Embelin Suppresses Osteoclastogenesis Induced by Receptor Activator of NF-κB Ligand and Tumor Cells *In vitro* through Inhibition of the NF-κB Cell Signaling Pathway

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Abstract

Most patients with cancer die not because of the tumor in the primary site, but because it has spread to other sites. Common tumors, such as breast, multiple myeloma, and prostate tumors, frequently metastasize to the bone. It is now well recognized that osteoclasts are responsible for the osteolysis observed in bone metastases of the tumor. Receptor activator of NF-κB ligand (RANKL), a member of the tumor necrosis factor superfamily and an activator of the NF-κB signaling pathway, has emerged as a major mediator of bone loss, commonly associated with cancer and other chronic inflammatory diseases. Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), derived from the Ayurvedic medicinal plant *Embelia ribes*, has been shown to bind and inhibit X-linked inhibitor of apoptosis protein and inhibit inflammatory pathways. We investigated whether embelin could inhibit osteoclastogenesis-associated bone loss induced by RANKL and by tumor cells *in vitro*. We found that embelin suppressed the RANKL-induced differentiation of monocytes into osteoclasts. This benzoquinone also suppressed the osteoclastogenesis induced by multiple myeloma and by breast cancer cells. This effect of embelin correlated with the suppression of NF-κB activation and inhibition of IκB degradation. Furthermore, by using an inhibitor of the IKK or NF-κB essential modulator (NEMO), the regulatory component of the IKK complex, we showed that the NF-κB signaling pathway is mandatory for RAW 264.7 cell differentiation into osteoclasts. Thus, embelin, an inhibitor of RANKL-induced NF-κB activation has great potential as a therapeutic agent for osteoporosis and cancer-linked bone loss. *Mol Cancer Res; 8*(10); 1425–36. ©2010 AACR.

Introduction

Bone loss is one of the major problems associated with aging, arthritis, cancer, and other chronic inflammatory diseases. This bone loss is the result of disruption in the bone homeostatic mechanism that normally maintains a balance between bone resorption by osteoclasts and bone formation by osteoblasts. Postmenopausal osteoporosis, for example, is due to an increase in osteoclast number after the cessation of menses; in other types of osteoporosis (glucocorticoid or male osteoporosis), a marked depression of osteoblast formation is observed (1). Efforts to improve the outcomes of treatment for bone loss have recently focused on the molecular details of this mechanism. The activation of osteoclasts, multinucleated cells derived from hematopoietic cells, is regulated by various molecular signals, of which the receptor activator of NF-κB (RANK) ligand (RANKL), a member of the tumor necrosis factor superfamily, is one of the best-studied cytokines. RANKL is a membrane protein residing on osteoblasts and their precursors that activates osteoclast formation by stimulating its receptor RANK through its interaction with an adaptor molecule, tumor necrosis factor receptor–associated factor 6 (TRAF6; ref. 2). The downstream intracellular signaling pathways include TRAF6-dependent activation of the IκBα kinases (IKK), which phosphorylate and degrade the inhibitor of NF-κB, IκBα (2). Once IκBα is degraded, NF-κB translocates to the nucleus and activates the transcription of specific genes. The clinical relevance in cancer extends well beyond primary bone cancers. Bone is one of the most common sites for tumor metastasis. Bone metastases, which invariably lead to hypercalcemia, bone pain, fractures, and nerve compression, increase the morbidity and mortality in patients with cancer (3). Breast cancer, which is the second leading cause of cancer death in women, metastasizes to the bone in more than 80% of patients with advanced disease (3).
In patients with prostate cancer, bone metastasis occurs in about 70% of the patients. In patients with lung, colon, kidney, thyroid, and stomach carcinoma, bone metastasis has been reported in 15% to 30% patients (4). It has now been well defined that osteoclasts, not tumor cells, are principally responsible for the osteolysis observed in bone metastases and directly lead to the bony pathologies observed in these patients (5).

The search for treatments of bone loss has naturally included inhibitors of the RANKL cell signaling pathway. One potential inhibitor is embelin, from the fruit of the Embelia ribes Burn., plant (Myrsinaceae; called false black pepper in English, Vidanda in Sanskrit, and Babrang in Hindi languages). Embelin has been used for thousands of years to treat fever, inflammatory diseases, and a variety of gastrointestinal ailments (6). More than 4 decades ago, the active component from this plant was isolated and named embelin (ref. 7; see structure in Fig. 1A) and was later chemically synthesized (8). Embelin has been shown to have antitumor, anti-inflammatory, and analgesic properties (9), and our group has previously shown that embelin abolished activation of NF-κB and suppressed expression of a variety of proliferative, metastatic, and antiapoptotic gene products (10). This novel NF-κB blocker also enhanced the apoptosis induced by cytokine and chemotherapeutic agents (10). As a result, we hypothesized that embelin modulates RANKL-induced signaling and osteoclastogenesis. Our test of the hypothesis indicates that embelin inhibits RANKL-induced NF-κB activation through inhibition of the IKK complex and suppresses osteoclastogenesis induced by RANKL and tumor cells.

Materials and Methods

Reagents

A 100 mmol/L solution of embelin (Sigma-Aldrich; Fig. 1A), a benzoquinone, was prepared in 100% DMSO, stored at −20°C, and diluted as needed in cell culture medium. DMEM/F12, RPMI 1640, DMEM, fetal bovine serum, 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen. RANKL protein was purchased from R&D Systems. Embelin was prepared in 100% DMSO, stored at −20°C, and diluted as needed in cell culture medium.

Cell lines

RAW 264.7 (mouse macrophage) cells were kindly provided by Dr. Bryant Darnay (Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX). Rabbit polyclonal antibodies to IkBα were purchased from Imgenex. Antibody against p-IκBα was purchased from Cell Signaling Technology. Anti-IKKα and anti-IKKβ antibodies and NEMO (NF-κB essential modifier; IKKγ)-binding domain peptide (NBP) were a kind gift from Imgenex. Antibody against p-IκBα/β was purchased from Cell Signaling Technology, and phospho-extracellular signal-regulated kinase (ERK)-1/2 and caspase-3 antibodies were from Santa Cruz Biotechnology. Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates were purchased from Bio-Rad. Antibody against β-actin and leukocyte acid phosphatase kit (387-A) for tartrate-resistant acid phosphatase (TRAP) staining were purchased from Sigma-Aldrich. Protein A/G-agarose beads were obtained from Pierce. [γ-32P]ATP was purchased from ICN Pharmaceuticals.

Osteoclast differentiation assay

RAW 264.7 cells were cultured in 24-well plates at a density of 10 × 10⁴ per well and allowed to adhere overnight. The medium was then replaced, and the cells were treated with 5 nmol/L RANKL for 5 days. All samples were subjected to TRAP staining using leukocyte acid phosphatase kit (Sigma-Aldrich). For coculture experiments with tumor cells, RAW 264.7 cells were seeded at 5 × 10⁴ per well and allowed to adhere overnight. The following day, U266 or MDA-MB-231 cells, at 1 × 10⁵ per well, were added to the RAW 264.7 cells, which were then treated with embelin and cocultured for 5 days before being subjected to TRAP staining. For conditioned medium experiments, RAW 264.7 cells were seeded at 10 × 10⁴ per well and allowed to adhere overnight. The following day, the medium was replaced with 4/5 of RAW 264.7 medium (DMEM/F12) and 1/5 of conditioned medium from U266 and MDA-MB-231 cells. For the latter, cultured U266 and MDA-MB-231 cells were centrifuged and the supernatant was used. Then, RAW 264.7 cells were cultured for 5 days and subjected to TRAP staining.

Cell proliferation assay

Cell proliferation was assessed by a modified tetrazolium salt MTT assay as described previously (13). In brief, 2,000 cells were incubated with various concentrations of embelin, in triplicate, for 1, 3, and 5 days in 96-well plates at 37°C. Thereafter, an MTT solution was added to each well. After 2 hours of incubation at 37°C, lysis buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and then the absorbance was measured at 570 nm using a 96-well multiscaner (MRX Revelation, Dynex Technologies).

Electrophoretic mobility shift assays for NF-κB

Nuclear extracts were prepared as described previously (14). Briefly, nuclear extracts from RANKL-treated cells were incubated with a [γ-32P]-end-labeled 45-mer double-stranded DNA probe corresponding to the NF-κB binding site of the TNF-α promoter.
NF-κB oligonucleotide (15 μg protein with 16 fmol DNA) from the HIV long terminal repeat 5'-TTGTTACAA
GG-GACTTTC CGCTG GGGACTTTC CAGGGG- GAGGCGTGG-3 (boldface indicates NF-κB-binding sites) for 30 minutes at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized with a Storm820, and radioactive bands were quantified using a densitometer and ImageQuant software (Amersham). For conditioned medium experiments, RAW 264.7 cells were seeded at 2 × 10⁶ per well and allowed to adhere overnight. The following day, the medium was replaced with 4/5 of RAW 264.7 medium (DMEM/F12) and 1/5 of conditioned medium from U266 and MDA-MB-231 cells. For the latter, cultured U266 and MDA-MB-231 cells were centrifuged and the supernatant was used. Then, RAW 264.7 cells were cultured for 24 hours and assessed by electrophoretic mobility shift assay (EMSA) for NF-κB activity.

FIGURE 1. Embelin inhibits RANKL-induced osteoclastogenesis. A, structure of embelin. B, RAW 264.7 cells (10 × 10^3/mL) were incubated with medium, RANKL (5 nmol/L), or RANKL and embelin (15 μmol/L) for 3, 4, and 5 d and then stained for TRAP expression. TRAP-positive cells were photographed. Original magnification, ×100. C, RAW 264.7 cells (10 × 10^3/mL) were incubated with either medium or RANKL (5 nmol/L) along with 5, 10, and 15 μmol/L embelin for 3, 4, or 5 d and then stained for TRAP expression. Multinucleated osteoclasts (i.e., those containing three nuclei) were counted. Cells exposed to medium alone were used as a control (C). D, RAW 264.7 cells (2 × 10^7/0.1 mL) were incubated with medium (Ctrl) or with 5, 10, or 15 μmol/L embelin for 1, 3, and 5 d, and then cell proliferation was assessed by the MTT method.
Western blot analysis

To determine the levels of protein expression in the cytoplasm and nucleus, we prepared extracts (14) and fractionated them by 10% SDS-PAGE. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes, blotted with each antibody, and detected with a chemiluminescence reagent (GE Healthcare).

Trypan blue exclusion assay

Cells were harvested from six-well plates by treatment with 0.2% trypsin-EDTA, centrifuged, and suspended with 1 mL of culture medium. The cell suspension was mixed with an equal volume of 0.4% isotonic trypan blue solution. Total cell number and the fraction of nonviable, dye-accumulating cells were counted after 2 minutes in a Fuchs-Rosenthal hemocytometer under a light microscope.

IKK assay

To determine the effect of embelin on RANKL-induced IKK activation, an IKK assay was done following a method described previously (12). Briefly, the IKK complex from whole-cell extracts (600 μg protein) was precipitated with antibody against IKKα followed by treatment with protein A/G-agarose beads. After 2 hours of incubation, the beads were washed with lysis buffer and assayed in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L MgCl2, 2 mmol/L DTT, 20 μCi [γ-32P]ATP, 10 mmol/L unlabeled ATP, and 2 μg of substrate glutathione S-transferase-IκBα (amino acids 1-54). After incubation at 30°C for 30 minutes, the reaction was terminated by boiling in SDS sample buffer for 5 minutes. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a PhosphorImager.

Immunoprecipitation assay

To determine the effect of embelin on RANKL-induced IKK complex formation, protein A/G-agarose beads were first incubated with IKKδ antibody for 2 hours; then, the beads were washed with lysis buffer and incubated with whole-cell extracts (600 μg protein) of treated cells overnight at 4°C. The following day, the beads were again washed with lysis buffer and boiled in SDS sample buffer for 5 minutes. Finally, the supernatant was analyzed on 10% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and blotted with anti-IKKα or anti-IKKβ antibody.

Results

The present studies were designed to investigate the effect of embelin on osteoclastogenesis induced by RANKL and tumor cells and on RANKL-induced NF-κB activation. RAW 264.7 (murine macrophage) cells, a well-established system for osteoclastogenesis, were used for this study.

Embelin inhibits RANKL-induced osteoclastogenesis

Because RANKL is one of the major cytokines that induces osteoclastogenesis, we used RANKL to induce differentiation of RAW 264.7 cells and investigated whether embelin can modulate this differentiation. RAW 264.7 cells were incubated with RANKL for different days and allowed to differentiate into osteoclasts. Then, the effect of embelin on RANKL-induced osteoclastogenesis was evaluated after 3, 4, and 5 days. The morphologic observations clearly showed differentiation of cells after addition of RANKL, and the benzoquinone suppressed this differentiation (Fig. 1B). The extent of suppression was measured by counting the number of TRAP+ osteoclasts per well (Fig. 1C) after 3, 4, and 5 days. We found that RANKL induced osteoclast differentiation in a time-dependent manner, with a maximum of TRAP+ osteoclasts per well at day 5 (Fig. 1C). On the other hand, embelin dose-dependently decreased the number of TRAP+ osteoclasts per well, with an almost complete inhibition at 15 μmol/L at all days examined (Fig. 1B and C). To exclude that this observation was due to a reduction in cell proliferation by embelin, we analyzed the proliferation of RAW 264.7 cells treated with 5, 10, and 15 μmol/L embelin at days 1, 3, and 5. As shown in Fig. 1D, embelin did not significantly affect the proliferation of RAW 264.7 cells.

Embelin acts at an early step in the RANKL-induced osteoclastogenesis pathway

Complete osteoclast differentiation of RAW 264.7 cells takes up to 5 days after RANKL stimulation. To identify at what step embelin acts in this differentiation pathway, RAW 264.7 cells were initially treated with RANKL and embelin was added 1, 2, 3, or 4 days later (Fig. 2A and B). This result suggests that embelin affects the proliferation of RAW 264.7 cells act directly or indirectly (by secreting certain cytokines) on these tumors.

Embelin inhibits osteoclastogenesis induced by tumor cells

Given that osteoclastogenesis is commonly linked with breast cancer and multiple myeloma, we next investigated whether embelin inhibits osteoclastogenesis induced by these tumors.

RAW 264.7 cells were coincubated with breast cancer (MDA-MB-231; Fig. 3A) or multiple myeloma (U266; Fig. 3B) cells and allowed to differentiate for 5 days. As shown in Fig. 3A, MDA-MB-231 cells induced differentiation of RAW 264.7 cells into osteoclasts, and embelin completely inhibited this differentiation. However, at days 3 and 4 after RANKL addition, osteoclast formation was no longer completely prevented by embelin (Fig. 3A and B). The result suggests that embelin acts at an early step in the osteoclast differentiation pathway. Embelin alone had no effect on cell viability (Fig. 2A, left).
the medium of cultured U266 or MDA-MB-231 cells (conditioned medium) for 5 days. The result presented in Fig. 3A and B (right) clearly indicates that conditioned medium alone is sufficient to activate osteoclastogenesis in RAW 264.7 cells, indicating that certain cytokines secreted by tumor cells are responsible for osteoclast differentiation. Furthermore, treatment with embelin inhibits osteoclastogenesis induced by conditioned medium (Fig. 3A and B, right).
Embelin inhibits RANKL-induced NF-κB activation

How embelin inhibits osteoclast differentiation induced by RANKL and tumor cells was investigated. One major mechanism that has been associated with osteoclastogenesis is activation of the NF-κB pathway. Therefore, we investigated whether conditioned medium from U266 and MDA-MB-231 cells induces NF-κB activation in RAW 264.7 cells. As shown in Fig. 3C, conditioned medium from U266 and MDA-MB-231 cells effectively activates NF-κB in RAW 264.7 cells, indicating that this transcription factor is involved in osteoclast differentiation by tumor cells.

FIGURE 3. Embelin inhibits osteoclastogenesis induced by tumor cells. A, RAW 264.7 cells (10 × 10^3/mL) were incubated in the presence of MDA-MB-231 cells (1 × 10^3/mL; left and right) or conditioned medium from MDA-MB-231 cells (right), exposed to embelin (15 μmol/L) for 5 d, and then stained for TRAP expression. Multinucleated osteoclasts (i.e., those containing three nuclei) were counted (right). B, RAW 264.7 cells (10 × 10^3/mL) were incubated in the presence of U266 cells (1 × 10^3/mL; left and right) or conditioned medium from U266 cells (right), exposed to embelin (15 μmol/L) for 5 d, and then stained for TRAP expression. Multinucleated osteoclasts (i.e., those containing three nuclei) were counted (right). C, RAW 264.7 cells (2 × 10^3/1 mL) were incubated in the presence of conditioned medium from MDA-MB-231 and U266 cells for 24 h and then assessed for NF-κB activity by EMSA. Fold value is based on the value for medium (control), arbitrarily set at 1.
On the other hand, we studied the effect of embelin on RANKL-induced NF-κB activation in RAW 264.7 cells by EMSA (Fig. 4A). RAW 264.7 cells were pretreated with different concentrations of embelin for 12 h, treated with 10 nmol/L RANKL for 30 min, and then examined for NF-κB activation by EMSA. Fold value is based on the value for medium (control), arbitrarily set at 1. The cell viability (CV) was determined by the trypan blue exclusion assay. A, RAW 264.7 cells (1.5 × 10⁶/mL) were incubated with different concentrations of embelin for 12 h, treated with 10 nmol/L RANKL for 30 min, and then examined for NF-κB activation by EMSA. Fold value is based on the value for medium (control), arbitrarily set at 1. B, RAW 264.7 cells (1.5 × 10⁶/mL) were incubated with 30 μmol/L embelin for 12 h, treated with 10 nmol/L RANKL for the indicated times, and then examined by for NF-κB activation by EMSA. Fold value is based on the value for medium (control), arbitrarily set at 1. C, RAW 264.7 cells (1.5 × 10⁶/mL) were incubated with 30 μmol/L embelin for 12 h and treated with 10 nmol/L RANKL for the indicated times. Cytoplasmic extracts were examined for IκBα degradation by Western blot using an anti-IκBα antibody. Anti-β-actin antibody was used as a loading control.

Embelin inhibits RANKL-induced IκBα degradation and phosphorylation

Because the translocation of NF-κB to the nucleus succeeds the proteolytic degradation of IκBα, we next sought to determine whether embelin-induced NF-κB inhibition was due to inhibition of IκBα degradation. Therefore, we examined the NF-κB expression level after different stimulation times by RANKL in the nucleus by EMSA (Fig. 4B) and IκBα degradation in the cytoplasm by Western blot (Fig. 4C). As shown in Fig. 4B, RANKL activated NF-κB within 10 minutes, and embelin inhibited this activation. In accordance with EMSA results, Western blot analysis showed that RANKL induced IκBα degradation in control cells after 10 minutes and returned to normal level within 60 minutes (Fig. 4C), and cells pretreated with embelin for 12 hours showed no degradation of IκBα.

Given that IκBα phosphorylation is necessary for IκBα degradation, we investigated the effect of embelin on IκBα phosphorylation by using the proteasome inhibitor N-acetyl-leu-leu-norleucinal, which prevents RANKL-induced IκBα degradation (Fig. 5A). Western blot analysis showed that RANKL plus N-acetyl-leu-leu-norleucinal treatment induced phosphorylation of IκBα at serines 32 and 36, and that embelin pretreatment inhibited this activation in RAW 264.7 cells. Embelin alone did not induce phosphorylation of IκBα and only RANKL alone induced degradation of IκBα (Fig. 5A, middle). These results clearly indicate that embelin inhibits both RANKL-induced NF-κB activation and IκBα degradation and phosphorylation.
Embelin inhibits RANKL-induced IKK activation

Because IKK is required for RANKL-induced phosphorylation of IκBα and embelin inhibits the phosphorylation of IκBα, we determined the effect of embelin on RANKL-induced IKK activation (Fig. 5B and C). Results of the immune complex kinase assay showed that RANKL strongly induced the phosphorylation of glutathione S-transferase-IκBα by IKK within 2 minutes, and that embelin completely inhibited this phosphorylation. Neither RANKL nor embelin affected the expression of IKKα or IKKβ proteins (Fig. 5B). Given that activation of the IKK complex requires phosphorylation of its subunits IKKα/β, we next investigated whether embelin also inhibited the phosphorylation of IKKα/β (Fig. 5C, top) and/or prevented the binding of IKKα/β to the regulatory subunit IKKγ (Fig. 5C, bottom). We first pretreated RAW 264.7 cells with embelin and then stimulated them with RANKL. The result of Western blot with p-IKKα/β antibody (Fig. 5C, top) shows that embelin almost completely inhibits the phosphorylation of IKKα/β. On the other hand, an immunoprecipitation assay (Fig. 5C, bottom) investigating whether embelin disrupts IKK complex formation indicates that embelin pretreatment also induces a decrease in the binding of the IKKα/β subunits to the IKKγ subunit, suggesting that at least these two mechanisms are important for the inhibitory effect of embelin on IKK activation.

Inhibition of osteoclastogenesis by embelin is NF-κB specific

Given that RANKL-induced osteoclastogenesis is triggered by two main signaling pathways, namely, the NF-κB and the mitogen-activated protein kinase (MAPK) pathways, we investigated whether embelin pretreatment also affects the MAPK pathway. However, as shown in Fig. 5D, embelin

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**FIGURE 5.** Embelin suppresses RANKL-induced IκBα degradation and phosphorylation through inhibition of IKK activity. A, RAW 264.7 cells (1.5 × 10⁶/mL) were pretreated with embelin (30 μmol/L) for 12 h, incubated with N-acetyl-leu-leu-norleucinal (ALLN; 50 μg/mL) for 30 min, and then treated with RANKL (10 nmol/L) for 15 min. Cytoplasmic extracts were prepared and then analyzed by Western blot with either anti-phospho-IκBα or anti-IκBα antibodies. Anti-β-actin antibody was used as a loading control. B, RAW 264.7 cells (3 × 10⁶/mL) were pretreated with embelin (40 μmol/L) for 12 h and then incubated with RANKL (10 nmol/L) for the indicated times. Whole-cell extracts were immunoprecipitated using an antibody against IKKα and analyzed by an immune complex kinase assay using recombinant glutathione S-transferase (GST)-IkBα as described in Materials and Methods. To examine the effect of embelin on the level of IKK proteins, whole-cell extracts were analyzed by Western blot using anti-IKKα and anti-IKKβ antibodies. C, top, RAW 264.7 cells (1.5 × 10⁶/mL) were pretreated with embelin (40 μmol/L) for 12 h and then incubated with RANKL (10 nmol/L) for the indicated times. Whole-cell extracts were immunoprecipitated using an antibody against IKKα and analyzed by an immune complex kinase assay using recombinant glutathione S-transferase (GST)-IkBα as described in Materials and Methods. To examine the effect of embelin on the level of IKK proteins, whole-cell extracts were analyzed by Western blot using anti-IKKα and anti-IKKβ antibodies. C, top, RAW 264.7 cells (1.5 × 10⁶/mL) were pretreated with embelin (40 μmol/L) for 12 h and then incubated with RANKL (10 nmol/L) for the indicated times. Whole-cell extracts were immunoprecipitated using an antibody against IKKα and analyzed by Western blot using anti-IKKα antibody. Anti-IKKβ antibody was used as a loading control. C, bottom, RAW 264.7 cells (3 × 10⁶/mL) were pretreated with embelin (40 μmol/L) for 12 h and then incubated with RANKL (10 nmol/L) for the indicated times. Whole-cell extracts were immunoprecipitated using an antibody against IKKγ and then analyzed by Western blot using an anti-IKKγ antibody. Anti-IKKα antibody was used as a loading control. D, RAW 264.7 cells (1.5 × 10⁶/mL) were pretreated with embelin (40 μmol/L) for 12 h and then incubated with RANKL (10 nmol/L) for the indicated times. Whole-cell extracts were analyzed by Western blot using an anti-p-IκBα/β antibody. Anti-IKKα antibody was used as a loading control. E, RAW 264.7 cells (10 × 10³/mL) were incubated with 5, 10, or 15 μmol/L embelin and RANKL (5 nmol/L) for 5 d. Whole-cell extracts were analyzed by Western blot using an anti-procaspase-3 antibody. Anti-β-actin antibody was used as a loading control.
has no effect on p-ERK1/2, indicating that inhibition of osteoclastogenesis by embelin is probably specific to the NF-κB signaling pathway.

**Embelin induces apoptosis in mature osteoclasts**

It is known that osteoclastogenesis can be inhibited by a drug either by targeting signaling pathways involved in osteoclast differentiation, such as the RANKL pathway, or by inducing apoptosis of osteoclasts. To verify whether embelin induces apoptosis in RAW 264.7 cells, we treated cells with embelin and stimulated them with RANKL for 5 days. As shown in Fig. 5E, embelin induced apoptosis of mature osteoclasts, indicated by the activation of caspase-3.

**A NEMO binding domain peptide inhibits RANKL-induced osteoclastogenesis**

To further ascertain the specificity of embelin, we specifically inhibited NF-κB activation. The IKK complex, which is responsible for NF-κB activation, contains primarily the kinases IKKα and IKKβ and the regulatory kinase IKKγ, also known as the NF-κB essential modulator (NEMO). Whereas the serine kinases IKKα and IKKβ target the serines 32 and 36 of the IκBα protein, NEMO regulates the IKK complex activity through its binding to the COOH-terminal region of the IKKα and IKKβ subunits, called the NEMO binding domain. In this regard, a cell-permeable peptide that blocks the NEMO binding domain would inhibit the association of NEMO with the IKK complex and consequently suppress NF-κB activation and, most likely, osteoclastogenesis.

To determine the effect of the NEMO binding domain peptide (NBP) on RANKL-induced osteoclastogenesis, we pretreated RAW 264.7 cells with 100 μmol/L NBP for 2 hours and then treated the cells with RANKL for 5 days (Fig. 6). Our results showed that the peptide, which targets the NEMO binding domain, and thus inhibits IKK complex activity, strongly inhibited osteoclast formation (Fig. 6A and B). Furthermore, when we treated nuclear extracts from RAW 264.7 cells with 100 μmol/L NBP for 2 hours and then with RANKL for 30 minutes, RANKL-induced NF-κB activation was completely inhibited (Fig. 6C). These results confirm that NF-κB is responsible for osteoclast differentiation of RAW 264.7 cells, and that inhibition of NF-κB either by embelin or NBP prevents osteoclastogenesis.

**Discussion**

In this study, we investigated the effect of embelin, derived from the Ayurvedic medicinal plant *E. ribes*, on osteoclastogenesis induced by RANKL and tumor cells and on RANKL-induced NF-κB activation. Our results indicated that embelin suppressed RANKL-induced osteoclastogenesis in a dose- and time-dependent manner and completely prevented osteoclast differentiation induced by tumor cells in vitro. We established that this benzoquinone inhibited NF-κB activation by inactivating the IKK complex and thus prevented the subsequent inhibition of IκBα phosphorylation and degradation.

The role of the NF-κB cell signaling pathway in osteoclast differentiation has been shown by numerous studies. Mice lacking the NF-κB subunits p50 and p52 and mice deficient in IKKβ show severe osteopetrosis caused by failure of osteoclast formation (15, 16). Furthermore, Abu-Amer and colleagues (17) reported that an IκB super-suppressor blocked osteoclast differentiation and activation. The same
group reported that the dominant-negative IκB protein, which lacks the NH₂-terminal phosphorylation site, lowered NF-κB activation and reduced recruitment of osteoclasts (18).

Our results indicate that embelin inhibits NF-κB activation by inactivating the IKK complex through inhibition of the phosphorylation of IKKα/β and prevention of the binding of the IKKγ subunit to the IKKα/β subunits. How embelin inhibits the phosphorylation of IKKα/β is, however, not clear. Numerous kinases have been implicated in the activation of IKK, including AKT; MAPK/ERK kinase kinases 1, 2, and 3; glycogen synthase kinase-3β; and NF-κB-inducing kinase (NIK; ref. 19). Previously, our group has shown that NIK-induced NF-κB activation is blocked by embelin (10). Thus, it is possible that embelin inhibits RANKL-induced IKK activation through inhibition of NIK.

Osteoclastogenesis is dependent on RANKL under physiologic and pathologic conditions (20, 21). Although the mechanism by which RANKL induces osteoclastogenesis is not completely understood, it is known that RANKL-induced activation of NF-κB and MAPK pathways as well as upregulation of NFATc2 expression is required for osteoclastogenesis (16). Furthermore, RANKL, the best-known osteoclast inducer, has been implicated in bone metastasis resulting from a wide spectrum of tumor types including breast, prostate, neuroblastoma, multiple myeloma, thyroid, renal, and lung (5). Pharmacologic studies using various RANKL inhibitors suggest that RANKL within tumor-associated bone is a central pathway responsible for increased osteoclastogenesis and accelerated bone resorption (5).

To determine whether embelin, besides inhibiting RANKL-induced NF-κB activation, would also inhibit RANKL-induced osteoclastogenesis, we cocultured RAW 264.7 cells with embelin and RANKL for 5 days. Our results indicated that embelin effectively inhibits RANKL-induced osteoclastogenesis. In addition, a kinetic study indicated that embelin acts at an early step in the differentiation process of the osteoclasts. To further confirm that inhibition of the NF-κB signaling pathway is responsible for the arrest of the osteoclastogenesis process in our system, we used a cell-permeable peptide that targets the NEMO binding domain of the IKKα and IKKβ kinases and thus prevents NF-κB activation. This NBP has been shown to inhibit osteoclastogenesis in vitro (22, 23). In addition, previous studies showed that pharmacologic or genetic inactivation of IKKα and/or IKKβ is sufficient for inhibition of osteoclastogenesis and prevention of inflammation and osteolytic induced bone loss (24-27).

Our results showed that this NF-κB inhibitor completely blocked RANKL-induced osteoclastogenesis in the same manner as did embelin. Interestingly, the inhibitory effect of 100 μmol/L NBP on osteoclastogenesis was as potent after 5 days as was 15 μmol/L embelin, which suggests that embelin is a more potent inhibitor of osteoclastogenesis, at least in vitro.

Binding of RANKL to its receptor RANK activates two major signaling pathways leading to osteoclastogenesis, the NF-κB pathway through TRAF6 and the mitogen-activated protein kinase (MAPK) pathway (28). In the MAPK pathway, active ERK can directly phosphorylate c-Fos, and active c-Jun-NH₂-terminal kinase phosphorylates c-Jun (29). Our studies, however, show that embelin does not affect the MAPK pathway, indicated by no change in the p-ERK1/2 level after RANKL stimulation. This suggests that the inhibitory effect of embelin on osteoclastogenesis is probably specific to NF-κB inhibition.

Therapeutic inhibition of bone resorption is generally reached by reducing the differentiation rate of osteoclasts either directly by acting on osteoclast precursors or indirectly by downregulating the expression and production of RANKL (30). Another major strategy to decrease bone resorption is to increase or accelerate the death rate of osteoclasts (31, 32). Indeed, our results show that embelin induces apoptosis in mature osteoclasts. However, whether embelin induces apoptosis due to inhibition of NF-κB is not clear.

Malignant tumors with the skeleton as the primary site as well as metastatic bone lesions are considered a major health problem, affecting more than 350,000 patients in the United States annually. Among them, 70% to 95% of patients with multiple myeloma and up to 75% of patients with advanced breast cancer or prostate cancer develop bone metastasis (33, 34). This osteoclast-mediated bone destruction is a major complication in metastatic breast cancer and multiple myeloma.

To establish the effect of embelin on osteoclastogenesis induced by these tumor cells, we used breast cancer and multiple myeloma cells that are known to express RANKL (35, 36) and exhibit constitutive NF-κB activation (37, 38). Our group previously found that embelin can inhibit constitutive NF-κB activation in U266 cells (10). In this study, embelin completely inhibited osteoclastogenesis induced by these tumors, indicating that embelin is an attractive agent for the treatment of patients with metastasis to the bone. To date, several studies have shown that myeloma cells enhance osteoclast formation and activity through upregulation of RANKL (39-41) or by expressing RANKL themselves (42, 43). Similarly, our studies with RAW 264.7 cells cocultivated with conditioned medium from U266 or MDA-MB-231 cells confirm that certain factors secreted by tumor cells stimulate osteoclast differentiation and clearly show that these factors activate NF-κB. Furthermore, treatment with embelin inhibits osteoclastogenesis induced by conditioned medium from U266 or MDA-MB-231 cells, suggesting that embelin acts directly on osteoclasts and not on the proliferation of U266 or MDA-MB-231 cells.

Embelin, which is routinely used in traditional medicine, and thus should have minimum toxicity, could be a safe treatment for patients with secondary bone lesions associated with various cancers (22, 44, 45), including breast cancer, and those associated with other diseases, such as osteoporosis, Paget’s disease, and rheumatoid arthritis (46-49).

Bisphosphonate drugs, potent inhibitors of osteoclast formation and activity, are the current standard of care and are...
most widely used for the treatment of cancer-induced osteolytic diseases. Their efficacy in abating pain and prolonging time to significant skeletal complications has been shown in the treatment of breast cancer–induced bone diseases (46). However, there is increasing recognition of the increased risk of severe osteonecrosis of the jaw in cancer patients receiving bisphosphonates (47–49), with devastating consequences for affected patients (50). Thus, it is therefore prudent to develop other antiresorptive strategies to combat lytic bone lesions in patients with metastatic cancer, including breast cancer metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Embelin Suppresses Osteoclastogenesis Induced by Receptor Activator of NF-κB Ligand and Tumor Cells In vitro through Inhibition of the NF-κB Cell Signaling Pathway

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