Bing De Ling®, a Chinese herbal formula, inhibits cancer cells growth via p53

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1. ABSTRACT

Bing De Ling® is a Chinese herbal formula that has been used to treat cancer patients for more than a decade. However, the molecular mechanisms behind its anti-tumor efficacy are still elusive. Here, we show that Bing De Ling® inhibits cell proliferation in ovarian cancer epithelial cell lines, OV2008 and C13. It induces G1/S arrest in a p53-dependent manner in that this effect is attenuated in OV2008 cells transfected with dominant-negative p53 plasmid. Moreover, we show that Bing De Ling® up-regulates p53 transcriptional activities as well as its downstream target genes, such as p21Cop1, MDM2, and MDMX. In addition, Bing De Ling® inhibits MDMX-p53 interaction which may result in stabilization and activation of p53. Collectively, our results suggest that the anti-tumor activity of Bing De Ling® may be in part due to activation of p53.

2. INTRODUCTION

Complementary and alternative medicines (CAM), including Chinese herbal medicine, have a long cultural history in the treatment of human diseases in the East (1). In the past few decades, CAM has become increasingly popular in the United States. It now has been a therapeutic option for cancer patients. Despite their widespread use, the molecular mechanisms underlying these herbal therapies remain elusive.

Bing De Ling®, a Chinese herbal formula, is based on modern knowledge of its ingredients' biochemical activities and traditional Chinese herbal formulatory principles to boost the body's immune responses and resistance to viral infection and to preserve homeostatic balance (2). A study from Yu's group has shown that Bing De Ling® elevates interleukin-2 (IL-2) and interferon-γ (IFN-γ) production in splenocytes and enhances...
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macrophage and lymphokine-activated killer cell cytotoxicity in a murine model (2). These data suggest a potential of using Bing De Ling® as a novel therapy for viral infections, cancer, and other disorders that may respond to immunologic stimulation. More recently, the same group has reported that Bing De Ling® enhances antimicrobial effects and ameliorates weight loss induced by the chemotherapeutic agent 5-Fluorouracil in a mouse CT26 tumor model (3). These results indicate that Bing De Ling® is a beneficial adjunctive medicine when used along with chemotherapy drugs.

During a decade-long practice, one of the authors (RJZ) has observed that some tumors cease to grow in cancer patients taking Bing De Ling®. Based on this observation, we hypothesize that Bing De Ling® is able to directly inhibit tumor cells proliferation. Bing De Ling® is composed of seven Chinese herbs among which the crude extract of Rheum palmatum possesses antiproliferative activity on eight cancer cell lines (4). Flow cytometry analysis of breast cancer cells exposed to this extract suggests that it arrests cells in the G2/M phase of the cell cycle. Emofin, a natural derivative isolated from Rheum palmatum, has been reported to interfere with the growth of several human cancers including liver and lung cancers. In lung adenocarcinomas, Emofin induces apoptosis through a reactive oxygen species-dependent mitochondrial signaling pathway (5). Emofin, also identified as a tyrosine kinase inhibitor, suppresses tumor cell migration through down-regulation of the phophatidylinositol 3-kinase (PI3K)-Cdc42/Rac1 pathway (6). In addition, this herb sensitizes HER-2/neu-overexpressing lung cancer cells to chemotherapeutic drugs and represses transformation and metastasis-associated properties of HER-2/neu-overexpressing breast cancer cells (7, 8).

However, long-term usage of single herbs such as rhubarb root from which Emofin is isolated often causes severe toxicity including stomach cramps and diarrhea unless the ratio of the herbs in the formula of Bing De Ling® is carefully adjusted. Seeking to elucidate the potential combinatorial mechanism of Bing De Ling® action in ovarian cancer, we now report that this formula drug arrests the growth of ovarian epithelial cancer cell lines OV2008 and C13 in a p53-dependent manner.

3. MATERIALS AND METHODS

3.1. Herbal medicine

Bing De Ling® solution consists of Astragalus root (Astragalus membranaceus), rhubarb root (Rheum palmatum), white astr trickles (Atractylodes macrocephala), isatis root (Isatis tinctoria), scutellaria root (Scutellaria baicalensis), dogberry (Cormus officinalis), and shield fern root (Dryopteris erawirikizoma) at a concentration of 0.1815 g/ml of water.

3.2. Cell lines and plasmids

Ovarian cancer epithelial cell lines, OV2008 and C13 were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were cultured in 199/MCDB105 medium (Sigma, St. Louis, MO) with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 5% fetal bovine serum and incubated at 37°C with 5% CO2.

Ovarian epithelial cancer cell line, OVCA23, was also obtained from ATCC, but was cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml), 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate, and 10 μg/ml bovine insulin. The breast cancer cell line MDA-MB-231 was obtained from ATCC and was cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). HCT116p53 (+/+ ) and HCT116p53 (-/-) colon cancer cell lines (kind gifts from Dr. Bert Vogelstein) were cultured in DMEM medium (Mediatech, Inc. Manassas, VA). Dominant-negative p53 (DN-p53) plasmid is a kind gift from Dr. Robert Weinberg, and BP-100 plasmid is described in Peng et al. (9).

3.3. MTT assays

Cell proliferation was evaluated by MTT assay. Briefly, cancer cells were plated in 96-well flat bottom plates at a density of 2x10³ cells/well, and after 24 h the medium was changed, then the Bing De Ling® solution was added to the wells in serial dilutions (1:5, 1:10, 1:20, 1:50). Deionized water in similar volumes was added to the control wells. At day 3, the cells were incubated in 3-(4,5-dimethylthizol-ol-x)-2, 5-diphenyltetrazolium bromide (MTT) solution for 4 h, then supplemented with 100 μl of dimethyl sulfoxide (DMSO) and shaken for 15 min. The absorbance of Bing De Ling®-exposed cultures was measured using a multi-well spectrophotometer at a test wavelength of 550 nm and a reference wavelength of 630 nm. Results were calculated as percentage of absorbance in unexposed control cultures.

3.4. Cell cycle analysis

OV2008 cells were harvested and resuspended in a single cell suspension by syringing with a 21G needle three times; aliquots of 1 x 10⁶ cells were centrifuged, resuspended in 100 μl citrate buffer and stored at -80°C prior to analysis. Cell suspensions were digested by adding 450 μl of 0.003% trypsin solution and left at room temperature for 10 minutes. To prevent further degradation 0.05% (w/v) trypsin inhibitor solution and 0.01% (w/v) RNase A were added in a final volume of 375 μl. The cell suspensions were mixed and left at room temperature for 10 min. Finally, cells were stained by adding 416 μl/ml ice-cold propidium iodide (PI)/1.16 mg/ml spermine tetrahydrochloride solution in a final volume of 250 μl and samples were left on ice in the dark for an additional 10 min prior to flow cytometry analysis.

3.5. Immunoblotting analysis

Whole-cell extracts were prepared by adding lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 20 mM Na2HPO4, 0.1% SDS, 1% Triton-100, and protease inhibitor cocktail) containing 100 mM NaCl. Protein concentrations of all samples were determined using Bradford reagent (Bio-Rad). Proteins in the supernatant were resolved on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies followed by antimouse or rabbit IgG, horseradish peroxidase (HRP)
conjugated secondary antibodies (GE healthcare) and the interested proteins were visualized by Chemiluminescent Detection Kit (Pierce). Anti-p27Kip1 (sc-528), p53 (DO-1) (sc-6243), p21Cip1 (sc-6246) antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-MDM2 monoclonal antibodies (3G9) and anti-MDMX monoclonal antibodies (8C6) were previously described (10). An anticyclin D1 antibody was purchased from BD Biosciences.

3.6. Transfection and luciferase reporter assays
All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For reporter assays, cells grown in 60 mm dishes were harvested with 300 μl of passive lysis buffer (Promega). Protein concentrations of all samples were determined using Bradford reagent (BioRad), and the relative light unit values were measured in a luminometer using the Luciferase kit from Promega. All assays were carried out in triplicates.

3.7. ELISA (enzyme-linked immunosorbent assay)
GST-MDM2 (1-150), GST-MDMX (1-200) and His6-p53 were expressed in E. coli and purified by binding to glutathione-agarose and Ni2+NTA beads under non-denaturing conditions. ELISA plates were incubated with 5 ng His6-p53 in phosphate buffered saline (PBS) for 16 hrs. After washing with PBS+0.1% Tween 20 (PBST), the plates were blocked with PBS+5% non-fat dry milk+0.1% Tween 20 (PBSMT) for 0.5 hr. GST-MDM2 (1-150) (4 nM) or GST-MDMX (1-200) (4 nM) were mixed with compounds (Bing De Ling®, pdi or nutlin, respectively) in PBSMT+10% glycerol+10 mM DTT and added to the wells. The plates were washed with PBST, incubated with anti-MDM2 antibody (4B2) or anti-MDMX antibody (8C6) in PBSMT for 1 hr, followed by washing and incubation with HRP-rabbit-anti-mouse IgG antibody for 1 hr. The plates were then developed by incubation with TMB peroxidase substrate (KPL) and measured by absorbance at 450 nm.

4. RESULTS AND DISCUSSION

4.1. Bing De Ling® induces growth inhibition in p53-positive ovarian cancer surface epithelial cells
To determine the anti-proliferative activity of Bing De Ling®, three different ovarian cancer cell lines, OV2008, OVCAR3, C13, one breast cancer cell line, MDA-MB-231 as well as colon cancer cell lines, HCT116p53 (+/+) and HCT116p53 (-/-) were treated with different dosages of Bing De Ling® for three days and MTT assays were performed (Figure 1). Among these cell lines, OV2008 and C13 were sensitive to Bing De Ling®. At 1:20 dilution or 0.009 g/ml, Bing De Ling® suppressed cell growth by half when compared to control in OV2008 cells (Figure 1A). Compared to its effect on OV2008 cells, Bing De Ling® inhibited C13 cells growth to lesser extent but this inhibition was still statistically significant (Figure 1B) while it was ineffective on OVCAR3 and MDA-MB-231 which lack functional p53 (Figure 1C and 1D). Significantly, the Bing De Ling® sensitive cell lines harbored wild type p53, while Bing De Ling® resistant cell lines lacked wild type p53 (11-13). However, Bing De Ling® did inhibit the growth of colon cancer cells HCT116p53 (+/+) and HCT116p53 (-/-) (Figure 1E and 1F), indicating that Bing De Ling®’s p53-dependent growth inhibitory effect may be specific for ovarian surface epithelial cells. A similar but lesser growth inhibitory effect of Bing De Ling® was noted on p53-positive immortalized non-tumorigenic ovarian epithelial IOSE80, IOSE121 and breast epithelial MCF-10A cells, indicating that Bing De Ling® affects tumor cells more than normal cells (data not shown). The sensitivity of cisplatin-resistant C13 cells to Bing De Ling® may have significant clinical relevance. Cisplatin is a first line chemotherapy drug on ovarian cancer but most patients eventually relapse with drug-resistant tumors (14). Therefore, Bing De Ling® may prove useful as an alternative or adjunctive agent in relapsing ovarian cancer patients.

4.2. Bing De Ling® induces G1 arrest in OV2008 cells
To determine whether Bing De Ling®-induced growth inhibition is due to apoptosis or cell cycle arrest, we performed flow cytometry analysis (FACS). Briefly, Bing De Ling® was added to the OV2008 cells at a concentration of 0.009 g/ml in cell culture medium for three days. Cells were then harvested for cell cycle analysis (Figure 2). From the above results, Bing De Ling® increased G1 phase of OV2008 from 26.98% to 40.38%, reduced S phase from 62.19% to 54.03% and G2/M phase from 10.83% to 5.39%. No sub-G1 population was detected in the analysis suggesting that apoptosis was not involved (Figure 2B). To assess whether Bing De Ling®-induced G1 arrest also occurs in other types of tumors cells, HCT116p53 (+/+) and HCT116p53 (-/-) colon carcinoma cells were exposed to Bing De Ling® at a concentration of 0.009 g/ml in cell culture medium for three days. Post Bing De Ling® treatment, both cell lines showed marked G2/M but not G1 arrest, suggesting that Bing De Ling® may have a different impact on different cell types (data not shown).

4.3. Bing De Ling® regulates the protein levels of cell cycle-related genes
To examine the mechanisms by which Bing De Ling®-induced G1 arrest, we set out to determine whether Bing De Ling® alters the expression levels of proteins which control the cell cycle. The increased levels of cyclin/cdk inhibitor p27Kip1 are a hallmark for G1 arrest while the up-regulation of G1 cyclin, cyclin D1, is an indicator for cells entering the cell cycle. As shown in Figure 3A, levels of p27Kip1 were dramatically increased on day 5 post Bing De Ling® treatment and remained sustained on day 6, while levels of cyclin D1 gradually decreased after Bing De Ling® treatment. A decrease in cyclin D1 levels associated with an increase of p27Kip1 supports the notion that Bing De Ling® induces G1 arrest in OV2008 ovarian cancer cell line. Consistent with these observations, Scutellaria baicalensis, one of the components of Bing De Ling®, suppresses expression of cyclin D1 in LNCap cells, resulting in G1 phase arrest (15). In addition, along with other cyclin-dependent kinase inhibitors such as p16INK4a and p21Cip1, p27Kip1 is regarded as a key effector of cellular senescence and whether Bing De Ling® plays a role in cancer cells senescence warrants further investigation (16). In addition, Bing De Ling® also
regulates cell cycle-related genes, cyclin D1 and p27kip1 in p53 deficient HCT116p53 (-/-) cells (Figure 3B), which may account for Bing De Ling®-mediated cell cycle arrest in this cell line. However, the mechanisms by which Bing De Ling®-induced growth inhibition in cells lacking p53 remain elusive.

4.4. Bing De Ling® upregulates p53 protein levels, its transcriptional activities and its downstream target genes

Because both OV2008 and C13 cell lines harbor the wild type p53 and their growth was suppressed by Bing De Ling®, we hypothesized that Bing De Ling® induces cell cycle arrest via p53. To this end, we set out to detect the p53 protein levels and its downstream target genes after Bing De Ling® treatment. Consistent with this hypothesis, p53 and its down-stream target genes such as p21cip1 were sharply increased in both OV2008 and C13 cells after Bing De Ling® treatment (Figure 4A and 4B). Apart from p21cip1, the levels of another p53 downstream gene, murine double minute (MDM2), were increased in C13 cells but not in OV2008 cells, suggesting that p53 transcriptional activity may be regulated differently in these two cell lines (Figure 4A and data not shown). We also examined another
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**Figure 2.** Bing De Ling® induces G1 arrest in OV2008 cells. OV2008 cells were treated with or without Bing De Ling® at a concentration of 0.009 g/ml in culture medium for 3 days and cell cycle profiles were determined by flow cytometry. A) Table showing cell cycle distribution. B) Representative flow cytometry profiles.

p53 downstream gene Gadd45, which mediates p53-dependent G2/M arrest (17). As expected, no induction of Gadd45 levels was observed in Bing De Ling®-treated OV2008 cells, suggesting that Bing De Ling®-induced cell cycle arrest via p53/p21Cip1 rather than p53/Gadd45 signaling (data not shown). To validate our conclusion that Bing De Ling® activates p53, OV2008 cells were transfected with BP100, a plasmid harboring a luciferase gene driven by a promoter containing p53 binding sites (9). Six hours post transfection, OV2008 cells were treated with 0.009 g/ml Bing De Ling® for 24 hrs and luciferase activities were determined by luciferase assay. Bing De Ling® enhanced the luciferase activity by two fold suggesting that it up-regulated p53's transcriptional activity (Figure 4C).

It is well established that p53 exerts its function in G1 arrest via p21Cip1 (18). It is conceivable that the inhibitory effect of Bing De Ling® on ovarian cancer cells may be partly due to *Rheum palmatum* since this herb exhibits antiproliferative properties through p53 and its downstream p21Cip1 pathway (19).

**4.5. Bing De Ling® induces growth inhibition in OV2008 ovarian carcinoma cells via p53**

To further determine whether Bing De Ling®-induced cell cycle arrest through p53, OV2008 cells were transfected with a dominant-negative p53 (DN-p53) construct and cells were selected with 50 μg/ml hygromycin to enrich the cell population harboring DN-p53 (DN-p53 plasmid contains a hygromycin resistance gene) for 7 days. Cells were then treated with Bing De Ling® at 0.009 g/ml for 4 days and flow cytometry was performed to determine the cell cycle distribution (Figure 5). Upon Bing De Ling® treatment, cells transfected DN-p53 did not increase the G1 population (53.42% vs. 52.54%) at variance with control cells (51.80% vs. 60.74%). Bing De Ling®'s growth inhibitory effect was attenuated in OV2008 cells with DN-p53, suggesting that growth suppression by Bing De Ling® requires p53.

**4.6. Bing De Ling® disrupts MDMX-p53 interaction in vitro and in vivo**

MDM2 and MDMX negatively regulate the stability and transcriptional activity of p53 by binding to its N-terminus (20). MDM2 possesses E3 ligase activity and
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Figure 3. Bing De Ling® regulates expression of cell cycle-related genes. A) OV2008 cells were treated with Bing De Ling® with the indicated concentration for 0, 1, 2, 3, 4, 5 or 6 days. Cells were lysed and Western blotting analyses were performed with the indicated antibodies. Anti-β-actin Western blotting analysis was carried out to ensure equal loading of proteins. B) HCT116p53 (+/+) and HCT116p53 (-/-) cells were treated with 0.009 g/ml Bing De Ling® for 0, 1, 2 or 3 days. Cells were lysed and Western blotting analyses were performed with anti-p27*kip1, anti-cyclin D1 or anti-β-actin antibodies.

Figure 4. Bing De Ling® upregulates p53 protein levels and activates transcription of its downstream target genes. A) OV2008 cells were left untreated or treated with Bing De Ling® for 1, 2 and 3 days. Cells were then harvested and Western blotting analyses were performed with the indicated antibodies. B) C13 cells untreated or treated with Bing De Ling® for 1, 2 and 3 days. Western blotting analyses were performed with the indicated antibodies. C) OV2008 cells were transfected with empty vector or BP100-luc. Six hour post transfection, Bing De Ling® was added to the culture medium and 24 hour after Bing De Ling® treatment, luciferase activities were measured by a luciferase assay kit (Promega).
promotes p53 turnover. Unlike MDM2, MDMX does not harbor intrinsic E3 ligase capability. MDMX may inhibit p53 transcriptional activity by formation of inactive MDMX-p53 complexes and both MDM2 and MDMX may cooperatively inactivate p53 (21, 22). Regulation of MDMX expression appears important to ensure a proper p53 response under stress conditions, at least following genotoxic stress. Treatment of cells with ultraviolet radiation (UV)-C, ionizing radiation (IR) or adriamycin results in degradation of MDMX (23, 24). As shown in Figure 4B, in C13 cells, upon Bing De Ling® treatment, MDMX levels were decreased. These data indicate that Bing De Ling® may act as a DNA-damaging agent and down-regulate MDMX levels which in turn activate p53.

It is intriguing that the levels of MDM2, an E3 ligase, that promotes the degradation and nuclear export of p53, are also increased along with the levels of p53 in C13 cells (25-28) (Figure 4B). As it appears that MDM2-mediated p53 turnover is blocked, one possibility is that Bing De Ling® inhibits MDM2-p53 interaction. To test this idea, we performed ELISA assay to examine the ability of Bing De Ling® to disrupt the binding of MDM2-p53. As shown in Figure 6A, the IC50 for inhibiting MDM2-p53 binding is ~2 mg/ml. We also examined the capability of Bing De Ling® to disrupt MDMX-p53 interaction. As shown in Figure 6B, the IC50 for inhibiting MDMX-p53 binding is ~1 mg/ml. Because Bing De Ling® is a formula drug, the concentration of the active compound may be very low. We thus conclude that Bing De Ling® inhibits MDM2-p53 and MDMX-p53 binding which in turn safeguards p53 against MDM2-mediated degradation and activates p53. As positive controls, a small-molecule MDM2 inhibitor, nutlin 3, and a newly identified high-affinity MDM2 and MDMX binding peptide, pDI, were used in the ELISA assays (17, 29). As expected, nutlin 3 suppressed the MDM2-p53 binding but not MDMX-p53 binding, while pDI disrupted both MDM2-p53 and MDMX-p53 binding (Figure 6C and 6D).

To determine whether Bing De Ling® disrupts MDM2-p53 or MDMX-p53 interaction in cells, C13 cells were treated with vehicle control (H₂O) or Bing De Ling® (0.009 g/ml) for two days. Because the levels of p53, MDM2 and MDMX are dramatically changed by Bing De Ling® in C13 cells (Figure 4B), the proteasome inhibitor, MG132, was added to the culture medium 6 hr prior to harvest to ensure minimal changes in the levels of the
**Figure 6.** Bing De Ling® inhibits MDMX-p53 interaction. Bing De Ling® was tested in an ELISA assay of GST-MDM2 and GST-MDMX binding to immobilized His-p53 (panels A and B). As controls, nutlin 3 and pDI were also tested for their ability to disrupt MDM2-p53 and MDMX-p53 interaction (panels C and D). E) C13 cells were treated with vehicle control or Bing De Ling® (0.009 g/ml) for 2 days. MG132 (10 µM) was added to the culture medium 6 hr prior to harvest. P53 was immunoprecipitated by anti-p53 antibodies (mAb1801, generated by J. Chen) followed by immunoblotting with anti-MDMX antibodies. The levels of input of MDMX and p53 in vehicle control or Bing De Ling®-treated samples were indicated by direct Western blotting analysis. Anti-β-actin Western blotting analysis was also performed to ensure the equal loading of proteins.
above proteins. P53 proteins were immunoprecipitated with anti-p53 antibodies and MDM2 or MDMX bound to p53 were detected by Western blotting analysis using anti-MDM2 or anti-MDMX antibodies. Our results show that MDMX-p53 interaction is abrogated by Bing De Ling® treatment (Figure 6E), while MDM2-p53 interaction seems not to be affected by Bing De Ling® (data not shown). Therefore, it is conceivable that Bing De Ling® activates p53 by inhibition of MDMX-p53 interaction.

Collectively, our study demonstrates that Bing De Ling® has an anti-proliferative effect on p53-positive ovarian epithelial cancer cells, OVC2008 and C13. Rather than inducing apoptosis, Bing De Ling® arrests cells in G1 and this effect is p53-dependent. Moreover, Bing De Ling® activates p53 transcriptional activities and up-regulates p53 downstream target genes. In addition, Bing De Ling® may activate p53 by down-regulating MDMX levels and disruption of MDMX-p53 binding. Our study thus identifies a molecular mechanism that underlies the Bing De Ling®-induced anticancerogenic effect and suggests a complementary therapeutic role for this compound in ovarian epithelial cancer.

5. ACKNOWLEDGEMENTS

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6. REFERENCES


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**Abbreviations:** DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; EGTA: ethylene bis (aminomethylene)-N,N,N′,N′-tetraacetic acid; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HER-2: Human Epidermal growth factor Receptor 2; Rac1: ras-related C3 botulinum toxin substrate 1; Cdc42: cell division cycle 42

**Key Words:** Ovarian Cancer, p53, Cell Cycle, MDMX.

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