KSP inhibitor SB743921 inhibits growth and induces apoptosis of breast cancer cells by regulating p53, Bcl-2, and DTL

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Kinesin spindle protein (KSP) is a microtubule-associated motor protein that is specifically expressed by mitosis cells. It is highly expressed in various types of tumors including hematolymphomas and solid tumors. Chemical KSP inhibition has become a novel strategy in the development of anticancer drugs. SB743921 is a selective inhibitor for KSP, which is a mitotic protein essential for cell-cycle progression. Although SB743921 has shown antitumor activities for several types of cancers and entered into clinical trials, its therapeutic effects on breast cancer and mechanisms have not been explored. In this study, we tested the antitumor activity of SB743921 in breast cancer cell lines and partly elucidated its mechanisms. KSP and dendriticless E3 ubiquitin–protein ligase homolog (DTL) are overexpressed in breast cancer cells compared with noncancer tissues. Chemical inhibition of KSP by SB743921 not only reduces proliferation but also induces cell-cycle arrest and leads to apoptosis in breast cancer cells. Treatment of MCF-7 and MDA-MB-231 breast cancer cell lines with SB743921 results in decreased ability of colony formation in culture. SB743921 treatment also causes a KSP accumulation in protein level that is associated with cell arrest. Furthermore, we showed that SB743921 treatment significantly reduces the expression of bcl-2 and cell cycle-related protein DTL, and upregulates p53 and caspase-3 in breast cancer cells. Taken together, these data indicated that SB743921 can be expected to be a novel treatment agent for breast cancers. Anti-Cancer Drugs 27:863–872

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Introduction

Cytotoxic chemotherapy and targeted therapy are the standard procedures for the treatment of breast cancer after surgery [1,2]. Several chemotherapy agents and target drugs such as trastuzumab, tamoxifen, rastuzumab emtansine, inhibitors targeting phosphatidylinositol 3-kinase, mammalian target of rapamycin, and cyclin-dependent kinase (CDK) have been developed successfully and used widely in clinical therapy for breast cancers [3–7]. However, the 5-year survival rates of patients with advanced breast cancer at diagnosis are still low. Thus, novel drug development may provide more treatment options for patients with advanced breast cancer.

The spindle plays an essential role in mitosis, which is a highly regulated process during cell division. Kinesins are ATP-dependent mitotic motor proteins that play essential roles in mitotic spindle function and are potential targets in the novel antimitotic cancer therapeutics [8,9]. Kinesin spindle protein (KSP), also known as Eg5, is a homotetrameric Bim C/kinesin-5 family member that facilitates centrosome separation as well as bipolar spindle assembly and elongation and is specifically expressed by mitosis cells [10,11]. Its aberrant expression has been found in a variety of tumors such as lymphoma, leukemia, and solid tumors [12,13]. Chemical inhibition of KSP has emerged as a novel and validated therapeutic strategy against cancers and provides new opportunities for the development of anti-breast cancer therapeutics [14]. Several new KSP inhibitors have been identified in recent years and, some of these, such as ispinesib (SB-715992), ARRY-520, SB743921, SB743921, and MK-0731, have entered into clinical trials for the treatment of myeloid leukemia and solid tumors.
such as hepatocellular carcinoma [15–19]. SB743921 is a novel KSP inhibitor that disrupts functional mitotic spindle formation in cell mitosis by hydrolyzing ATP [12]. It showed a strong inhibitory effect on wide-spread tumors such as leukemia, multiple myeloma, lymphoma, and solid tumors [12,13,19,20]. Furthermore, SB743921 also has the ability to overcome chemoresistance of CML cells by suppressing ERK and AKT activity [13]. KSP inhibitors have become promising antitumor agents in various types of cancers; however, little is known about KSP expression in breast cancers and the potential of KSP inhibitors in the treatment of breast cancer patients.

Multiple classes of regulatory molecules, such as cyclins and CDKs, determine the cell-cycle progression. Several transcription factors, such as P53, function as inducers or inhibitors of the CDKs. P53-induced expression of p21 (WAF1/CIP1/Sdi1) leads to G1 arrest and apoptosis [21]. Eukaryotic cell-cycle transitions are driven by specific protein targets, which are regulated by E3 ubiquitin ligase-catalyzed ubiquitylation [22]. DTL is a ubiquitin–protein ligase complex, also called the CRL4 (CDT2) complex, that mediates the polyubiquitination and subsequent degradation of cell-cycle regulators such as cyclin CDT1, CDKNA/p21(CIP1), and SETD8 [23,24]. Given the important roles of DTL in cell-cycle control, DNA damage response, and DNA synthesis, we hypothesize that SB743921 disrupts cell cycle, which might alter the expression levels of P53 and DTL gene besides targeting KSP protein. In this work, we investigated the cytotoxic effects of SB743921 on breast cancer cells and its effects on bcl-2, DTL, and P53 gene expression.

Materials and methods

Cell lines and chemicals

Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and maintained in DMEM medium supplemented with 10% fetal bovine serum and 2 mmol/l L-glutamine. Both cell lines were cultured in a monolayer in a 37°C incubator and 5% with 100% humidity. SB743921 (Selleck Chemicals, Houston, Texas, USA) were dissolved in DMSO to a concentration of 1 mmol/l and stored at −20°C. The tumor specimens from nine breast cancer patients were obtained according to protocols and ethical requirements approved by the Institutional Review Board at Changhai Hospital. All patients (ranging in age from 37 to 70 years) were diagnosed with invasive ductal carcinoma at II or III stages. Specimens were obtained immediately after surgical resection and the noncancerous tissue was dissected under a microscope and stored at −80°C for further analysis.

Real-time quantitative PCR

The mRNA level of KSP, DTL, caspase-3, and Bax of breast cancer cells were determined by real-time reverse-transcription PCR analysis. Briefly, total RNA was isolated using the RNEasy method according to the manufacturer’s protocol [25]. Total RNA (2 µg) from each sample was subjected to reverse transcription using the superscript first-strand cDNA synthesis kit (Thermo Scientific, Waltham Massachusetts, USA) according to the manufacturer’s instructions. Real-time PCR reactions were then carried out in a total of 15 µl reaction mixture: 2.5 µl of cDNA, 7.2 µl of 2× SYBR Premix Ex Taq [TaKaRa Biotechnology Co. Ltd (Dalian, China)], 0.3 µl of ROX-IL, 1.0 µl of each 10 pmol/l forward and reverse primers, and 4.0 µl of H₂O. The PCR program was initiated by 30 s at 95°C before 40 thermal cycles, each for 3 s at 95°C and 30 s at 60°C. Data were analyzed using the comparative Cₐ method and were normalized by β-actin expression in each sample. The sequences of PCR primers for KSP, DTL, caspase-3, and Bax are listed in Table 1.

Table 1 Primers used in this study

| Gene   | Primers                                                                 |
|--------|-------------------------------------------------------------------------|
| β-Actin| Sense 5'-CGGGGAAATCGTGCCTGAC-3'                                         |
|        | Antisense 5'-GGAGAGGGGCGAGGGAGGAG-3'                                   |
| Ksp    | Sense 5'-TCTGGAACAGGATCTGAAACTGGA-3'                                   |
|        | Antisense 5'-CCTGTTGCTAGTTCTCAACGATGTTG-3'                             |
| DTL    | Sense 5'-AACCAAGACCACTACCATGGCTTT-3'                                   |
|        | Antisense 5'-GGAGATGGGTAGGGATACAAAC-3'                                 |
| Caspase-3| Sense 5'-TCTCCTACCCTGCTGTTAC-3'                                       |
|        | Antisense 5'-AATAAGGACCCCTCCCTCATACA-3'                                |
| Bax    | Sense 5'-GCCACGTTGCCGTCGTCAC-3'                                       |
|        | Antisense 5'-GCCACGTTGCCGTCGTCAC-3'                                   |

Colonizing assay

Breast cancer cell line MCF-7 and MDA-MB-231 cells were trysinized, washed, and suspended in culture medium. A total of 2000 cells were seeded in triplicate in six-well plates. Cells were incubated for 7 days at 37°C under a 5% CO₂ atmosphere, the colonies were stained with Giemsa (Solarbio, Beijing, China), and colony numbers were counted.

Cell-cycle analysis

The MCF-7 and MDA-MB-231 cells were treated with SB743921 at different concentrations. After culture in a 5% CO₂ atmosphere at 37°C for 24 h, cells were trysinized and PBS was washed and then fixed in ice-cold 70% ethanol. Cells (1 × 10⁶) were stained with a propidium iodide solution (20 µg/ml propidium iodide) and DNA content data were acquired on a FACS Caliber and analyzed using the Modifit software package (CBD Company, Franklin Lakes, New Jersey, USA).

Apoptosis assay

The MCF-7 and MDA-MB-231 cells were treated with different concentrations of SB743921 for 24 h. Both nonadherent and adherent cells were collected and washed. Then, cells were stained using the Annexin V Apoptosis Kit (eBioscience, San Diego, California, USA) according to the manufacturer’s instructions. Briefly, 1 × 10⁶ cells were resuspended in 100 µl of 1× binding
buffer with 5 μl Annexin V-FTTC. After incubation at room temperature for 20 min, samples were stained by propidium iodide and detected by flow cytometry within 1 h.

CCK-8 assays for cell proliferation
The MCF-7 and MDA-MB-231 cells were trysinized, washed, and seeded in a 96-well culture at a concentration of 2 × 10³ cells/well. These cells were treated with SB743921 at different concentrations. After incubation for 0, 24, 48, 72, and 96 h, the supernatant was removed and cell viability was detected using Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Absorbance at 450 nm was measured using a microplate reader. The proliferation assays were performed independently and repeated at least three times.

Western blot assay
The cells were treated with SB743921 at different concentrations. After washing with ice-cold PBS, protein was extracted from cells by suspending in RIPA buffer (1× PBS, 1% Nonidet NP-40, 0.1% SDS) containing a cocktail of protease inhibitors for 30 min on ice. Lysates were clarified by centrifugation and assayed for protein concentration using the Pierce BCA Protein Assay kit (Thermo Scientific). Total protein (30 μg) in cell lysates was separated by electrophoresis on a SDS gel, transferred to a polyvinylidene difluoride membrane, and blotted with 5% milk in TBS Tween 20 (TBS-T). The membranes were incubated with primary antibodies anti-KSP (1 : 1000), anti-P53 (1 : 1000), anti-Bax (1 : 1000), and antitubulin (1 : 1000) and subsequently incubated with secondary antibodies conjugated with peroxidase. The signal was then detected using the chemiluminescence detection system.

Statistics
Data obtained from multiple experiments were reported as the mean ± SEM. Significance levels were determined using Student’s t-test and analysis of variance.

Results
KSP and DTL are overexpressed in breast cancer cell lines
To explore the possibility of KSP inhibition in the treatment of breast cancer cells, we determined the KSP mRNA level in tumor tissues from breast cancer patients and T47D, MCF-7, and MDA-MB-231 breast cancer cell lines using quantitative PCR. The KSP mRNA was significantly increased in breast cancer tissues compared with adjacent noncancerous tissues (Fig. 1a). The cell line MDA-MB-231 with highly aggressive and metastatic characteristics [26] showed the highest levels of KSP expression (Fig. 1b). DTL is a substrate-specific adapter of a DCX (DDB1-CUL4-X-box) E3 ubiquitin–protein ligase complex that is required for the cell-cycle process [27]. We further checked the DTL expression in breast cancer tissues and cell lines. The DTL is upregulated in primary breast cancer tissues (Fig. 1c) and cell lines (Fig. 1d). It is indicated that both KSP and DTL are highly expressed in breast cancer cells and may be considered targets for breast cancer therapy.

SB743921 inhibits the proliferation of breast cancer cells
SB743921 is the specific inhibitor of KSP and currently in clinical trials for the treatment of several types of tumors. The toxicity effects of SB743921 on breast cancer cells were evaluated in vitro using a CCK-8 assay. Treatment of breast cancer cell lines MDA-MB-231 (Fig. 2a) and MCF-7 (Fig. 2b) with SB743921 results in significantly decreased growth. It is indicated that SB743921 has a concentration-dependent antitumor activity in MDA-MB-231 and MCF-7 cells. Colony formation represents the growth ability of breast cancer cells. The colony formation assays were performed to evaluate the long-term effect of SB743921 on the growth of breast cancer cell lines. Following 7 days of SB743921 treatment, very few colonies of MDA-MB-231 (Fig. 2c and d) and MCF-7 cells (Fig. 2e and f) were observed. This highlighted the antitumor effects of SB743921 on in-vitro colony-forming ability of breast cancer cells.

SB743921 induces cell-cycle arrest and apoptosis in breast cancer cells
The KSP protein is specifically expressed by mitosis cells. To investigate the effect of SB742921 on the cell-cycle process and the survival of breast cancer cells, the MDA-MB-231 and MCF-7 cells were exposed to SB743921 at a concentration of 0, 1, and 5 nmol/l for 24 h and assayed for cell-cycle distribution. The cell-cycle analysis showed that the G0/G1 phase was decreased and the G2/M phase was significantly increased in both MDA-MB-231 (Fig. 3a and b) and MCF-7 (Fig. 3c and d) cells treated with SB743921. SB743921 induced a concomitant accumulation of cells in the G2/M phase.

The apoptosis-promoting activity of SB743921 was further evaluated in both MDA-MB-231 and MCF-7 breast cancer cell lines. The apoptosis of SB743921-treated cells was determined through DAPI staining and flow cytometry detection. As shown in Fig. 3, the treatment of MDA-MB-231 (Fig. 3e) and MCF-7 (Fig. 3f) cells with SB743921 resulted in significantly increased apoptosis. The morphological changes in MDA-MB-231 (Fig. 3f) and MCF-7 (Fig. 3b) cells treated with SB743921 showed apoptotic characteristics such as the presence of apoptotic bodies, cell shrinkage, and nuclear condensation. It is suggested that SB743921 induced G2/M cell-cycle arrest, followed by apoptosis in MCF-7 cells.

SB743921 downregulates DTL and induces P53 expression in breast cancer cell lines
To determine the mechanisms by which SB 743921 induces apoptosis and cell arrest in breast cancer cells, we
detected the expression of cell-cycle regulators such as P53, caspase-3, KSP, and DTL in MCF-7 and MDA-MB-231 cells treated with SB743921. Treatment of MDA-MB-231 (Fig. 4a and b) and MCF-7 (Fig. 4e and f) cells with SB743921 results in an increase in cell arrest that is accompanied by KSP accumulation. As Bcl-2 is overexpressed in most breast cancers and acts as a survival factor in the regulation of apoptosis, whereas Bax belongs to the BCL-2 protein family and acts as an antiapoptotic regulator in breast cancers [28]. Thus, it is of interest to determine whether they are influenced by SB 743921 in breast cancer cells. SB743921 treatment decreases BCL-2 and increases Bax expression in protein levels of MDA-MB-231 (Fig. 4a) and MCF-7 (Fig. 4e) cells. SB743921 treatment also induces P53 expression in MDA-MB-231 cells (Fig. 4a). Supportively, the apoptosis-related molecule caspase-3 was upregulated (Fig. 4c and g). DTL expression is elevated in primary breast cancer tissues and in proliferative cancer cells. For detailed mechanism studies, the mRNA levels of cycle regulator DTL were further evaluated in SB743921-treated breast cancer cells. We confirmed that SB743921 treatment decreased DTL expression in both MDA-MB-231 (Fig. 4d) and MCF-7 cells (Fig. 4h). Given its critical role of DTL in the regulation of cell-cycle progression, its downregulation might contribute toward SB743921-induced cell-cycle arrest.

**Discussion**

Chemical KSP inhibition has attracted considerable attention in the search for novel therapeutic drug candidates in anticancer therapy [29]. Accumulating evidence has shown that KSP was expressed by mitosis cells and associated with malignancy as well as drug resistance of tumors [13,14]. Previous studies have reported that KSP/Eg5 is highly expressed in various types of tumors, including hematomalignances and solid tumors [30–32]. In this study, using real-time reverse-transcription PCR,
we showed that high expression of KSP was detected in breast cancer tumor tissue. We also found that the inhibition of KSP by SB743921 impairs cell-cycle progression and induces apoptosis and cell death in breast cancer lines. It is noteworthy that the effects of SB743921 on breast cancer cell growth arrest were associated with downregulation of DTL and upregulation of p53 levels.

Several small-molecule KSP inhibitors including SB743921 have been developed and entered into clinical trials for the
SB743921 induces cell-cycle arrest and apoptosis of breast cancer cells. (a–d) The MDA-MB-231 (a, b) and MCF-7 (c, d) were treated with SB743921 at different concentrations indicated for 24 h; the cell cycle and apoptosis were analyzed using PI staining. The percentage of cell-cycle distribution of MDA-MB-231 (a) and MCF-7 (c) cells, the representative flow cytometry plot MDA-MB-231 (b) and MCF-7 (d) are shown. *P < 0.01, **P < 0.01, compared with the DMSO control. (e–h) The MDA-MB-231 (e, f) and MCF-7 (g, h) treated with SB743921 at different concentrations indicated for 24 h; the apoptosis was analyzed using DAPI and Annexin-V staining. The percentage of Annexin-V+ cells of MDA-MB-231 (e) and MCF-7 (g) cells and the representative morphological changes of MDA-MB-231 (f) and MCF-7 (h) cells are shown. **P < 0.01, compared with the DMSO control.

Treatment of cancers [33,34]. Previous reports have indicated that SB743921 has shown a wide range of anticancer activities against a variety of malignancies including cervical carcinoma, lymphoma, multiple myeloma, and myeloid leukemia [13,19,20]. In this study, we evaluated the potential antitumor effects of SB743921 on breast cancer cells. In agreement with previous findings in other types of tumors, KSP expression is also unregulated in tumor tissues from patient samples, suggesting the potential targeting KSP for therapeutic intervention in breast cancers. We show that SB743921 has significant cytotoxic activity, which presents as inhibition of the growth and colony-forming ability of breast cancer cell lines. In addition, we showed that SB743921 specifically induces apoptosis in both MCF-7 and MDA-MB-231 cells. It is encouraging that inhibition of KSP/Eg5 protein represents a novel therapeutic venue for disrupting the mitotic spindle apparatus in breast cancers, as some KSP inhibitors have been reported to show inhibitory activity even against drug-resistant cancer [24], suggesting that these compounds could become a new treatment option for overcoming chemoresistance in breast cancers.

The mechanism studies showed that chemical inhibition of KSP effectively induces cell-cycle arrest and apoptosis.
by mitochondria-mediated pathways [35,36]. Thus, it is understood that SB743921 treatment leads to an accumulation of KSP protein in breast cancer cells. Because P53 is involved in the mitochondrial apoptotic pathway, we further confirmed that SB743921 induced P53 expression in MDA-MB-231 cells. The level of anti-apoptotic protein Bcl-2 was downregulated and pro-apoptotic Bax and caspase-3 were significantly increased in response to SB743921 treatment. Our data showed that SB743921 influences the characteristics of breast cancer cells through activation of the p53 pathway and repression of BCL-2 antiapoptotic protein. In keeping with the notion of p53 expression and bcl-2 repression in SB743921-treated cells, we further determined the changes of cell-cycle regulator DTL expression in SB743921-treated cells. The DTL complex, also called the CRL4 (CDT2) complex, which mediates the polyubiquitination and subsequent degradation of several cycle regulators such as CDT1, CDKN1A/p21 (CIP1), and SETD8, is required for cell-cycle control, DNA damage response, and DNA synthesis [37,38]. In the cancer cells, the DTL complex also promotes the Lys-164 monoubiquitination of PCNA, and is thereby being involved in PCNA-dependent translesion DNA synthesis [39]. Given the important roles of DTL in cell-cycle progression, we also investigated the KSP protein and DTL mRNA levels in breast cancer cells treated with SB743921. SB743921 treatment results in the accumulation of KSP proteins and reduced DTL expression, suggesting their critical roles in the development of breast cancer [23].

Cancer therapies may be rendered ineffective because of the survival of cancer stem cells and chemoresistances [40,41]. Multiple pathways, including growth factors, stress, exosomes, and those originating from microenvironmental stimuli, have been proposed to mediate the survival of cancer stem cells and chemoresistance of
It is generally accepted that KSP is expressed by mitosis cells and integrate signals from multiple pathways to regulate cell proliferation of breast cancer cells. Thus, KSP inhibition may become a very effective strategy for improving sensitivity and overcoming chemoresistance of breast cancers. Our previous works have provided evidence for KSP inhibition to overcome TKI resistance in CML cells [13]. The data in this study support the concept that SB743921 is a potentially promising novel drug for the treatment of advanced breast cancers. It is would be of interest to test the combination of KSP inhibitors with current treatments to overcome chemoresistance in the future.

**Summary**

KSP is highly expressed in breast cancer cells. SB743921 showed considerable antiproliferative and proapoptotic efficacy through regulating multiple functional effectors in the progression of cycles such as p53, DTL, and Bcl-2. On the basis of these findings, SB743921 could be a
promising candidate as an antitumor agent for the treatment of advanced breast cancers.

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Conflicts of interest
There are no conflicts of interest.

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