**Plectranthus amboinicus** Attenuates Inflammatory Bone Erosion in Mice with Collagen-induced Arthritis by Downregulation of RANKL-induced NFATc1 Expression

YU-CHIEH HSU, CHIA-PI CHENG, and DEH-MING Chang

**ABSTRACT.** **Objective.** *Plectranthus amboinicus* has been known to treat inflammatory diseases or swelling symptoms. We investigated whether *P. amboinicus* exhibited an inhibitory effect on osteoclastogenesis in *vivo* and inflammatory bone erosion in collagen-induced arthritis (CIA) mice, an animal model of rheumatoid arthritis. We attempted to identify the active component of *P. amboinicus* involved in regulation of osteoclastogenesis.

**Methods.** We treated M-CSF- and RANKL-stimulated murine bone marrow-derived macrophages (BMM) and RANKL-induced RAW264.7 cells with different concentrations of *P. amboinicus* or rosmarinic acid, a phytopolyphenol purified from *P. amboinicus*, to monitor osteoclast formation by TRAP staining. The mechanism of the inhibition was studied by biochemical analysis such as RT-PCR and immunoblotting. CIA mice were administered gavages of *P. amboinicus* (375 mg/kg) or placebo. Then clinical, histological, and biochemical measures were assessed to determine the effects of *P. amboinicus* on synovial inflammation and bone erosion by H&E staining of the inflamed joints and ELISA.

**Results.** Rosmarinic acid strongly inhibited RANKL-induced NF-κB activation and nuclear factor of activated T cells c1 (NFATc1) nuclear translocation in BMM, and also inhibited RANKL-induced formation of TRAP-positive multinucleated cells. A pit formation assay and the CIA animal model showed that *P. amboinicus* significantly inhibited the bone-resorbing activity of mature osteoclasts.

**Conclusion.** We postulated that rosmarinic acid conferred the inhibitory activity on *P. amboinicus* for inhibition of osteoclastogenesis via downregulation of RANKL-induced NFATc1 expression. Our results indicated the possibility of *P. amboinicus* as a new remedy against inflammatory bone destruction. (J Rheumatol First Release July 1 2011; doi:10.3899/Jrheum.101223)

**Key Indexing Terms:**
COLLAGEN-INDUCED ARTHRITIS
OSTEOCLAST
PLECTRANTHUS AMBOINICUS
NUCLEAR FACTOR OF ACTIVATED T CELLS c1

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by the presence of synovitis, bone erosion concomitant with pannus formation, and the destruction of joint cartilage. An increasing body of evidence has demonstrated that osteoclasts play a pivotal role in the bone resorption in inflammatory joint diseases. Multinucleated giant cells with the phenotypic features of osteoclasts predominate at erosion sites in RA and animal models of collagen-induced arthritis. Further, it has been reported that mice lacking osteoclasts were resistant to arthritis-induced bone erosion. These findings indicate the application of herbal compounds that inhibit osteoclastogenesis at inflammatory sites might be promising in treatment of RA. However, no study has to date reported the regulatory effects of *Plectranthus amboinicus* on osteoclastogenic differentiation.

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Hsu, et al: *P. amboinicus* inhibits osteoclastogenesis
Bone mass in adults is intricately regulated by a balance between osteoclast-mediated bone resorption and osteoblast-induced bone formation. Perturbations in this balance lead to diseases such as osteoporosis caused by an abnormally high bone metastasis with enhanced bone-resorbing activity of osteoclasts. Therefore, upregulated activation of osteoclasts is the target for therapeutic intervention in pathological bone loss. The differentiation into multinucleated osteoclasts from hematopoietic monocyte/macrophage precursor cells requires 2 cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL). Osteoblasts produce these 2 cytokines in membrane-bound or secreted form when activated by interleukin 1 (IL-1), prostaglandin E2, and vitamin D3. RANKL expressed in osteoblasts is the key cytokine involved in the differentiation from precursor cells into osteoclasts, whereas M-CSF secreted by osteoblasts provides a survival signal for osteoclasts. Binding of RANKL to its receptor RANK activates tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which is critical for the RANK-induced activation of the NF-κB and mitogen-activated protein kinases (MAPK). Active extracellular signal-regulated kinase (ERK) can directly phosphorylate c-Fos and active c-Jun-N-terminal kinase (JNK) phosphorolysates c-Jun. Thus AP-1 transcription factor, a heterodimer composed of a Fos family (c-Fos, FosB, Fra-1, Fra-2) and a Jun family (c-Jun, JunB, JunD) protein, can be a target of ERK and JNK in response to RANKL in osteoclast precursor cells. In addition, nuclear factor of activated T cells 1 (NFATc1), the key transcription factor for osteoclastogenesis, is strongly induced by RANKL. Accumulating evidence suggests that NFATc1 directly regulates some osteoclast-specific genes such as TRAP, calcitonin receptor, cathepsin K, β3 integrin, and OSCAR.

*P. amboinicus* is known as a medicinal plant that has been used for abrasions and lacerations, for burns, coughs and colds, and also for conjunctivitis. The major components of *P. amboinicus* as an aqueous extract are Δ3-carene, γ-terpinene, camphor, and carvacrol. In Taiwan, *P. amboinicus* is a folk herb used to treat inflammatory diseases or swelling symptoms. A recent study revealed that treatment with *P. amboinicus* mitigated the arthritic severity in collagen-induced arthritis (CIA) rats. However, how *P. amboinicus* is involved in the regulation of inflammatory bone erosion in CIA remains elusive.

We assessed the effects of *P. amboinicus* and rosmarinic acid (RosA), a phytopolypheon, on RANKL-induced osteoclastogenesis in RAW 264.7 cells and bone marrow-derived macrophages (BMM). Moreover, we evaluated the in vivo efficacy of *P. amboinicus* in CIA mice to make a direct comparison with in vitro data obtained in the analog preparation from BMM.

**MATERIALS AND METHODS**

*Mice and reagents.* DBA/1J mice were purchased from the Jackson Laboratory and bred under conventional conditions at the Animal Center of the National Defense Medical Center, Taiwan. All experimental procedures were approved by the Institute Animal Care and Use Committee in Taiwan. The murine monocye/macrophage RAW 264.7 cell line was obtained from the Food Industry Research and Development Institute, Taiwan. *P. amboinicus* and RosA were kindly provided by Dr. Chi-Huey Wong (Academia Sinica, Taiwan). RANKL and M-CSF were purchased from Peprotech (London, UK). Anti-NF-κB p65, anti-ΙκBα, anti-p38, anti-ERK, anti-phospho-p38, and anti-phospho-ERK antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin was from Sigma Chemical Co. Anti-NFATc1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries Ltd.

*Purification of rosmarinic acid from *P. amboinicus.* RosA was purified from crude *P. amboinicus* extract as described. *P. amboinicus* crude leaf juice was centrifuged at 10,000 × g for 30 min, and the supernatant was filtered. The filtrate was lyophilized to yield a dry powder. The powder was then reconstituted in 50% acetonitrile-water mixture with 0.1% trifluoroacetic acid and purified by RPC18-HPLC. The fraction was monitored at 214 nm. A linear gradient from 90% solvent A (98% water, 2% acetonitrile, 0.1% trifluoroacetic acid) to 20% solvent B (90% acetonitrile, 10% water, 0.1% trifluoroacetic acid) in 30 min was used to purify RosA. The column was reequilibrated with 90% solvent A for 20 min prior to each injection. Peaks were further collected by a mobile phase CH312SB Advantec fraction collector. RosA was found in the peak with TR = 17.5 min as a single compound identified by mass spectrometry. The structure of RosA was identified by NMR spectroscopy (Figure 1). It was determined that 1 g of *P. amboinicus* crude dry powder can yield 8.9 mg of RosA.

*MTT assay.* Cells (1 × 10⁶ cells/well) were seeded in a 96-well plate with medium supplemented with 10% fetal bovine serum (FBS) and treated with various concentrations of *P. amboinicus* and RosA for 24 h, and then washed 3 times with phosphate-buffered saline (PBS) and treated with medium containing 500 µg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 30 min at 37°C. Cells were then washed with PBS and solubilized in 100 µl DMSO. The intracellular purple formazan concentrations were determined at 550 nm in an ELISA plate reader.

*Bone marrow macrophage culture and osteoclast differentiation.* Bone marrow cells from DBA/1J mice were obtained for osteoclast progenitor cell preparation. Bone marrow cells were isolated from tibias and femurs of 8-week-old male mice by flushing the bone marrow cavity with PBS. The
cells were centrifuged at 1000 x g for 10 min and exposed to 0.5 ml of hypotonic ACK buffer [0.15 mM NH₄Cl, 1 mM KClO₃, and 0.1 mM EDTA (pH 7.4)] at room temperature for 5 min to remove the red blood cells. They were incubated with α-MEM (Gibco BRL) containing penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated FBS for 12 h to separate the floating and adherent cells. The floating cells were collected, suspended in α-MEM, counted, seeded onto a 96-well plate (4 x 10⁴ cells/well), and cultured in α-MEM in the presence of 30 ng/ml M-CSF for 3 days to form macrophage-like osteoclast precursor cells. After the floating cells, including the lymphocytes, were removed by aspiration, the adherent osteoclast precursors were cocultured with activated lymphocytes in the presence of 30 ng/ml M-CSF and 100 ng/ml RANKL as well as *P. amboinicus* and RosA for 5 days to generate osteoclasts. On Day 3, the medium was replaced with fresh medium containing M-CSF, RANKL, *P. amboinicus*, and RosA.

**Cell culture of murine RAW264.7.** The murine monocye/macrophage cell line RAW264.7 was cultured with Dulbecco modified Eagle’s medium (Gibco BRL) containing 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). All cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C. To induce osteoclast differentiation, RAW264.7 cells were suspended in α-MEM containing 10% FBS, 2 mM L-glutamate, 100 U/ml penicillin, and 100 µg/ml streptomycin; seeded 10⁴ cells/dish in a 96-well plate; and cultured with 100 ng/ml soluble RANKL for 5 days. The medium was changed on Day 3.

**Tartrate-resistant acid phosphatase (TRAP) staining.** Cells were washed with PBS and fixed with 3.7% formaldehyde for 30 min. After washing with PBS, cells were incubated at 37°C in a humid and light-protected incubator for 1 h in the reaction mixture of the Leukocyte Acid Phosphatase Assay kit (Cat. 387, Sigma), as directed by the manufacturer. Cells were washed 3 times with distilled water and TRAP-positive multinucleated cells containing 5 or more nuclei were counted under a light microscope and photographed.

**Pit formation assay.** To assess the effect of *P. amboinicus* and RosA on RANKL-induced bone resorption, BMM were seeded onto 20 mm² dentine slices (Cat. 3988, Corning) in 24-well plates, 10⁵ cells/well. All cultures were incubated in triplicate, and cells were replenished every 3 days with fresh medium containing test chemicals. Then the dentine slice was treated with 1 N NH₄OH with sonication for 5 min. Resorption pits on the dentine slices and then incubated with 30 ng/ml M-CSF and 100 ng/ml RANKL in the presence or absence of RosA. The distribution of NFATc1 protein 24 h after stimulation was assessed according to published protocols. Coverslips were removed, washed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with 5% bovine serum albumin, and incubated overnight with a specific anti-NFATc1 monoclonal antibody (1:50; Santa Cruz Biotechnology). Cells were washed in PBS incubated 2 h with FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). After the immunostaining procedures, cells were nuclear-stained with DAPI (Sigma). Fluorescence was visualized using a Leica fluorescence microscope. The percentage of cells displaying nuclear staining was then quantified; 100 cells per group were measured from 3 separate coverslips per group.

**CIA mouse experiments.** DBA/1J mice, 6–8 weeks old, were housed in polycarbonate cages and fed with standard mouse chow and water ad libitum. Mice were maintained under climate-controlled conditions under 12 h light–dark cycle. Twelve mice were divided into 3 groups as follows: (1) CIA; (2) CIA plus vehicle; (3) CIA plus *P. amboinicus* (375 mg/kg).

**Induction of CIA and *P. amboinicus* treatment.** CIA was induced as described with minor modifications. Briefly, male DBA/1J mice were given an intradermal injection of 100 µg bovine type II collagen emulsified in complete Freund’s adjuvant (1:1, wt/vol; Chondrex, Redmond, WA, USA) into the middle region of the tail. Two weeks later, mice were given a booster intradermal injection of 100 µg bovine type II collagen emulsified in incomplete Freund’s adjuvant (1:1, vol/vol; Chondrex) into the base of the tail. *P. amboinicus* was dissolved in saline. After the second immunization, development of rheumatic symptoms was observed approximately on Day 22 and mice were administered test compounds by gavage after symptoms first occurred for 4 weeks. Mice were sacrificed under CO₂ euthanasia on Day 50 and hind leg joints were collected for histopathological assessments.

**Determination of marine IL-1β and TNF-α protein levels.** For quantitative analysis of IL-1β and TNF-α protein expression in serum, ELISA kits (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer’s instructions. Marine IL-1β and TNF-α protein levels were measured in mouse serum and the reaction was quantified in an ELISA microplate reader.

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**Table 1.** PCR was performed with mouse-specific primers.

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<tr>
<td>5’-ACA GTC TTT TGG GTG GCA GTA ATG T-3’ (reverse)</td>
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Hsu, et al: *P. amboinicus inhibits osteoclastogenesis*
Clinical assessments of arthritic index and paw thickness in CIA mice. A blinded independent observer with no knowledge of the treatment protocol performed evaluation of joint inflammation. The severity of arthritis in each footpad was quantified daily by a clinical score measurement. The level of arthritic inflammation of each paw was graded 0 to 4 according to Campo, et al. The maximum arthritic index of 4 paws was summed for each animal and ranged from 0 to 16 (0 = no disease; 16 = highest possible score). The degree of paw swelling was measured using a caliper. The increased thickness of paws was compared with normal mice without collagen immunization.

Histopathological assessment of arthritis. Sections were evaluated blindly as described. Ankle joints were stored in 10% neutral buffered formalin for at least 24 h prior to placement in decalciﬁer. After 1 day of decalciﬁcation, the digits were trimmed and the ankle joint was transected in the longitudinal plane to give approximately equal halves. These were processed for parafﬁn embedding, sectioned, and stained with hematoxylin and eosin for inﬂammation and bone erosion. Multiple sections were prepared to ensure that the distal tibia with both cortices was present and that abundant distal tibial medullary space was available for evaluation. Bone destruction was graded on a scale of 0–5, ranging from no damage to complete loss of the bone structure. Ankles of CIA mice were also scored 0–5 for inﬂammation (0 = normal; 1 = minimal inﬁltration of inﬂammatory cells in periarticular tissue; 2 = mild inﬁltration; 3 = moderate inﬁltration, with moderate edema; 4 = marked inﬁltration, with marked edema; and 5 = severe inﬁltration, with severe edema). Cartilage damage was not scored in the adjuvant arthritis model because we have found this generally to be a minor feature not reliable for evaluation of potential treatment effects.

Statistical analysis. Data were expressed as means ± SD. Statistical analyses were performed by a paired 2-tailed Student t-test assuming equal variances. Values of p < 0.05 were considered to indicate statistical significance.

RESULTS

Effects of P. amboinicus and RosA on RANKL-induced osteoclast differentiation. We screened for natural compounds that could potentially inhibit osteoclastogenesis by evaluating the activity of TRAP, a specific marker of osteoclast differentiation, using osteoclast precursor cell line RAW264.7. RosA decreased the number of multinucleated osteoclasts in a dose-dependent manner (Figure 2C, 2D). The effects of P. amboinicus and RosA on osteoclastogenesis were further conﬁrmed by using primary BMM. Murine BMM were formed from bone marrow cells within 3 days in response to M-CSF (30 ng/ml). BMM were cultured in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) together with or without various concentrations of P. amboinicus or RosA for 5 days. Both P. amboinicus and RosA inhibited osteoclast formation in a concentration-dependent manner when added throughout the entire culture period (Figure 2A, 2E). The IC50 values of P. amboinicus and RosA for TRAP-positive multinucleated osteoclast formation were 50 µg/ml and 40 µM, respectively (Figure 2B, 2F). RosA reduced the number of TRAP-positive multinucleated cells generated with 49% ± 4.7% and 84.1% ± 1.9% inhibition at 40 µM and 80 µM concentrations, respectively (Figure 2F). RosA 80 µM completely suppressed multinucleated osteoclast formation in RAW264.7 cells (Figure 2D). To examine the effect of P. amboinicus and RosA on cell growth, we treated cells with various concentrations of P. amboinicus or RosA for 24 h and measured cell growth by an MTT assay. P. amboinicus and RosA did not affect the cell growth rate of BMM and RAW264.7 (Figure 3A, 3B), sustaining substantial viability even when used at concentrations that signiﬁcantly inhibited osteoclast formation.

Effects of RosA on NFATc1-regulated gene expression during RANKL-induced osteoclast development. Osteoclast differentiation is associated with upregulation of specific genes in response to RANKL and RANK binding. The c-fos and NFATc1 genes have been reported to play a pivotal role in osteoclast differentiation, and NFATc1 regulates OSCAR expression. As well, DC-STAMP is essential for cell–cell fusion during RANKL-mediated osteoclastogenesis. Therefore, we examined the effects of RosA on the RANKL-induced regulation of c-fos and NFATc1 expression and assessed whether there were any effects on TRAP, OSCAR, DC-STAMP, and β3 integrin expression. BMM were pretreated with or without RosA and further stimulated with RANKL (100 ng/ml) in the presence of M-CSF (30 ng/ml) at various timepoints. Results revealed that c-fos and NFATc1 mRNA levels were increased in response to RANKL, but expression of both c-fos and NFATc1 was signiﬁcantly inhibited by RosA. TRAP, OSCAR, DC-STAMP, and β3 integrin mRNA expression was also signiﬁcantly inhibited by RosA in a concentration-dependent manner (Figure 4). This raises the possibility that RosA may inhibit osteoclast differentiation through inhibition of RANKL-induced c-fos and NFATc1 expression.

Effects of RosA on RANKL-induced MAPK activation in osteoclasts. Reports have indicated that RANKL induces the activation of 3 well known MAPK (ERK, JNK, and p38) in osteoclast precursor cells and RAW264.7 cells. These kinases (especially ERK and JNK) also participate in c-Fos and c-Jun activation in osteoclast precursors. Based on the ﬁndings that RosA suppressed RANKL-induced c-fos mRNA expression, we next investigated whether MAPK were involved in the inhibition of osteoclastogenesis by RosA in BMM. RANKL (100 ng/ml) markedly activated p38 and ERK phosphorylation measured at the early time-point of 30 min. Results indicated that RANKL-induced phosphorylation was inhibited by RosA in a concentration-dependent manner (Figure 5A).

Effect of RosA on RANKL-activated 1xB/IFN-κB. Activation of NF-κB is important in the activation and survival of mature osteoclasts, as well as osteoclastogenesis. Recent studies have suggested that NF-κB is another upstream transcription factor modulating NFATc1 expression. Activity of NF-κB is regulated by its inhibitor, 1xB, which forms a complex with NF-κB in the cytoplasm. 1xB degradation through an ubiquitin/proteasome pathway unmask the nuclear localization signal motif of NF-κB, which allows the transcription factor to translocate from the cytoplasm into the nucleus. To investigate the molecular mechanism of...
RosA-mediated inhibition of osteoclast differentiation, we examined the effect of RosA on changes in IκB and NF-κB after RANKL stimulation. As shown in Figure 5C, RosA reduced RANKL-induced IκB degradation within 30 min in a concentration-dependent manner. Further, nuclear translocation of NF-κB p65 was investigated. Results showed that...
RosA blocked IκB degradation and concomitantly suppressed the subsequent nuclear translocation of NF-κB p65 (Figure 5B).

**Effects of RosA on RANKL-induced nuclear translocation and expression of NFATc1.** Binding of RANKL to RANK activates several transcription factors responsible for promoting osteoclastic gene expression. These are not all activated within the same timeframe: early-response factors, such as NF-κB, are activated before late-response factors such as NFATc1. NFATc1 is one of the key transcription factors involved in osteoclast differentiation by RANKL. In the inactive state, NFATc1 is retained in the cytoplasm. When being activated, NFATc1 is translocated from the cytoplasm into the nucleus. Therefore, we examined the effect of RosA on the nuclear translocation of NFATc1 in RANKL-activated BMM by immunofluorescent staining. As shown in Figure 6A, the majority of NFATc1 was located primarily in the cytoplasm. Upon RANKL stimulation, nuclear accumulation of NFATc1 was markedly increased by RANKL stimulation for 24 h compared with the levels in cells without RANKL treatment. This elevated level of nuclear NFATc1 was reduced by RosA in a concentration-dependent manner. To further confirm whether RosA suppressed NFATc1 nuclear translocation at the protein level, we performed Western blotting to analyze NFATc1 in nuclear extracts and cytoplasmic extracts. The data showed that NFATc1 in whole-cell extracts and nuclear extracts increased dramatically after 24 h treatment by RANKL. However, treatment of RosA inhibited NFATc1 expression and its nuclear translocation (Figure 6B), suggesting that RosA suppressed RANKL-induced NFATc1 nuclear translocation and activation during osteoclastogenesis in BMM.

**Effects of P. amboinicus and RosA on bone resorption in cell cultures.** To examine the effects of RosA on osteoclastic bone resorption, resorption pit formation was assessed. BMM were plated onto dentine slices, and then differentiated into osteoclasts by RANKL treatment for 5 days in the absence or presence of P. amboinicus or RosA. After culture for 5 days, many resorption pits were formed on the bone slices. RANKL-evoked bone resorption was dose-dependently diminished by concurrent addition of P. amboinicus (Figure 7A) or RosA (Figure 7B) in BMM. These results indicated that both P. amboinicus and RosA prevented osteoclastic bone resorption by inhibiting osteoclastogenesis.

**Effects of P. amboinicus treatment on arthritis scores and paw swelling.** Arthritis developed roughly 3 weeks after the primary immunization with type II collagen. P. amboinicus significantly reduced the mean arthritis scores (Figure 8A) and paw swelling (Figure 8B) compared with the vehicle group.

**Effects of P. amboinicus treatment on histological findings.** After receiving P. amboinicus (375 mg/kg) by gavage for 4 consecutive weeks, mice were sacrificed to assess the effects of P. amboinicus on synovial inflammation and bone erosion by H&E staining and ELISA. The P. amboinicus–treated mice had much less infiltration of inflammatory cells, synovial hyperplasia, and articular bone destruction in inflamed joints than the vehicle-treated mice. Our data showed that P. amboinicus markedly reduced CIA-evoked synovial inflammation and bone destruction (Figure 9A). Studies have shown that regulation of the production of proinflammatory cytokines during CIA is critical in the control of synovial inflammation and tissue destruction. To elucidate how P. amboinicus ameliorates CIA, we investigated the effects of P. amboinicus on the levels of TNF-α and IL-1β in serum. Blood was withdrawn from the heart on Day 50, and the levels of the 2 cytokines were measured by ELISA. Consistent with the joint-swelling result, marked
Figure 4. Effects of RosA on NFATc1-mediated mRNA expression. Murine bone marrow-derived macrophages (BMM) were pretreated with or without RosA for 1 h and with RANKL (100 ng/ml) for the indicated time periods. Total RNA was isolated with TRIzol, and 1 µg of total RNA was used to transcribe cDNA. cDNA was used as a template for PCR with mouse-specific primers. Data represent means ± SD of more than 3 cultures. *p < 0.05, **p < 0.01, significantly different from values after treatment with RANKL alone.
decreases in the levels of TNF-α and IL-1β were observed in the mice treated with *P. amboinicus* compared with the control mice (Figure 9B). These results suggested that *P. amboinicus* inhibited the production of proinflammatory cytokines in the CIA mice.

**DISCUSSION**

The major remedies currently used in osteoporosis treatment primarily include estrogen replacement therapy along with bisphosphonates (e.g., alendronate, risedronate), selective estrogen receptor modulators, and calcitonin. However,
these therapies are associated with undesired effects such as breast cancer, endometritis, thromboembolism, hypercalcemia, gastrointestinal problems, and hypertension. Although a panel of drugs has been developed for the treatment of RA, therapies for RA are not satisfied with these marketed drugs.
P. amboinicus, belonging to the Lamiaceae family, is a perennial with a 3- to 10-year lifespan; it is distributed in tropical Africa, Asia, and Australia. It is used as a food, additive, fodder, and especially as a medicine in treating a wide range of diseases46. We examined the effects of P. amboinicus on osteoclast differentiation and identified the active ingredient. The crude extract of P. amboinicus was further separated into 16 fractions using HPLC. After the crude extract was fractionated, all the fractions were subjected to TRAP activity assay to determine which fraction consisted of active compounds that exert an inhibitory effect on osteoclastogenesis. Our data suggested that RosA, a phytopolyphenol purified from fraction no. 10, inhibited RANKL-induced osteoclast formation as effectively as the

Figure 7. Effects of P. amboinicus and RosA on resorption pit formation. Murine bone marrow-derived macrophages (BMM) were cultured on bone slices with various concentrations of P. amboinicus (A) or RosA (B) in the presence of RANKL (100 ng/ml). After culturing for 5 days, the dentine slices were recovered for Mayer’s hematoxylin staining to visualize resorption pits; arrows indicate pit area. Percentages of the resorbed area were determined using US NIH Image software. Data represent the means ± SD of more than 4 slices. *p < 0.05 and **p < 0.01, significantly different from values after treatment with RANKL alone.
crude *P. amboinicus* extract did in BMM. These phenomena were not attributed to the cytotoxicity of *P. amboinicus* and RosA. Other compounds of fractions no. 8 and no. 9 showed inhibitory activities as well (data not shown).

RosA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. RosA can be found in numerous plants, including species of the *Boraginaceae* and the *Lamiaceae*. Previous studies revealed that RosA has several antiinflammatory and antiallergic properties. Youn, *et al.* demonstrated that RosA suppressed synovitis in CIA mice and that it may be beneficial for treatment of RA. Moreover, it has been reported that osteoclast formation is enhanced in CIA rats. However, the effects of RosA on bone metabolism are not yet known.

Bone is restructured at such a high speed that approximately 10% of the total bone content is replaced every year in human adults. Osteoclast differentiation is a multistep process that involves cell proliferation, commitment, fusion, and activation. Under normal conditions, the RANKL–RANK axis appears to be essential for osteoclastogenesis. The signaling mechanism of RANKL has been studied extensively. Binding of RANKL to RANK strongly activates the NF-κB by degradation of IκBα, allowing it to translocate into the nucleus, where it enhances transcription of target genes. Our data showed that RosA suppressed the activation and nuclear translocation of NF-κB by the inhibition of IκB degradation.

In addition to NF-κB, RANKL also activates a series of

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**Figure 8.** Effects of *P. amboinicus* on arthritis scores and paw swelling with time course in CIA mice. Mice were administered placebo and *P. amboinicus* by gavage 3 times a week for 4 weeks. Arthritis scores (A) and paw thickness (B) were measured at 3-day intervals. *P. amboinicus* markedly reduced the mean arthritis scores and paw swelling. Data represent means ± SD of 4 mice. *p < 0.05, in comparison with relevant controls.
major intracellular signaling transduction pathways including JNK, ERK, p38 MAPK, and transcriptional factors such as AP-1 and NFATc1. NFATc1 is a key mediator of osteoclastogenesis that autoamplifies and conducts the expression of osteoclast-specific genes including TRAP, calcitonin receptor, OSCAR, and cathepsin K. We found that RosA decreased the phosphorylation of ERK and p38 in response to RANKL. Further, our data demonstrated that RosA also significantly suppressed not only RANKL-induced c-fos expression but also NFATc1 nuclear transport and further induction. It has been shown that c-Fos induces NFATc1 expression and that c-Fos and NFATc1 cooperatively regulate osteoclastogenesis in response to RANKL stimulation. Thus, we suggested that the inhibition of RANKL-induced c-fos expression by RosA is a relevant factor in the suppression of downstream NFATc1 signaling pathways. As a result.

Figure 9. Effects of *P. amboinicus* on clinical evaluation and levels of serum IL-1ß and TNF-α in CIA mice. A: Representative histological sections of ankle joints were stained with H&E. Synovial inflammation and bone erosion were scored with the criteria described in Methods. There was a significant reduction in both synovial inflammation and bone erosion. B: Serum IL-1ß and TNF-α measured on Day 50. Data represent means ± SD of 4 mice. **p < 0.01, in comparison with relevant controls. A representative result of at least 3 independent experiments is shown.
of downregulation of NFATc1 expression, the NFATc1-mediated osteoclastogenic genes such as TRAP, DC-STAMP, and OSCAR were also concomitantly inhibited by RosA. Considering these data together, we postulated that RosA conferred inhibitory activity on *P. amboinicus* for inhibition of osteoclastogenesis via downregulation of RANKL-induced NFATc1 expression.

RA is an autoimmune disease accompanied by hyperplasia of the cartilage lining caused by infiltration of inflammatory cells, ultimately resulting in joint damage. Various cytokines are known to be involved in RA, particularly proinflammatory cytokines such as TNF-α, IL-1β, and interferon-γ that are produced by macrophages, dendritic cells, and T cells. Our data showed that *P. amboinicus* significantly ameliorated CIA and diminished TNF-α and IL-1β production in CIA mice, consistent with a study by Chang, et al. *P. amboinicus* suppressed the osteoclast formation in BMM and the bone erosion in CIA mice.

We provided the first evidence that RosA, an active ingredient from *P. amboinicus*, had inhibitory effects on RANKL-stimulated osteoclast differentiation and pit formation on dentine slices in vitro. We also provided molecular signaling pathways for this inhibition, involving MAPK and transcription factors such as NF-kB, NFATc1, and c-Fos, which act on genes involved in osteoclast differentiation. We found that RosA blocked RANKL-stimulated activation of MAPK and NF-kB/IκB signaling pathways, and subsequently suppressed NFATc1 expression. Finally, RosA inhibited osteoclast differentiation via downregulation of NFATc1-modulated gene expression. Together, our data might decipher the possible molecular mechanisms by which *P. amboinicus* mitigates CIA-evoked bone destruction, and might make *P. amboinicus* a potential novel therapy for bone disorders such as RA and osteoporosis by fine-tuning RANKL-induced osteoclast differentiation and functions.

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