple activities. *Nat Rev Cancer.* 2009;9(6):400–414.
21. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature*. 2001;411(6835):342–348.
22. Casati C, Dalerba P, Rivoltini L, et al. The apoptosis inhibitor protein survivin induces tumor-specific CD8+ and CD4+ T cells in colorectal cancer patients. *Cancer Res*. 2003;63(15):4507–4515.
23. Guzinska-Ustymowicz K, Pryczynicz A, Kemona A, et al. Correlation between proliferation markers: PCNA, Ki-67, MCM-2 and antiapoptotic protein Bcl-2 in colorectal cancer. *Anticancer Res*. 2009;29(8):3049–3052.
24. Nurse P. Ordering S phase and M phase in the cell cycle. *Cell*. 1994; 79(4):547–550.
25. Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science.* 1988;282(5393):1497–1501.
26. Bartek J, Lukas J. Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol*. 2001;13(6):738–747.
27. Muntean AG, Pang L, Poncz M, et al. Cyclin D-Cdk4 is regulated by GATA-1 and required for megakaryocyte growth and polyploidization. *Blood*. 2007;109(12):5199–5207.
28. Harakeh S, Abu-El-Ardat K, Diab-Assaf M, et al. Epigallocatechin-3-gallate induces apoptosis and cell cycle arrest in HTLV-1-positive and-negative leukemia cells. *Med Oncol*. 2008;25(1):30–39.
29. Kessel D, Luo Y. Cells in cryptophycin-induced cell-cycle arrest are susceptible to apoptosis. *Cancer Lett*. 2000;151(1):25–29.
30. Purohit A, Hejaz HA, Walden L, et al. The effect of 2-methoxyoestrone-3-O-sulphamate on the growth of breast cancer cells and induced mammary tumours. *Int J Cancer.* 2000;85(4):584–589.
31. Zafonte BT, Hulit J, Amanatullah DF, et al. Cell-cycle dysregulation in breast cancer: Breast cancer therapies targeting the cell cycle. *Front Biosci*. 2000;5:D938–D961.
32. Xiong Y, Hannon GJ, Zhang H, et al. p21 is a universal inhibitor of cyclin kinases. *Nature*. 1993;366(6456):701–704.