Abstract. The bone is the most common metastatic site of breast cancer. Bone metastasis causes pain, pathologic fractures, and severely reduces the quality of life. Breast cancer causes osteolytic bone metastasis, which is dependent on osteoclast-mediated bone resorption. While current treatments rely on palliative anti-resorptive agents, there is a need to develop a drug based on potential alternative therapies. This study is the first to determine that wedelolactone (WDL), a natural coumarin isolated from plants, can inhibit breast cancer-mediated osteoclastogenesis. Osteoclasts were generated from human CD14+ monocytes cultured with M-CSF/RANKL and WDL suppressed human osteoclast differentiation and activity in vitro in a dose-dependent manner. Moreover, WDL inhibited the upregulation of osteoclasts stimulated by MDA-MB-231 breast cancer cells. The activity of WDL on osteoclasts and breast cancer-mediated osteoclastogenesis was associated with the inhibition of Akt/mammalian target of the rapamycin signaling pathway (mTOR). Blocking Akt and mTOR by specific inhibitors significantly decreased osteoclast differentiation and bone resorption. Furthermore, WDL regulated breast cancer-enhanced interaction of osteoblasts and osteoclasts by decreasing M-CSF expression in MDA-MB-231-stimulated osteoblasts. Thus, this study suggests that WDL may be a potential natural agent for preventing and treating bone destruction in patients with bone metastasis due to breast cancer.

Introduction

Breast cancer is the most common female cancer in the world and bone metastasis occurs in up to 70-80% of patients with advanced breast cancer (1,2). Bone metastasis of breast cancer typically leads to osteolysis and results in bone destruction and skeletal-related events, including severe bone pain, pathologic fractures, spinal cord and nerve compression syndromes, and life-threatening hypercalcemia, which are a common cause of morbidity and mortality (3). Current treatments for bone metastasis have limited efficacy and are only palliative (4). Side-effects like renal toxicity and osteonecrosis of the jaw are considered clinically significant, potentially painful, and debilitating enough to decrease life quality of breast cancer patients (2,5,6). Thus, it is important that therapeutic strategies are developed for the prevention and treatment of bone metastasis in breast cancer.

The spread of breast cancer to the bone begins with the initiation of bone demolition, the entry of malignant cells in the bone marrow, and subsequently, an increase in osteolysis (7). Bone metastasis is characterized by the complicated interaction between cancer cells and bone micro-environment (i.e., osteoclasts, osteoblasts and stroma cells) (7). Osteolytic bone metastasis is driven by the ‘vicious cycle’ of cancer-mediated upregulation of osteoclasts and bone stroma-induced cancer progression (8). Osteoclasts are large, multi-nucleated cells derived from hematopoietic precursor cells of the monocyte/macrophage lineage (9). Their differentiation is mediated by two cytokines, including macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) (10). RANKL is crucial for osteoclast function by binding to its receptor RANK, while M-CSF maintains the proliferation and survival of osteoclasts, and upregulates RANK receptor expression in osteoclast precursor cells (11).

In addition to osteoclasts, breast cancer cells can also interact with osteoblasts to support osteoclast formation (12). Breast cancer cells increase the expressions of osteoclast differentiation factors, M-CSF and RANKL, and decrease osteoprotegerin (OPG) level in osteoblasts, leading to osteoclastogenesis (13-15). Therefore, molecular targeting of both cancer-mediated osteoclastogenesis or mending the imbalance of osteoblast-osteoclast interaction can reduce bone destruction (osteolytic bone metastasis).

Wedelolactone (7-methoxy-5,11,12-trihydroxy-coumestan, Fig. 1A) (Sigma-Aldrich, St. Louis, MO, USA) is a natural
coumarin isolated from *Wedelia chinensis* and *Eclipta prostrata*, which are traditionally used for hair and skin health and for preventing liver damage due to alcohol overdose (16-19). Previous studies show that WDL has diverse pharmacologic effects such as anti-hepatotoxic, anti-androgenic, and anti-human immunodeficiency activities (19-21). Recent research has highlighted the antitumor effects of WDL. Combinations of active compounds in *W. chinensis*, including WDL, apigenin, and luteolin, have been found to suppress the growth of prostate cancer cells in vitro and in vivo (20,22). Treatment with WDL inhibits the growth of pituitary adenoma cells and mammary carcinomas in vitro (23,24). Moreover, WDL can induce caspase-dependent apoptosis in prostate cancer cells via downregulation of PKCe (25).

This study demonstrates that WDL inhibits MDA-MB-231-mediated osteoclastogenesis through the downregulation of the Akt/mTOR signaling pathway. Moreover, WDL downregulates M-CSF expression in MDA-CM-stimulated osteoblasts and inhibits osteoblast-mediated osteoclastogenesis.

**Materials and methods**

**Cell culture.** Human breast cancer MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MDA-MB-231 cells were cultured in standard modified essential medium (α-MEM) [α-MEM supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin]. Human primary osteoblasts were obtained from Lonza (Walkersville, MD, USA) and cultured in osteoblast growth medium.

For conditioned medium (CM) collection, the MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or various concentrations of WDL for 12 h. After washing, fresh α-MEM was added and cultured for another 24 h. The supernatants were collected, filtered (0.22 mm), and defined as MDA-CM or WDL-MDA-CM. Osteoblasts were cultured with MDA-CM or WDL-MDA-CM (20%) for another 24 h and the supernatants were then collected and filtered (0.22 mm). The collected supernatants were grouped as osteoblast-CM (OB-CM), MDA-OB-CM and WDL-MDA-OB-CM.

**Osteoclast differentiation.** Human peripheral blood, obtained from healthy adult volunteers, was collected in syringes isolated using CD14+ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec), according to the manufacturer’s instructions.

For conditioned medium (CM) collection, the MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or various concentrations of WDL for 12 h. After washing, fresh α-MEM was added and cultured for another 24 h. The supernatants were collected, filtered (0.22 mm), and defined as MDA-CM or WDL-MDA-CM. Osteoblasts were cultured with MDA-CM or WDL-MDA-CM (20%) for another 24 h and the supernatants were then collected and filtered (0.22 mm). The collected supernatants were grouped as osteoblast-CM (OB-CM), MDA-OB-CM and WDL-MDA-OB-CM.

**Enzyme-linked immuno-sorbent assay (ELISA).** The levels of M-CSF were assessed by M-CSF ELISA kit (R&D Systems, Minneapolis, MN, USA). The RANKL and OPG levels of osteoblast were quantified using the DuoSet ELISA kits (R&D Systems).

**Statistical analysis.** Data were expressed as means ± SD. Statistical comparisons were made using analysis of variance. Significant differences (p<0.05) between two test groups were analyzed by Student’s t-test.

**Results**

**WDL exhibits direct inhibitory effect on osteoclast differentiation and bone resorption activity.** To investigate if novel agents can inhibit osteoclasts, the effects of various phytoestrogens, including calycosin, formononetin, neobavaisoflavone, ononin, pinoresinol, and wedelolactone (WDL), on osteoclast differentiation were assessed. The addition of RANKL and M-CSF caused the formation of numerous TRAP-positive multi-nucleated osteoclasts (Fig. 1B). The differentiation of osteoclasts was significantly inhibited by WDL but other agents failed to affect osteoclastogenesis. Treatment of WDL decreased osteoclastogenesis in a dose-dependent manner (Fig. 1C). Moreover, WDL treatment substantially reduced osteoclastic bone resorption in a dose-dependent manner (Fig. 1D). These findings suggested that WDL inhibited osteoclast differentiation and bone resorption activity in vitro.

**WDL inhibited breast cancer-mediated osteoclastogenesis.** The effect of WDL on breast cancer-mediated osteoclasto-
genesis was evaluated (Fig. 2A). MDA-MB-231-CM markedly increased osteoclastogenesis (TRAP-positive multi-nucleated osteoclasts) (Fig. 2B). This stimulatory effect of breast cancer MDA-MB-231 cells was significantly decreased by WDL. MDA-MB-231-CM also enhanced the bone resorption activity of osteoclasts and this effect of breast cancer was also reduced by WDL (Fig. 2C). These results indicated that WDL inhibited MDA-MB-231 cell-mediated osteoclastogenesis and osteoclast activity.

WDL decreased osteoclastogenesis by inhibiting Akt/mTOR. The molecular mechanism of WDL on osteoclastogenesis was investigated. The addition of M-CSF/RANKL increased the phosphorylation of Akt, mTOR, ERK1/2 and p38. The targets of mTOR, p70S6K, and 4EBP4 were also activated by M-CSF/RANKL, showing that Akt/mTOR signaling cascade was triggered in osteoclastogenesis. The activation of osteoclast inducers (M-CSF and RANKL) on Akt/mTOR signaling was inhibited by WDL (Fig. 3A).

The role of Akt/mTOR on osteoclastogenesis and bone resorption was further supported by using both Akt (Akt inhibitor IV) and mTOR inhibitors (rapamycin), which decreased osteoclastogenesis and its activity (Fig. 3B and C). In addition, WDL not only decreased M-CSF/RANKL-mediated Akt/mTOR activation, but also reduced the reinforcing effect of breast cancer on this signaling pathway (Fig. 3D). These results revealed that WDL inhibited the Akt/mTOR signaling pathway, which was critical in osteoclastogenesis induced by M-CSF/RANKL and MDA-MB-231 cells.

WDL decreased the stimulatory effect of breast cancer on M-CSF expression in osteoblasts. Osteoblasts are known to play a critical role on the deterioration of bone metastasis by increasing M-CSF and RANKL, and decreasing OPG expression (13-15). As such, the effect of WDL on the secretion of M-CSF, RANKL, and OPG in osteoblasts after stimulation of breast cancer was assessed. MDA-MB-231-CM markedly increased the expressions of M-CSF and RANKL in osteo-

Figure 1. WDL decreased osteoclastogenesis. (A) The chemical structure of WDL. (B) The effect of various phytoestrogens in osteoclastogenesis. WDL decreased (C) osteoclast differentiation and (D) bone resorption. Each value is the mean ± SD of three independent experiments. The pit area was determined by AlphaEase FC software. *p<0.05, or significant difference with controls, as analyzed by Student’s t-test.
blasts (Fig. 4A and B). Furthermore, MDA-MB-231-CM also decreased OPG expression in osteoblasts (Fig. 4C). WDL decreased the stimulatory effect of breast cancer on the production of M-CSF, although it did not affect the expressions of RANKL and OPG in osteoblasts (Fig. 4).

**WDL reduces the enhancement effect of breast cancer on osteoblast-mediated osteoclastogenesis.** The activity of WDL on breast cancer-stimulated interaction of osteoblasts and osteoclasts was further investigated (Fig. 5A). Breast cancer MDA-MB-231 stimulated osteoblast-induced osteoclastogenesis, compared to results of the osteoclastogenesis cultured in unstimulated osteoblast supernatants (Fig. 5B). The stimulatory effect of MDA-MB-231 cells was inhibited by WDL treatment. Similarly, breast cancer MDA-MB-231 cells increased the promotion of osteoblasts on osteoclast bone resorption and this effect was prevented by WDL (Fig. 5C). These findings suggest that WDL inhibited the deterioration of osteoblasts on breast cancer-mediated osteolytic bone metastasis.

**Discussion**

Bone metastasis is a devastating development in patients with breast cancer because once this occurs, morbidity and
Figure 3. WDL decreases osteoclastogenesis by inhibiting Akt/mTOR signaling. (A) WDL reduced M-CSF/RANKL-mediated Akt signaling activation. Akt (Akt inhibitor IV) and mTOR (rapamycin) inhibitors decreased (B) osteoclast differentiation and (C) bone resorption. (D) WDL inhibited MDA-MB-231-mediated Akt/mTOR activation. Each value is the mean ± SD of three independent experiments. The pit area was determined by AlphaEase FC software. *p<0.05, or significant difference with controls, as analyzed by Student's t-test.
mortality rates are markedly increased (2,4). Drugs that target osteoclastogenesis, such as bisphosphonates or anti-RANKL antibody (denosumab), decrease the incidence of skeletal complications and have been prescribed for the inhibition of cancer-mediated bone destruction (6). However, 30-50% of patients on such therapies still develop new bone metastasis, skeletal complications, and disease progression, emphasizing the need for new therapies (2,4,6). The present study is the first to demonstrate that WDL can inhibit breast cancer-induced osteolytic bone metastasis by decreasing the stimulatory effect of breast cancer on osteoclasts and breast cancer-mediated interaction of osteoblasts and osteoclasts.

Evaluation of osteoclast differentiation is characterized in breast cancer bone metastasis (4,12). An increase in osteoclastic bone resorption leads to the release of bone-derived growth factors that promote the growth and progression of breast cancer cells (4,12). Inhibiting osteoclast function provides a potential approach to preventing bone metastasis. Isolated from Eclipta prostrata and Wedelia chinensis, WDL belongs to the coumarin category of phytoestrogens. This is the first study that investigated and determined that WDL exhibits a direct inhibitory effect on osteoclast differentiation and bone resorption activity.

In addition, WDL also inhibits breast cancer MDA-MB-231-mediated osteoclastogenesis and bone degradation. This finding suggests that WDL possesses therapeutic potential in the treatment of breast cancer bone metastasis. The impact of metastasized cancer cells on the bone interrupts the interaction between the activities of osteoclasts and osteoblasts (26). Osteoblasts perform vital roles in the regulation of skeleton physiology because they not only act as precursors of osteocytes but are also dual regulators of osteoclast differentiation (27,28). Osteoblasts stimulate osteoclast formation from osteoclast precursors by producing M-CSF and RANKL (29). In contrast, osteoblasts inhibit osteoclastogenesis via secreting OPG, a decoy receptor for RANKL to block the binding of RANKL to its receptors on pre-osteoclasts, RANK (13).

Cancer can alter the expressions of M-CSF, RANKL, and OPG in osteoblasts, facilitating cancer-associated osteolytic bone metastasis (14,30). In this study, breast cancer MDA-MB-231 cells increased the expressions of M-CSF and RANKL but decreased OPG expression, thereby increasing osteoclastogenesis and the bone resorption activity. This upregulation of M-CSF by MDA-MB-231 is prevented by WDL, resulting in a decrease in breast cancer-associated osteoclastogenesis and bone resorption. This study revealed that WDL can mend the imbalanced interaction of osteoblasts and osteoclasts in breast cancer-mediated skeletal microenvironment.

Akt is a critical mediator of cell proliferation, survival and differentiation in a variety of cell types (31). Akt has been identified as being involved in the regulation of osteoclast survival and differentiation, whereas its deficiency in osteoclasts results in impaired bone resorption (32,33). mTOR, a downstream molecule of Akt, has also been shown to be involved in the regulation of osteoclast survival (34). Inhibition of the mTOR pathway by rapamycin, an mTOR inhibitor, decreases the number of TRAP-positive multi-nucleated osteoclasts in the chondro-osseous junction in rats (35).

In this study, osteoclast differentiation induced by M-CSF/RANKL increased the activation of Akt and mTOR signaling cascade. Inhibitors of both Akt and mTOR markedly suppressed osteoclast differentiation and bone resorption activity, suggesting that the Akt/mTOR pathway plays a crucial role in osteoclastogenesis and osteoclast functions. Moreover, breast cancer MDA-MB-231 cells augment the activation of
Akt/mTOR signaling, resulting in enhanced osteoclastogenesis. WDL not only blocks M-CSF/RANKL-induced Akt/mTOR activation, but also prevents the reinforcing effect of breast cancer on this signaling pathway. These data suggest that WDL is an Akt/mTOR inhibitor, targeting both the induction of osteoclast differentiation and the cancer inducible activation of Akt/mTOR.

In conclusion, WDL has protective potential against breast cancer-induced bone destruction by directly decreasing cancer cell mediated osteoclast differentiation and bone resorption and by restoring the balance of osteoblast-osteoclast interaction. Taken together, WDL possesses potential and dual ameliorating effects on breast cancer-associated osteolytic bone metastasis.

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References

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