Telocinobufagin inhibits the epithelial-mesenchymal transition of breast cancer cells through the phosphoinositide 3-kinase/protein kinase B/extracellular signal-regulated kinase/Snail signaling pathway

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Received May 30, 2017; Accepted February 13, 2018

DOI: 10.3892/ol.2018.8349

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Key words: telocinobufagin, epithelial-mesenchymal transition, invasion, migration, snail

Abstract. Telocinobufagin (TBG), an active ingredient of Venenumbufonis, exhibits an immunomodulatory activity. However, its antimetastatic activity in breast cancer remains unknown. The present study investigated whether TBG prevents breast cancer metastasis and evaluated its regulatory mechanism. TBG inhibited the migration and invasion of 4T1 breast cancer cells. Furthermore, TBG triggered the collapse of F-actin filaments in breast cancer. The epithelial-mesenchymal transition (EMT) markers, vimentin and fibronectin, were downregulated following TBG treatment. However, E-cadherin was upregulated following TBG treatment. Snail, a crucial transcriptional factor of EMT, was downregulated following TBG treatment. Signaling pathway markers, including phosphorylated protein kinase B (P-Akt), p-mechanistic target of rapamycin (mTOR) and p-extracellular signal-regulated kinase (ERK), were decreased following TBG treatment. The same results were obtained from in vivo experiments. In conclusion, in vitro and in vivo experiments reveal that TBG inhibited migration, invasion and EMT via the phosphoinositide 3-kinase (PI3K)/Akt/ERK/Snail signaling pathway in breast cancer.

Introduction

Breast cancer is a common malignancy that causes cancer-associated mortality in females worldwide (1,2). Despite the improved examination and therapeutic interventions in early breast cancer, the prognosis for patients with metastatic breast cancer continues to require significant improvement (3). Although numerous approaches have been applied to the treatment of patients with breast cancer, their clinical efficiency remains unsatisfactory (4,5). Therefore, novel potential adjuvants, particularly those able to increase the curative rates in these patients, should be developed.

Traditional Chinese Medicines (TCMs) are widely used in the United States and Western Europe. TCMs are considered safe and effective, since TCM formulas have been utilized for thousands of years in China (6). Although the precise assessment of TCM is difficult, it is administered to patients with chronic diseases, including cancer (7), autoimmune diseases (8), asthma (9) and acquired immune deficiency syndrome (10). Chan Su (Venenumbufonis), a TCM originating from the dried white secretions of the auricular and skin glands of toads, has been used in China to treat numerous conditions, including sore throat, palpitations and even cancer (11,12). Telocinobufagin (TBG), which is isolated from Chan Su, possesses enriched pharmacological characteristics, including immunoregulation (13), anticancer (14) and inhibition of Na+/K+-ATPase activity (15). Furthermore, TBG also exhibits crucial antitumor characteristics, including restraining cell proliferation, inhibiting cell differentiation, disrupting the cell cycle, dominating tumor angiogenesis and accommodating immune repercussion (15).

Epithelial-mesenchymal transition (EMT) is a fundamental process in normal embryonic growth (16) and is associated with the stimulation of migration in cancer (17). However, EMT also serves a function in breast cancer and may induce the migration and invasion of cancer cells (18). During EMT, cells lose their epithelial features and gain mesenchymal
phenotypes. For instance, their adhesion, apico-basal polarity and E-cadherin levels are decreased, while their N-cadherin, vimentin, and fibronectin levels are increased. Consequently, phenotypic characteristics, including anchorage-independent growth and motility, are developed. EMT is often associated with the gene zinc-finger transcriptional repressor Snail (19). The Snail family of transcription factors comprises Snail, Slug, and Smuc (20). Snail, Slug, Twist, Zeb1 and Zeb2 are important transcription factors that regulate EMT and promote the formation and development of breast cancer (12,21). In the present study, Snail was decreased by TBG treatment, but Slug, Twist, Zeb1, and Zeb2 remained unchanged following administration of TBG.

In the present study, TBG inhibited the invasion and migration of breast cancer cells, triggered the collapse of F-actin filaments, downregulated the mesenchymal markers, including vimentin and fibronectin, and upregulated epithelial markers including E-cadherin, through the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/extracellular signal-related kinase (ERK)/Snail signaling pathway. TBG also downregulated Snail, a crucial transcriptional factor of EMT. The anti-metastatic activity of TBG was verified by establishing a spontaneous metastatic model of BALB/c mice.

Materials and methods

Cell lines. The 4T1 mouse cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The 4T1 cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA). The 4T1 cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

MTT assays. Cell viability was measured by MTT assay. 4T1 (5,000) cells were plated onto 96-well plates overnight. The cells were treated with various concentrations of TBG (0, 0.05, 0.1, 0.5, 1, 1.25 and 1.5 µg/ml) for 48 h. The cells were incubated with MTT solutions for 4 h at 37°C and DMSO was added to each well. The absorbance was measured at 570 nm using a multifunctional microplate reader (SpectraMax M5; Molecular Devices, LLC, Sunnyvale, CA, USA).

Wound healing assay. A total of 5x10⁵ 4T1 cells were plated onto 6-well plates overnight. Wounds were created by scratching with a 10-µl white pipette tip. Detached cells were washed using phosphate-buffered saline (PBS). TBG (Biopurify Phytochemicals Ltd., Chengdu, China) was dissolved in 0.05 µg/ml dimethyl sulfoxide (DMSO) concentration. A control group with an equal DMSO concentration of RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) was created. The different concentrations of TBG (0, 0.05 and 0.5 µg/ml) were used to culture cells for 24 h. The wound distances were measured under fluorescence microscope (LSM710; magnification, x20; Carl Zeiss GmbH, Jena, Germany). The wound area was imaged using ImageJ software 1.8.0 (National Institutes of Health, Bethesda, MD, USA). The same fields were measured again to survey the wound gap. The experiments were repeated at least three times.

Cell migration assay. In this procedure, 4x10⁴ 4T1 cells were added to the upper chamber with 0.05 µg/ml TBG and RPMI-1640 medium (Sigma-Aldrich; Merck KGaA), or the control treatment without FBS. A total of 0.5 ml RPMI-1640 medium with 10% FBS was added to the lower chamber. The chambers were incubated at 37°C for 48 h. The transmigrated cells were stained with 0.2% crystal violet at room temperature for 10 min. The invaded cells were imaged and counted under a fluorescence microscope (magnification, x200). The experiments were repeated at least three times.

Cell invasion assay. The cell invasion assay was conducted using a BD PET-track-etched membrane invasion chamber with 50 µl diluted Matrigel (BD Biosciences, San Jose, CA, USA) overnight. A total of 4x10⁴ 4T1 cells were added to the upper chamber with 0.05 µg/ml TBG and RPMI-1640 medium (Sigma-Aldrich; Merck KGaA), or the control treatment without FBS. A total of 0.5 ml RPMI-1640 medium with 10% FBS was added to the lower chamber. The chambers were incubated at 37°C for 48 h. The invaded cells were stained with 0.2% crystal violet at room temperature for 10 min. The invaded cells were imaged and counted under a fluorescence microscope (magnification, x200). The experiments were repeated at least three times.

Immunofluorescence. In this procedure, 4x10⁴ 4T1 cells were cultured in 24-well plates overnight, prior to 0.05 µg/ml TBG being added. The control group was cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA), with 10% FBS (Sigma-Aldrich; Merck KGaA) for 24 h. 4T1 cells were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min at the room temperature and permeabilized with 0.5% Triton X-100 for 20 min at the room temperature. Next, the cells were blocked with 1% bovine serum albumin (BSA) for 1 h at the room temperature. Cells were subsequently incubated with primary antibodies against the following: E-cadherin (1:100; UMB700076), vimentin (1:100; UMB70054), and Fibronectin (1:100; AM06754SU-N; all from OriGene Technologies, Inc., Rockville, MD, USA) at 4°C overnight, prior to being washed three times with PBS. Cells were then incubated with Dylight 488 (1:100; 200-482-211) and Dylight 649 (1:100; A23620), secondary antibodies including Dylight 488 AffiniPure Goat Anti-Mouse IgG (H+L) (1:50; A23210), and DyLight 649 AffiniPure Goat Anti-Mouse IgG (H+L) (1:50, A23610) in the dark for 1 h at 37°C. They were purchased from Amy Jet Scientific, Inc., Hubei, China. The cell nuclei were labeled with 4',6-diamidine-2-phenylindole (DAPI). Images was captured under a fluorescence microscope (LSM710; magnification, x100; Carl Zeiss GmbH, Jena, Germany).

Western blot analysis. 4T1 cells were cultured overnight, prior to the addition of 0.05 or 0.5 µg/ml TBG for 24 h at 37°C. Te control cells were cultured in a RPMI-1640 medium (Sigma-Aldrich; Merck KGaA), with 10% FBS (Sigma-Aldrich; Merck KGaA) for 24 h at 37°C. Cells were lysed in phenylmethanesulfonyl fluoride (100 mM, Beyotime Institute of Biotechnology, Haimen, China) in the fridge at 4°C, and then centrifuged at 4°C at 12,000 x g for 5 min. The protein concentration was determined using a BCA Protein
Assay kit (Amylet Scientific, Inc.). Proteins (20 μg) were separated by 10% SDS-PAGE followed by electro transfer onto polyvinylidene fluoride membranes. After blocking with 5% non-fat dry milk in TBST buffer at 37°C for 1 h, the membranes were incubated in TBST buffer with the antibodies against ERK (1:500; sc-135900), Slug (1:500; sc-166476), Twist1 (1:500; sc-6269), Zeb1 (1:500; sc10572) and Snail (1:1000; 3879) overnight at 4°C, which were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). The membranes were incubated in TBST buffer with the antibodies against Akt (1:1000; 4685), P-Akt (1:1000; 4060), m-TOR (1:1000; 2983), P-mTOR (1:1000; 5536), P-ERK (1:1000; 4370) and SnaIl (1:1000; 3879) overnight at 4°C, which were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The membranes were incubated in TBST buffer with the antibodies E-cadherin (1:1000; ab76055), Fibronectin (1:1000; ab2413) and vimentin (1:1000; ab92547), β-actin (1:2000; ab8266) overnight at 4°C, which were obtained from Abcam (Cambridge, UK). Membranes were then incubated in TBST buffer with the Anti-Rabbit IgG VHH Single Domain HRP (1:3000; ab191866) secondary antibodies and Anti-Mouse IgG1 VHH Single Domain HRP (1:3000; ab193651) secondary antibodies for 1 h at room temperature. All Membranes were developed with ECL reagents. by ECL Plus Western Blot Detection System kit (Amersham, Piscataway, NJ, USA). The blots were analyzed by IPP6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Observation of F-actin filaments. 4T1 cells (4x10^4) were plated onto sterile climbing plates in 24-well plates and were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA), supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA). The cells were treated with 0.05 μg/ml TBG at 1, 3, 6 and 12 h to observe the collapse of F-actin filaments. 4T1 cells were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min at room temperature. Subsequently, the cells were blocked with 10% BSA for 1 h at room temperature. The cells were incubated with 5 μM fluorescein isothiocyanate (FITC) phalloidin at 37°C for 1 h to reveal F-actin. The cell nuclei were labeled with DAPI for 40 min and reagent 2 at 37°C for 40 min. The specimens were imaged under a fluorescence microscope (magnification, x100). To observe lung micro metastasis, sections were stained in hematoxylin at room temperature for 5 min, and eosin at room temperature for 30 sec.

Results

TBG inhibits the migration and invasion of breast cancer cells. The molecular structure of TBG was obtained from the product manual of Chan Su (V. bufonis) and is presented in Fig. 1A. To determine the effect of TBG treatment on cellular proliferation, 4T1 cells were treated with varying concentrations of TBG. No significant proliferation and inhibition activity was detected by an MTT assay following administration of reasonable concentrations of the drug (0.05 and 0.5 μg/ml TBG). However, with an increase in the concentration of TBG, the proliferation of cells was affected (Fig. 1B). A scratch assay was used to assess the effects of TBG on cell migration. TBG was applied at 0.05 and 0.5 μg/ml. After 24 h, the migration abilities of 4T1 cells were restrained in a concentration-dependent manner (Fig. 1C). The wounded area of the control group was almost entirely occupied by the migrating cells after 24 h, which was in contrast to the relatively wider gap observed in the TBG-treated groups.

The influence of TBG on migration and invasion was assessed by conducting Transwell assays for 48 h. TBG was used at 0.05 and 0.5 μg/ml TBG controlled the migration and invasion of 4T1 cells in a concentration-dependent manner. The number of migrating cells in the high-concentration group was reduced compared with that in the control group (Fig. 1D-F).
No significant differences were identified in the morphological characteristics of the cells compared with the treatment groups (Fig. 1G).

**TBG induces the disintegration of F-actin filament cytoskeleton in breast cancer cells.** The cells were treated with TBG at 1, 3, 6 and 12 h to observe the changes in the F-actin filaments. The F-actin filaments were observed by staining with FITC-labeled phalloidin, and the cell nuclei were labeled with DAPI. In the control group, the F-actin filaments exhibited a regular arrangement and were evenly distributed in the cytoplasm. In the cells treated with TBG, the structure of the F-actin filaments changed at 3 h, and F-actin filaments were destroyed at 12 h (Fig. 2A), suggesting that TBG triggered the collapse of the F-actin filament cytoskeleton in vitro, as well as increasing the contribution time (1, 3, 6 and 12 h).
Furthermore, F-actin levels were decreased relative to β-actin following TBG treatment (Fig. 2B).

**TBG regulates EMT markers.** EMT has been positively associated with the metastatic potential of tumor cells (17). To investigate whether TBG inhibits breast cancer cell migration and invasion via EMT, epithelial and mesenchymal markers, including E-cadherin, vimentin and fibronectin were investigated. The results revealed that vimentin and fibronectin were decreased in treated cells, but that E-cadherin was increased following TBG treatment (Fig. 3A). Immunofluorescence analysis was utilized to assess E-cadherin in the cell membrane and cytoplasm, and vimentin and fibronectin in the cytoplasm, which revealed the same results (Fig. 3B). Therefore, these results suggested that TBG enhanced the epithelial traits and inhibited the mesenchymal properties of breast cancer.

**TBG downregulates the transcription factor Snail via the Akt/ERK signaling pathway.** Several crucial transcription factors were observed in the 4T1 cells to determine which transcription factors, modulated by TBG, further regulated and controlled EMT. Snail was downregulated in 4T1 cells following TBG treatment (Fig. 3C). The other transcriptional factors, including Slug, Twist1, Zeb1 and Zeb2 exhibited no notable differences between the control group and the TBG treatment group (Fig. 3C).

The PI3K/Akt and ERK/mitogen-activated protein kinase (MAPK) signaling pathways are associated with EMT (22). Therefore, in order to reveal the underlying molecular mechanisms associated with TBG-inhibited EMT in 4T1 cells, markers including Akt, phosphorylated (p)-Akt, mechanistic target of rapamycin (mTOR), P-mTOR, ERK and p-ERK. The results identified that TBG inhibited p-AKT, P-mTOR and P-ERK in a concentration-dependent manner. AKT, mTOR and ERK were unchanged between the control group and the TBG treatment groups (Fig. 3D). Therefore, the data gathered revealed that TBG inhibited EMT in vitro via the Akt/ERK/Snail signaling pathway. **TBG inhibited tumor growth, metastasis and EMT in the mouse model via the Akt/ERK/Snail signaling pathway. An in vivo**
study was conducted to further evaluate the anti-metastatic effect of TBG in highly metastatic 4T1 mouse models. TBG was considered non-toxic at the doses used, since no body weight changes were observed in these mice (Fig. 4A). Notably, the single maximum tumor volume of the vehicle-treated mice was increased compared with that of the TBG-treated mice. Furthermore, the average tumor weight of the TBG-treated mice was less than that of the vehicle-treated mice (Fig. 4A). In the present study, multiple tumors in mammary fat pads were not observed. H&E staining was performed to confirm the pathological metastasis in the lungs, and the metastatic tumors were observed via microscopy (Fig. 4B). The mesenchymal biomarker E-cadherin was decreased, whereas the epithelial biomarkers vimentin and fibronectin were increased in vitro following TBG treatment. To determine their expression in primary tumor tissues immunohistochemical staining was conducted. The TBG treatment downregulated Snail, vimentin, fibronectin, P-Akt and P-ERK compared with the vehicle group (Fig. 4C and D). By contrast, E-cadherin was upregulated (Fig. 4C). The survival time of tumor-bearing mice was not measured or analyzed in the present study. Taken together, the results gathered indicated that TBG may be able to inhibit EMT by blocking the Akt/ERK/Snail pathway in vivo.

Discussion

Chan Su is probably one of the most extensively used TCMs owing to its pharmacological properties, including anti-cancer and immuno regulatory effects (12). TBG has potent anti-metastasis features (12) and the present study identified that TBG inhibited EMT in breast cancer.

Breast cancer migration and invasion are associated with EMT (23-25). Cell migration not only controls normal cellular processes, including tissue development, chemotaxis and wound healing (26,27), but is also involved in the invasion and metastasis of neoplasms (28). The results of the present study indicated that TBG significantly suppressed cell migration and invasion in
It was identified that lower doses of TBG-treatment inhibited cell migration and invasion, while higher doses of TBG inhibited cellular proliferation \textit{in vitro}. Furthermore, the results revealed that TBG at 10 and 20 µg/mouse inhibited average tumor volume, tumor weight and lung metastasis in these mice. In the present study, the TBG-mediated inhibition of cellular proliferation, migration and invasion was investigated, and the potential underlying molecular mechanism of heTBG-mediated anti-metastatic effect was evaluated using \textit{in vitro} and \textit{in vivo} models. However, the use of a single cell line is a limitation of the present study.

The polymerization and depolymerization of F-actin filaments and cell migration are also associated with neoplasm metastasis (29). The present study detected whether the disintegration of the F-actin cytoskeleton was associated with the anti-metastatic characteristics of TBG. It was identified that TBG resulted in the collapse of the F-actin cytoskeleton and further disrupted the polymerization and depolymerization of F-actin filaments.

When cancer cells undergo EMT, the epithelial phenotype is lost and a mesenchymal phenotype is obtained, further increasing the invasive and migratory properties (30). E-cadherin has a significant effect on the cell-to-cell adhesion that inhibits the invasion and metastasis of tumors (31). Fibronectin and vimentin are regarded as critical mesenchymal markers. The present study investigated whether E-cadherin was upregulated, and fibronectin and vimentin were downregulated following TBG treatment in 4T1 cells. Therefore, these data identified that TBG inhibits EMT in breast cancer cells.

The aberrant regulation of the transcription factor Snail is associated with EMT in multiple types of cancer (32). Snail is a transcription factor that serves a critical function in modulating genes, including E-cadherin, fibronectin and vimentin, which may be regulated at transcriptional and post-transcriptional levels via a complex signaling pathway (33). The experiments in the present study tested which transcription factors were inhibited with TBG.
treatment, and identified that TBG inhibited Snail, but that Slug, Twist1, Zeb1 and Zeb2 transcription factors were not notably altered.

The PI3K/Akt and MAPK/ERK signaling pathway serves a critical function in cell polarization, apoptosis, invasion and migration and are associated with the pathogenesis of multiple types of cancer (34-38). Notably, Ras-MAPK activates the relevant transcription factor Snail, which inhibits the transcription of E-cadherin and accelerates EMT (39,40). ERK cascades serve a function in a number of cellular processes, including proliferation, differentiation, migration and invasion (41). TBG repressed Snail via the PI3K/Akt and MEK/ERK signaling pathway. Therefore, TBG promoted E-cadherin expression, and suppressed vimentin and fibronectin expression through the Akt/ERK/Snail signaling pathway.

In summary, TBG suppressed EMT, metastasis, invasion and migration in vivo and in vitro. The present study revealed that TBG suppressed EMV via the Akt/ERK/Snail signaling pathway. The Akt/ERK/Snail signaling pathway was a promising target of TBG. According to the results of the present study, TBG, a small molecular agent, may have important implications for the effective treatment of patients with breast cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by the funds from the Scientific Foundation of Shandong Province (ZR2014HM086) and the Scientific Foundation of Shandong Province (ZR2015HL119).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YG and LS wrote the paper and conducted the experiments. ZC, XZ and FL conducted the western blot analysis. RW, JX and JZ conducted the immunohistochemistry and H&E staining. BZ and SL provided assistance with the experimental ideas and technical side of the present study.

Ethics approval and consent to participate

All animal experiments were implemented under the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the company. Animal protocols were approved by the guidelines built by the Animal Care Committee at Weifang Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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