ORIGINAL ARTICLE

Brucine Inhibits Bone Metastasis of Breast Cancer Cells by Suppressing Jagged1/Notch1 Signaling Pathways*

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ABSTRACT **Objective**: To examine the effects of brucine on the invasion, migration and bone resorption of receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclastogenesis. **Methods**: The osteoclastogenesis model was builded by co-culturing human breast tumor MDA-MB-231 and mouse RAW264.7 macrophages cells. RANKL (50 ng/mL) and macrophage-colony stimulating factor (50 ng/mL) were added to this system, followed by treatment with brucine (0.02, 0.04 and 0.08 mmol/L), or 10 μmol/L zoledronic acid as positive control. The migration and bone resorption were measured by transwell assay and *in vitro* bone resorption assay. The protein expressions of Jagged1 and Notch1 were investigated by Western blot. The expressions of transforming growth factor-β1 (TGF-β1), nuclear factor-kappa B (NF-κB) and Hes1 were determined by enzyme-linked immunosorbent assay. **Results**: Compared with the model group, brucine led to a dose-dependent decrease on migration of MDA-MB-231 cells, inhibited RANKL-induced osteoclastogenesis and bone resorption of RAW264.7 cells ($P<0.01$). Furthermore, brucine decreased the protein levels of Jagged1 and Notch1 in MDA-MB-231 cells and RAW264.7 cells co-cultured system as well as the expressions of TGF-β1, NF-κB and Hes1 ($P<0.05$ or $P<0.01$). **Conclusion**: Brucine may inhibit osteoclastogenesis by suppressing Jagged1/Notch1 signaling pathways.

KEYWORDS brucine, breast cancer, bone metastasis, Jagged1/Notch1 signaling pathway

Breast cancer is one of the most common malignant tumors, and up to 70% of patients with advanced breast cancers develop bone metastases.(1) Bone metastasis of breast cancer cells may result in bone fractures, severe pain, nerve compression, and hypocalcemia.(2) Bone homeostasis is maintained by the balance between osteoclasts and osteoblasts, and can be disrupted by breast cancer cells, resulting in metastatic tumor growth.(3) Breast cancer bone metastasis is closely associated with osteoclastogenesis and osteolytic bone metastasis,(4) however the precise molecular mechanism is not fully understood.

Osteoclastogenesis is an extremely complex process and is controlled by various signaling pathways. Osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK), receptor activator of nuclear factor-kappa B ligand (RANKL) are the crucial factors affecting bone balance, the OPG/RANKL/RANK pathway plays a predominant role in osteoclastogenesis. RANK receptor is expressed on the surface of human breast cancer cells and RANKL acts as a chemotactic factor for breast cancer cells to stimulate osteoclastogenesis.(5) OPG secreted by osteoblast plays a role in the regulation of osteoclast through inhibiting RANKL.(6) A constitutively active form of Notch1 reduces macrophage-colony stimulating factor (M-CSF) and enhances RANKL and OPG gene expression, resulting in an overall reduction of osteoclast formation in stromal cells.(7)

Transforming growth factor-β (TGF-β) plays an important role in bone metastasis of cancer cells. TGF-β1 is released in an active form upon tumor-induced osteoclastic bone resorption and stimulates bone metastatic tumor cells to secrete factors that further drive osteolytic bone destruction adjacent to the tumor.(8) These findings demonstrate that TGF-β1, a well known pro-metastatic cytokine, stimulates Jagged 1 expression in cancer cells to promote osteolytic bone metastasis.

Nuclear factor-kappa B (NF-κB) also plays a crucial role in the osteolytic bone metastasis of breast cancer by stimulating osteoclastogenesis through granulocyte-macrophage colony stimulating factor (GM-CSF)

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induction.[9] Dll4-Fc inhibits NF-κB activity in small cell lung cancer cells by suppressing Dll4-Notch signaling.[10] A recent study suggests that paracrine NF-κB activation promotes the expansion of cancer stem cells through the activation of Notch in basal-type breast cancer cells.[11] Thus deregulation of NF-κB may be related to Notch signaling on breast cancer bone metastasis.

Recently, the Notch signaling pathway has been found to be involved in breast cancer bone metastasis. Sethi, et al[12,13] reported that elevated expression of the Notch ligand Jagged1 was associated with breast cancer bone metastasis, and tumor-derived Jagged1 promoted osteolytic bone metastasis of breast cancer cells by activating the Notch pathway in the bone microenvironment. Moreover, Hes1, a downstream target gene of Notch signaling, was found to be up-regulated in osteosarcoma cells and the expression status of Hes1 correlated with the invasive and metastatic potential of osteosarcoma cells. Accordingly, blockade of Notch signaling with a small molecule inhibitor of γ-secretase reduced invasion in matrigel without affecting cell proliferation, survival, or anchorage-independent growth.[14] Therefore, targeting Notch signaling is a promising strategy for the prevention and treatment of bone metastasis of breast cancer. As another target of Notch signaling, interleukin-6 (IL-6) has a strong pro-tumorigenic activity due to its multiple effects on bone metabolism. It was proposed that Jagged1/Notch signaling may stimulate the release of IL-6 from osteoblasts to promote tumor proliferation.[4]

The increase of bone resorption by the coordination of these factors results in further production and release of growth factors, thereby promoting the growth and proliferation of tumor cells. Thus, deregulation of multiple signaling pathways may contribute to bone metastasis of breast cancer cells.

Brucine is a bitter alkaloid extracted from the *Strychnos nux-vomica* tree, found in Southeast Asia. Many studies have shown that brucine is an effective agent for the treatment of breast cancer.[15] It was reported that treatment of breast cancer MCF-7 cells with brucine resulted in inhibition of cell proliferation and induction of apoptosis, accompanied with up-regulation of Bax and caspase-3 while down-regulation of B-cell lymphoma-2 (BCL-2),[16,17] in animal experiments, brucine inhibited the growth of breast cancer mouse xenografts without obvious effect on mice survival.[18,19] Intriguingly, brucine was found to inhibit bone metastasis of breast cancer,[20] vascular endothelial growth factor (VEGF) expression and angiogenesis;[21,22] however, the precise mechanism of action is still unknown.

Due to its high bone metastasis characteristics, breast cancer cell line MDA-MB-231 is widely used in experimental study of bone metastasis in breast cancer.[23] In this study, we developed a RANKL-induced osteoclastogenesis model by co-culturing human breast tumor MDA-MB-231 cells and mouse RAW264.7 macrophages cells. The effects of brucine on the invasion, migration, bone resorption, and modulation of the Jagged1/Notch1 signaling pathways, as well as the expressions of TGF-β1, NF-κB, and Hes1 were examined.

METHODS

Chemical and Reagents

Brucine with purity of 91.7% was obtained from National Institutes for Food and Drug Control (Beijing, China); zoledronic acid was purchased from Novartis Pharma Ltd. (Switzerland, lot No. S0051). The tartrate resistant acid phosphatase (TRAP) staining kit (Sigma Aldrich, USA), Hes1 and NF-κB enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, Wuhan, China), TGF-β1 ELISA kit (eBioscience, San Diego, California, USA), RevertAid™ cDNA synthesis kit (ThermoScientific, Waltham, MA, USA), SYBR Green polymerase chain reaction (PCR) master (Invitrogen, Carlsbad, CA, USA), Western blotting luminol reagent, anti-RANKL and anti-β-actin antibodies (N-21, Santa Cruz, CA, USA), Jagged1 (1C4) rabbit mAb, Notch1 (C44H11) rabbit mAb, NF-κB p65 antibody, anti-rabbit IgG-horseradish peroxidase (HRP)-linked antibody, and rabbit anti-goat IgG-HRP-linked antibody (Cell Signaling Technology, Boston, MA, USA) were used.

Instruments

Microscope (Olympus, CK, Japan), scanning electron microscope (Hitachi, S-3400N, China), protein processor (Tanon, 6200; China), microplate reader (BIO-TEK Co., EXL800, USA), vertical electrophoresis apparatus (Tanon Co., 6600, China), and protein electrophoresis transfer apparatus (Tanon Co., EPS-300, China) were used.

Cells and Cell Culture

Human breast tumor MDA-MB-231 cells and mouse RAW264.7 macrophages cells were obtained

from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells cultured in Leibovitz's L-15 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Mouse RAW264.7 macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 ℃ in a humidified incubator containing 5% CO_2.

Co-culture of MDA-MB-231 and RAW264.7 Cells

MDA-MB-231 cells were plated in 75T culture plates overnight, followed by seeding RAW264.7 cells 4 times the number of MDA-MB-231 cells. At the same time, RANKL (50 ng/mL) and M-CSF (50 ng/mL) were added to this system, followed by treatment with different concentrations of brucine (0.02, 0.04 and 0.08 mmol/L), or 10 μmol/L zoledronic acid as positive control. The medium was replaced every two days. Six days later, the supernatant and cells were harvested and saved under –80 ℃ for further analysis.

Transwell Assay

In the transwell culture system, RAW264.7 cells were seeded in the lower chamber for 24 h, and then induced with 50 ng/mL RANKL and 50 ng/mL M-CSF. In the meantime, brucine (0.02, 0.04 and 0.08 mmol/L), or 10 μmol/L zoledronic acid were added to the medium for 5 days. On the 5th day, MDA-MB-231 cells were seeded onto transwell inserts (with a 8 μm pore size) and cultured for 24 h. Cells that had migrated to the lower surface of the membrane were subjected to crystal violet staining and observed with a light microscope. The migrated cells were counted using 5 randomly selected fields at 200× magnification.

TRAP Staining

The co-cultured MDA-MB-231 and RAW264.7 cells were first stimulated with 50 ng/mL RANKL and 50 ng/mL M-CSF, and then treated with different concentration of brucine (0.02, 0.04 and 0.08 mmol/L), or 10 μmol/L zoledronic acid for 6 days. The co-cultured cells were fixed in 4% paraformaldehyde, stained for TRAP using the TRAP staining kit following the manufacturer's protocol, and observed under light microscopy. TRAP positive multinucleated cells (>3 nuclei) were scored as osteoclast.

***In vitro* Bone Resorption Assay**

The bone resorption capacity of osteoclasts derived from RAW264.7 cells induced by RANKL was analyzed using the 50 μm thick bovine bone slice as previously described.[24] Briefly, co-cultured cells were plated on bovine cortical bone slices in 24-well plates, and treated with M-CSF (50 ng/mL) and RANKL (50 ng/mL) together with brucine (0.02, 0.04 and 0.08 mmol/L), or 10 μmol/L zoledronic acid. Cultures were maintained for 7 days to allow for bone resorption and then cells were removed from the bone slices with sonication. Bone slices were then subjected to scanning electron microscopy. At the same time, the slices were stained with toluidine blue, and resorption areas were observed under a light microscope and analyzed by Image-Pro Plus 6.0.

ELISA

The expression of the components of Jagged1/Notch signaling pathway, including TGF-β1, NF-κB and Hes1, was determined by ELISA. The supernatant of co-cultured cells after treatment with brucine and positive control drug were collected for the determination of TGF-β1, NF-κB and Hes1 levels using respective ELISA kit according to the manufacturer's instruction.

Western Blot Analysis

Total protein was extracted from cells using radio-immunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology). Equal amounts of protein extracts (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% w/v non-fat dry milk or 5% albumin from bovine serum (BSA) dissolved in Tris buffered saline plus Tween-20 [(triethanolamine buffered saline solution contain tween (TBS-T); 0.1% Tween-20; pH 8.3] at room temperature for 1 h, then incubated with primary antibodies at 4 ℃ overnight. The primary antibodies for immunoblotting included β-actin, Jagged1 and Notch1. After washing with TBS-T, membranes were incubated with HRP-labeled secondary antibodies (CST) for 1 h at room temperature. Immunobands were visualized using an enhanced chemiluminescence (ECL) kit (GE Healthcare, Waukesha, WI, USA) according to manufacture's instructions and exposed to X-ray films. β-Actin was used as a loading control.

Statistical Analysis

Statistical analyses were performed using Image

Pro Plus 6.0. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Differences were analyzed using multivariate repeated measures analysis of variance. Comparisons between two groups were done using least significant difference test. $P<0.05$ was considered as statistically significant difference.

RESULTS

Brucine Inhibited RANKL-Induced Migration of Breast Cancer Cells

In our preliminary experiments of co-culture system of MDA-MB-231 cells and RAW264.7, 0.02, 0.04 or 0.08 mmol/L brucine treatment for 72 h led to 80% of the survival rate of RAW264.7 cells. The transwell migration assay showed that RANKL treatment increased the migration of MDA-MB-231 cells compared with untreated control cells ($P<0.01$). Compared with the model group, zoledronic acid and brucine (0.02, 0.04 and 0.08 mmol/L) significantly reduced the migration of MDA-MB-231 cells ($P<0.01$). The effect of brucine was comparable to that of zoledronic acid ($P>0.05$). Moreover, brucine dose-dependently inhibited RANKL-induced migration of MDA-MB-231 cells ($P<0.0167$, Figure 1).

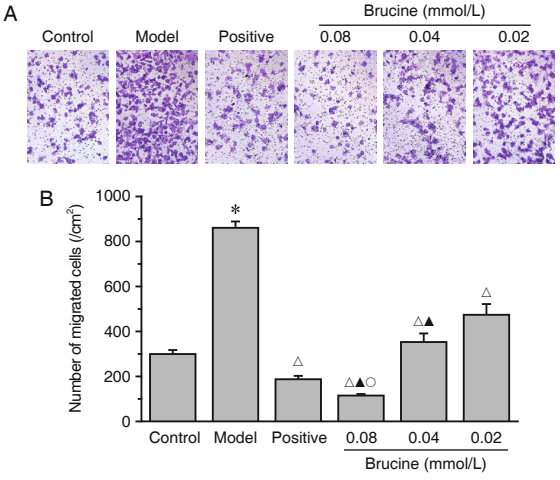

Figure 1. Brucine Inhibited the Migration of MDA-MB-231 Cells

Notes: (A) Migration tendency of MDA-MB-231 cells to RAW264.7 cells assessed by transwell assay (crystal violet staining, ×200). (B) Quantitation of the stained cells in A. $^{*}P<0.01$, *vs*. control group; $^{\triangle}P<0.01$, *vs*. model group; $^{\blacktriangle}P<0.0167$, *vs*. 0.02 mmol/L brucine group, $^{\bigcirc}P<0.0167$, *vs*. 0.04 mmol/L brucine group

Brucine Suppressed RANKL-Induced Osteoclastogenesis of RAW264.7 Cells

As shown in Figure 2, 50 ng/mL RANKL stimulation resulted in a dramatic enhancement of TRAP activity in RAW264.7 cells ($P<0.01$). Moreover, RANKL treated cells displayed many larger, red rose cytoplasm, characteristic of multinucleated osteoclast cells. Thus, MDA-MB-231 and RAW264.7 co-cultured cells treated with RANKL led to significantly higher osteoclast differentiation. Brucine clearly down-regulated TRAP activity in a dose-dependent manner. Moreover, compared with the RANKL group, under the treatment of varying concentrations of brucine, the number of osteoclasts was significantly reduced ($P<0.01$). In addition, similar inhibition of the maturation and differentiation of osteoclasts was observed in the presence of zoledronic acid.

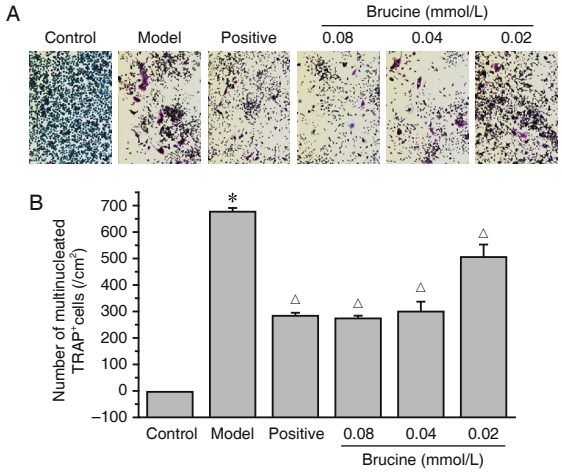

Figure 2. Effects of Brucine on Cell Differentiation of RAW264.7 Cells in MDA-MB-231 and RAW264.7 Cells Co-cultured System by RANKL

Notes: (A) Effects of brucine on osteoclast differentiation (TRAP staining, ×300); (B) Quantitation of stained cells in A. $^{*}P<0.01$, *vs*. control group; $^{\triangle}P<0.01$, *vs*. model group

Brucine Inhibited Osteoclastic Bone Resorption of RAW264.7 Cells Induced by RANKL

Light microscope analysis revealed violet round, oval, or irregular shapes, and boundary clear absorption lacunae (Figure 3A). Scanning electron microscopy demonstrated the changing trend of bone resorption lacunae, consistent with the results of light microscope (Figure 3B). Zoledronic acid significantly inhibited RANKL-induced bone resorption ($P<0.01$). Similarly, either 0.02 or 0.08 mmol/L brucine significantly suppressed RANKL-induced bone resorption ($P<0.01$, Figure 3C).

Brucine Reduced RANKL-Induced Expressions of TGF-β1, NF-κB and Hes1

As shown in Figure 4, RANKL stimulation for 24 h obviously up-regulated the expressions of TGF-β1, NF-κB and Hes1 ($P<0.01$). Zoledronic acid and 0.08 mmol/L brucine markedly inhibited the expressions of TGF-β1, NF-κB and Hes1 compared with the RANKL-treated group ($P<0.05$ or $P<0.01$).

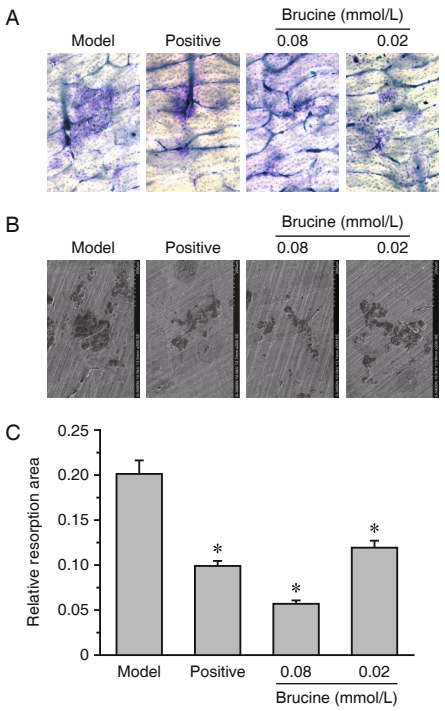

Figure 3. Brucine Suppressed Osteoclastic Bone Resorption of RAW264.7 Cells

Notes: (A) Osteoclasts on dentine slices and bone resorption pits by light microscopy (Toluidine blue staining, ×200). (B) Osteoclasts on dentine slices and bone resorption pits by scanning electronicmicroscopy (×200). (C) Percentage of bone resorption pits in each group. *P<0.01, *vs*. model group

Brucine Prevented the Induction of Protein Levels of Jagged1 and Notch1 by RANKL

As shown in Figure 5, RANKL increased the protein levels of Notch1 and Jagged1 compared with the control group (P<0.01). Zoledronic acid and brucine (0.02, 0.04 and 0.08 mmol/L) significantly inhibited the protein levels of Notch1 and Jagged1 compared with the model group (P<0.01).

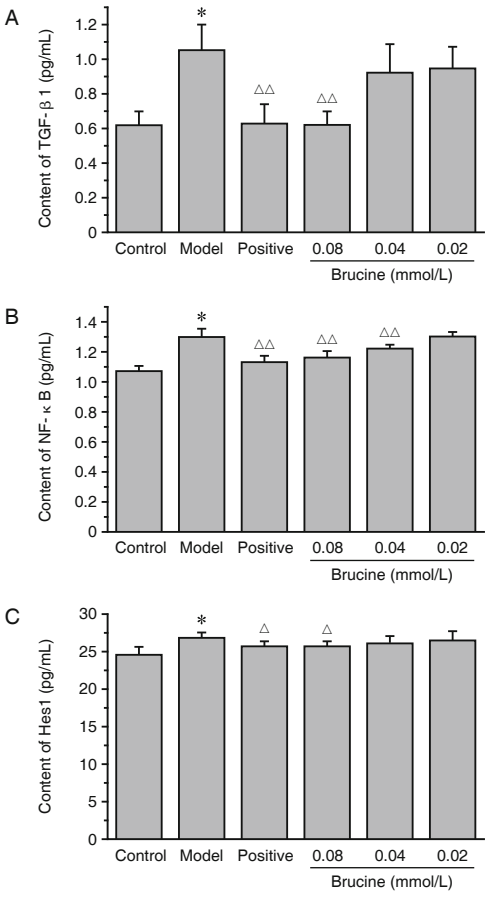

Figure 4. Effect of Brucine on TGF-β1, NF-κB and Hes1 Expressions in MDA-MB-231 and RAW264.7 Cells Co-cultured System by ELISA

Notes: *P<0.01, *vs*. control group; △P<0.05, △△P<0.01, *vs*. model group

DISCUSSION

In this study, we demonstrated that brucine inhibited RANKL-induced migration of MDA-MB-231 cells. Importantly, brucine suppressed the induction

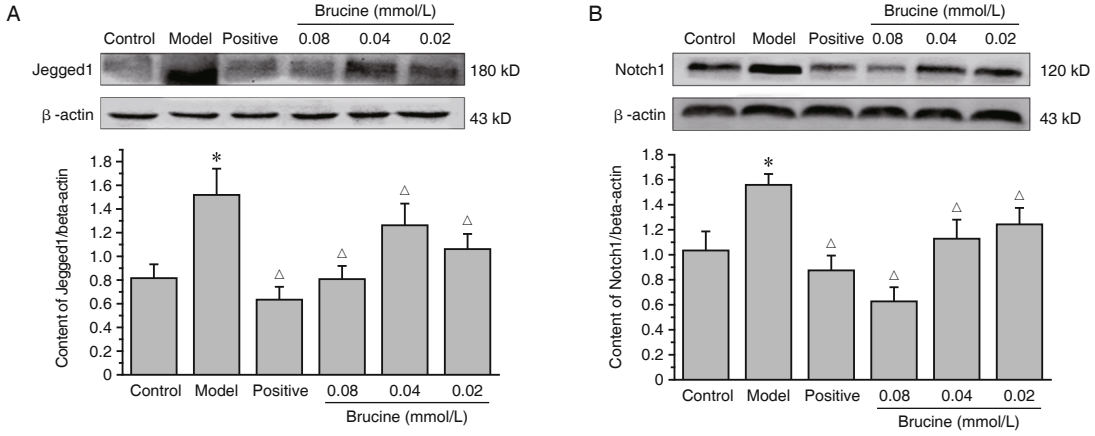

Figure 5. Effects of Brucine on Jagged1 and Notch1 Protein Expressions in MDA-MB-231 and RAW264.7 Cells Co-cultured System

Notes: Protein levels of Jagged1 (A) and Notch1 (B) determined by Western blot with β-actin as loading control. *P<0.01, *vs*. control group; △P<0.01, *vs*. model group

of osteoclastogenesis of RAW264.7 cells and osteoclastic bone resorption induced by RANKL. It was further showed that brucine reduced RANKL-induced expressions of TGF-β1, NF-κB and Hes1. Previous study suggested that Jagged1-expressing tumor cells may indirectly impact osteoclast activity by altering the expression of osteoblast-derived RANKL and OPG.[12] To test whether brucine alters the expressions of Jagged1 and Notch1, we treated co-cultured MDA-MB-231 and RAW264.7 cells with RANKL, brucine and zoledronic acid for 24 h, and determined protein levels of Jagged1 and Notch1 by Western blot. Brucine reduced the protein levels of Jagged1 and Notch1 induced by RANKL. These results suggest that brucine may inhibit osteoclastogenesis by suppressing Jagged1/Notch1 signaling pathways.

Notch signaling is an evolutionarily conserved pathway that profoundly impacts mammalian development by regulating cell survival, proliferation, and fate decision in a context-dependent manner. Moreover, it contributes to tissue maintenance and/or renewal in the central nervous system and can either promote or suppress cancer development such as breast cancer bone metastasis.[25]

The Jagged1/Notch1 pathway is an important cancer-promoting signaling and is up-regulated in breast cancer. The Jagged1/Notch1 pathway activates the maturation of pro-osteoclast to osteoclasts, thereby undermining the balance of the bone microenvironment. At the same time, TGF-β released by osteoclasts increases angiogenesis and accelerates cancer metastasis. Moreover, Notch signaling enhances the activity of NF-κB and expression the downstream target gene Hes1, ultimately enhancing NF-κB-mediated cell survival and promoting cell proliferation. Thus, the combined effects of a variety of signals lead to osteolysis and bone metastases.

RANKL plays a principle role in the regulation of osteoclasts. It has been reported that RANKL can stimulate mononuclear precursor cells to form multinucleated macrophages that have a bone resorption activity, leading to bone destruction. In addition, macrophages also promote the secretion of inflammatory cytokines and stimulate the expression of RANKL by osteoblasts thereby indirectly promoting RANKL-mediated osteoclastogenesis.[26]

A previous study showed that brucine could decrease the expression of VEGF in a nude mouse model of bone metastasis of breast cancer, suggesting that brucine may inhibit breast cancer bone metastasis possibly by inhibiting tumor angiogenesis.[27] Many studies have shown that elevated expression of Notch pathway ligands is associated with metastatic ability of breast cancer cells.[5,13] It was reported that high expression of Jagged1, the ligand of Notch1 was closely correlated with breast cancer bone metastasis in patient samples, and Notch signaling in long-bone osteoblasts indirectly regulated osteoclast differentiation by controlling the expression of RANKL and OPG.[25] In this study, RANKL stimulation of co-culture cells induced the expression of Jagged1 and Notch1, which was abolished by brucine treatment. Notch signaling in osteoblast-lineage cells has critical functions for bone homeostasis. Firstly, it maintains bone marrow mesenchymal progenitors through inhibition of osteoblast differentiation. Secondly, it regulates osteoclastogenesis from bone marrow macrophage precursors by modulating the production of RANKL and OPG by osteoblasts. Thus, inhibition of Jagged1 and Notch1 may be one of the mechanisms of the suppression of breast cancer cells to bone metastasis by brucine. During tumor progression, both tumor cells and stromal cells secrete a lot of TGF-β1, which promote tumor growth as well as inhibit host immune surveillance.[28] In this study, RANKL stimulation of co-cultured cells led to up-regulation of TGF-β1, which was inhibited by brucine treatment. Therefore, inhibition of TGF-β1 may contribute to the suppression of breast cancer cell bone metastasis by brucine.

In summary, in the MDA-MB-231 and RAW264.7 cell co-culture system, the expression levels of Jagged1 and Notch1 in the model group significantly increased, while TGF-β1, NF-κB and Hes1 increased to different extent in the supernatant when compared with the control group. Moreover, treatment with different concentrations of brucine significantly reduced the expression of Jagged1 and Notch1 proteins, and TGF-β1 and NF-κB levels in the supernatant of co-culture system in a dose-dependent manner, with optimal inhibition by 0.08 mmol/L brucine. Our findings suggest that brucine is a promising agent for the prevention and treatment of breast cancer cell bone metastasis and bone loss. Further studies need to be performed to verify that brucine can inhibit bone metastases of breast cancer *in vivo* and the protein and gene expression of Jagged1 and Notch1 are related

with bone metastases of breast cancer brucine treated.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Pei XH formed the conception of research; Hu KF designed the research; Hu KF, Kong XY, Zhong MC, Wan HY and Lin N performed experiments; Hu KF analyzed data, interpreted results of experiments and drafted manuscript; Kong XY prepared figures; Zhong MC edited and revised manuscript. All authors approved the final version of manuscript.

REFERENCES

1. Zhang Y, Ma B, Fan Q. Mechanisms of breast cancer bone metastasis. Cancer Lett 2010;292:1-7.
2. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2002;2:584-593.
3. Mundy GR. Mechanisms of bone metastasis. Cancer 1997;80:1546-1556.
4. Clezardin P. Therapeutic targets for bone metastases in breast cancer. Breast Cancer Res 2011;13:207.
5. Hofbauer LC, Kuhne CA, Viereck V. The OPG/RANKL/RANK system in metabolic bone diseases. J Musculoskelet Neuronal Interact 2004;4:268-275.
6. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3:537-549.
7. Fukushima H. Regulatory mechanisms of Notch signaling in osteoclast differentiation. J Oral Biosciences 2010;52:205-214.
8. Chiechi A, Waning DL, Stayrook KR, Buijs JT, Guise TA, Mohammad KS. Role of TGF-β in breast cancer bone metastases. Adv Biosci Biotechnol 2013;4:15-30.
9. Park BK, Zhang H, Zeng Q, Dai J, Keller ET, Giordano T, et al. NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nat Med 2007;13:62-69.
10. Kuramoto T, Goto H, Mitsuhashi A, Tabata S, Ogawa H, Uehara H, et al. Dll4-Fc, an inhibitor of Dll4-notch signaling, suppresses liver metastasis of small cell lung cancer cells through the downregulation of the NF-kappaB activity. Mol Cancer Ther 2012;11:2578-2587.
11. Zhang W, Grivennikov SI. Top Notch cancer stem cells by paracrine NF-kappaB signaling in breast cancer. Breast Cancer Res 2013;15:316.
12. Sethi N, Dai X, Winter CG, Kang Y. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. Cancer Cell 2011;19:192-205.
13. Sethi N, Kang Y. Notch signalling in cancer progression and bone metastasis. Br J Cancer 2011;105:1805-1810.
14. Zhang P, Yang Y, Zweidler-McKay PA, Hughes DP. Critical role of notch signaling in osteosarcoma invasion and metastasis. Clin Cancer Res 2008;14:2962-2969.
15. Zhao LM, Liu YG, Niu ZX. Anti-tumor effect on brucine. Chin J Cancer Treat (Chin) 2013;20:877-880.
16. Serasanambati M, Chilakapati SR, Manikonda PK, Kanala JR, Chilakapati DR. Anticancer effects of brucine and gemcitabine combination in MCF-7 human breast cancer cells. Nat Prod Res 2015;29:484-490.
17. Agrawal SS, Saraswati S, Mathur R, Pandey M. Cytotoxic and antitumor effects of brucine on Ehrlich ascites tumor and human cancer cell line. Life Sci 2011;89:147-158.
18. Deng XK, Yin W, Li WD, Yin FZ, Lu XY, Zhang XC, et al. The anti-tumor effects of alkaloids from the seeds of *Strychnos nux-vomica* on HepG2 cells and its possible mechanism. J Ethnopharmacol 2006;106:179-186.
19. Deng X, Yin F, Lu X, Cai B, Yin W. The apoptotic effect of brucine from the seed of *Strychnos nux-vomica* on human hepatoma cells is mediated via Bcl-2 and Ca^{2+} involved mitochondrial pathway. Toxicol Sci 2006;91:59-69.
20. Ma WJ, Li P. Effects of brucine on breast cancer bone metastasis in mice. Anhui Med Pharm (Chin) 2009;13:600-602.
21. Li P, Zhang M, Ma WJ, Sun X, Jin FP. Effects of brucine on vascular endothelial growth factor expression and microvessel density in a nude mouse model of bone metastasis due to breast cancer. Chin J Integr Med 2012;18:605-609.
22. Lowery FJ, Yu D. Growth factor signaling in metastasis: current understanding and future opportunities. Cancer Metastasis Rev 2012;31:479-491.
23. Schneider D, Liaw L, Daniel C, Athanasopoulos AN, Herrmann M, Preissner KT, et al. Inhibition of breast cancer cell adhesion and bone metastasis by the extracellular adherence protein of *Staphylococcus aureus*. Biochem Biophys Res Commun 2007;357:282-288.
24. Dempster DW, Moonga BS, Stein LS, Horbert WR, Antakly T. Glucocorticoids inhibit bone resorption by isolated rat osteoclasts by enhancing apoptosis. J Endocrinol 1997;154:397-406.
25. Bai S, Kopan R, Zou W, Hilton MJ, Ong CT, Long F, et al. NOTCH1 regulates osteoclastogenesis directly in osteoclast precursors and indirectly via osteoblast lineage cells. J Biol Chem 2008;283:6509-6518.
26. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 1999;25:255-259.
27. Cao L, Arany PR, Wang YS, Mooney DJ. Promoting angiogenesis via manipulation of VEGF responsiveness with notch signaling. Biomaterials 2009;30:4085-4093.
28. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99-146.

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