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Effects of cinnamoyloxy-mammeisin from geopropolis on osteoclast differentiation and *Porphyromonas gingivalis*-induced periodontitis

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Abstract

Background: Bone loss-related diseases such as rheumatoid arthritis, osteomyelitis, osteoporosis and periodontitis are associated with high rates of morbidity worldwide. These disorders are characterized by an imbalance between the formation and activity of osteoblasts and osteoclasts, leading to bone loss. In this context, we evaluated the effect of cinnamoyloxy-mammeisin (CNM), an anti-inflammatory coumarin found in *Melipona scutellaris* geopropolis, on key targets related to bone remodeling.

Methods: We investigated the *in vitro* effects of CNM on osteoclast differentiation and M-CSF +RANKL-induced osteoclastogenic markers expression. Additionally, the interference of CNM treatment on osteoclast activity was evaluated by zymography and resorption area. Finally, we assessed the capacity of the compound to mitigate alveolar bone loss *in vivo* in experimental murine periodontitis induced by *Porphyromonas gingivalis*.

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Conflict of interest

The authors have no conflicts of interest to declare.

Results: Treatment with CNM impaired osteoclast differentiation, as evidenced by a reduced number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells as well as the expression of osteoclastogenic markers upon M-CSF+RANKL-induced stimulation. Similarly, we observed reduced gelatinolytic and resorption capacity in M-CSF+RANKL-induced cells *in vitro*. Lastly, CNM attenuated alveolar bone loss in experimental murine periodontitis model.

Conclusion: CNM reduced M-CSF+RANKL-induced osteoclast differentiation and bone resorption, down regulating the expression of markers involved in osteoclast activity. These results were also observed *in vivo*, suggesting that CNM may be a promising treatment for bone loss diseases.

Keywords

cinnamoyloxy-mammeisin; osteoclastogenesis; RANKL; periodontitis

Introduction

Bone remodeling is a physiological process based on the equilibrium between the activity of osteoclasts and osteoblasts (Kim et al., 2015). A disturbance in this delicate balance, especially increased osteoclast activity, may lead to diseases such as rheumatoid arthritis, osteomyelitis, osteoporosis and periodontal disease (Ikeda and Takeshita, 2014). These disorders may originate from autoimmune, hormonal and infectious pathologies (Hayward and Fiedler-Nagy, 1987). The mediators involved on the onset of these diseases can induce osteoclastogenesis by accelerating the activity of the receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL). RANKL is a well-known osteoclastogenesis promoter that positively regulates the activation of kinases and transcription factors that lead to expression of osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP), V-ATPase, integrins, cathepsin K (CtsK), and metalloproteinases (MMP), at the end of the activation cascade (Leibbrandt and Penninger, 2008). These markers are thought to be important therapeutic targets in the development of new drugs against bone loss disorders such as periodontitis (Kajiya et al., 2010).

Natural products have been considered a promising source of biologically active molecules against several health disorders (Newman and Cragg, 2016), including bone loss-related diseases (Chiou et al., 2014, Thummuri et al., 2015, Kim et al., 2015). Yet, the therapeutic potential of some natural products such as geopropolis remains incompletely explored. Geopropolis is a mixture of resins, soil and wax collected by the stingless bee *Melipona scutellaris*, native to tropical areas (Velikova et al., 2000). Several studies from our group have reported its antimicrobial, anti-inflammatory and antiproliferative activities (Franchin et al., 2012, Franchin et al., 2013, da Cunha et al., 2013b, da Cunha et al., 2013a). Recently, (da Cunha et al., 2016), isolated and identified the 4-phenylcoumarin derivative cinnamoyloxy-mammeisin (CNM) as an abundant component and the major anti-proliferative constituent of geopropolis. Interestingly, CNM showed anti-inflammatory activity, by inhibiting MAPK, AP-1, and NF- κ B signaling pathways (Franchin et al., 2016b). Since those pathways participate in the osteoclastogenesis process, we hypothesized that CNM might have an effect on bone loss diseases.

The aim of this study was to investigate the effect of CNM on the osteoclastogenesis process using *in vitro* and *in vivo* models of bone loss.

Material and methods

Cinnamoyloxy-mammeisin from *M. scutellaris* geopropolis

CNM (C₃₄H₃₂O₇; Figure 1) was obtained as detailed by (da Cunha et al., 2016). The isolation and identification of CNM was performed as previously described and the purity (>95%) was verified by reverse phase high-performance liquid chromatography. CNM was isolated from geopropolis with a final yield of 0.5%.

Osteoclast Culture and TRAP staining

All experiments involving animals were conducted according to protocols approved by the Institutional Animal Care and Use Committee at the School of Pharmaceutical Science of Ribeirão Preto, University of São Paulo (process number 15.1.750.60.0). Bone marrow macrophages (BMM) from C57BL/6 mice were flushed from tibias and femurs and maintained for three days in α -minimal essential medium (α -MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 mg/mL of streptomycin (all from ThermoScientific, Carlsbad, CA, USA) in the presence of 30 ng/mL of murine macrophage colony-stimulating factor (M-CSF, R&D Systems). For the TRAP staining assay, non-adherent cells were removed and the remaining BMMs were seeded in appropriate cell plates and further cultured with both M-CSF (30ng/mL) and soluble murine RANKL (10ng/mL, R&D Systems) with or without different concentration of CNM for another three days. Cells were fixed and stained for TRAP with a specific commercial kit, following the manufacturer's instructions (Sigma-Aldrich 387A kit). TRAP-positive cells containing more than three nuclei/cell were considered osteoclasts and no distinctions were made between large and small osteoclasts. Nine fields were selected and counted for mature osteoclasts and were represented as multinucleated TRAP-positive (TRAP+) cells/well. To determine the osteoclast area we analyzed the images using ImageJ software (National Institutes of Health, Bethesda, MD). For substrate resorption, BMMs were plated onto specific hydroxyapatite-coated plates (OsteoAssay-Corning) with osteoclastogenic media as describe above. After four days, cells were removed with 5% sodium hypochlorite for 10 minutes and rinsed with distilled water as previously described (Barbour et al., 2014) Five wells were assessed per treatment in three different experiments.

Cell viability

BMMs were seeded in 96-well plates and cultured with osteoclastic media and different concentrations of CNM as described above. On the third day, thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) solution (5mg/mL) was added to each well. After 3h, the plate was centrifuged (200g/5 minutes), the medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan dye. The optical density (OD) was measured at 570nm using a microplate reader. The mean OD of the control group (RANKL) was set as 100%, and the experimental groups were compared to RANKL.

RT-qPCR

Total RNA was isolated from cell culture or maxillary tissue using the SV Total Isolation System kit (Promega) and quantified by spectrophotometer (NanoDrop-ThermoScientific) with an A260/280 ratio of 1.9 to 2.1. Complementary DNA was synthesized using HighCapacity kit (Applied Biosystems) using 500ng of total RNA. Quantitative real-time PCR (qPCR) was performed on StepOnePlus™ system using a predesigned TaqMan® primers/probes set (ThermoScientific-Applied Biosystems). The nuclear factor of activated T cells, calcineurin-dependent 1 (Nfatc1), tartrate-resistant acid phosphatase (Trap/Acp5), ATPase, H⁺ transporting, lysosomal V0 subunit D2 (V-ATPase), Ctsk, Integrin beta 3 (Itgb3) and matrix metalloproteinase 9 (MMP-9) was evaluated using glyceraldehyde 3-phosphate dehydrogenase (Gapdh) as an endogenous control (reference gene). The relative levels of mRNA expression were determined by the 2⁻ Cycle Threshold (2^{-CT}) method. The graphs show relative expression of the gene of interest normalized to the control population (e.g. RANKL/sham).

Western-blot analysis

BMMs were seeded in 24 well plates and cultured in α-MEM, 10% FBS, M-CSF (30 ng/mL), RANKL (10 ng/mL) and different concentration of CNM (2.5, 5 and 10µg/mL) for three days. The total cell lysates were obtained using RIPA buffer (Sigma-Aldrich) with a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). The protein concentration was determined using a BSA assay kit (ThermoScientific). Equal amounts of protein (5µg) were subjected to SDS/PAGE electrophoresis and further transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% non-fat milk for 1h at room temperature and incubated with specific antibodies against integrin αV, NFATc1, CtsK (all from Abcam) overnight at 4°C. After three washes with TBS-T, the membranes were incubated with an appropriate HRP-conjugated antibody for 2h at room temperature. Images were visualized and captured using Chemidoc-XRS system (Bio-Rad). Quantification of bands was performed with the densitometric program Image J using β-actin as a loading control. All gels were run under the same experimental conditions, and the results are representative of at least three independent experiments with similar results.

Gelatin zymography

Total cell lysates as described above (5µg) were subjected to standard gelatin zymography. Gelatinolytic activity of MMP-9 was assessed in 8% polyacrylamide gels copolymerized with 1% type 1 gelatin. After electrophoresis, the gels were washed twice with a 2.5% Triton X-100 solution (30 min each) and further incubated for 24 h in a buffer containing 50 mM Tris and 2.5 mM CaCl₂. Gels were staining with Coomassie blue solution and decolorized with 20% methanol and 10% acetic acid until clear bands had been visualized. The gel images were captured using a Chemidoc-XRS system (Bio-Rad) and quantified using the Image J software. A fold-change graph was made by dividing the means of the values for the treated cells (e.g., CNM group) by the mean of the values for the untreated cells (e.g., RANKL group).

Porphyromonas gingivalis-induced murine periodontitis

Male BALB/c mice were maintained in cages with controlled humidity and temperature, and light/dark cycles of 12 hours. *P. gingivalis* W83 was grown in pre-reduced brain heart infusion (BD-Difco, Sparks, MD, USA), supplemented with 1mg/mL hemin, and 0.5 mg/mL menadione (Sigma-Aldrich, St Louis, MO, USA) and incubated in a anaerobic jar (BD-Gaspak 85% N₂, 5% CO₂, 10% H₂) at 37°C for two days. Before oral infection, all animals received antibiotic treatment (sulfamethoxazole/trimethoprim 400, 80 mg/5mL) for seven days to reduce indigenous flora, followed by two days antibiotic-free. Animals were then orally inoculated with 100 µL of 10⁹ colony forming units (CFU) of *P. gingivalis* diluted in carboxymethylcellulose and phosphate buffered saline, five times with two days intervals. After the last inoculation animals received an intradermal injection in the dorsal region with 100 µg/Kg of CNM in phosphate buffered saline (PBS) with two days interval during thirty days (15 inoculations), as previously described (Franchin et al., 2016b, Franchin et al., 2016a). Sham-infected animals received the same volume of carboxymethylcellulose in PBS. Thirty days after the last inoculation, in order to evaluate alveolar bone loss, the maxilla were hemisected, exposed overnight to 3% hydrogen peroxide, and mechanically cleaned. To distinguish the cement-enamel junction, the mouse maxillae were stained with 0.3% methylene blue. The palatal faces of the molars were photographed with 2.5 x magnification using a stereomicroscope (ZEISS CL1500 ECO) and a digital camera (Canon EOS 1000D). The images were analyzed by ImageJ software. Quantitative analysis was used to measure the distomesial area (mm²) between the cement-enamel junction and the alveolar bone crest on the palatal side of the first and second molar as previous described (Barbour et al., 2014, Prates et al., 2014).

Statistical analyses

In all experiments, values are expressed as mean ±SEM. Differences between groups were tested for significance by One-way ANOVA with Bonferroni's multiple comparison tests using GraphPad Prism 5 software. A p-value of <0.05 was considered significant.

Results

Cinnamoyloxy-mammeisin (CNM) impairs RANKL-induced osteoclast differentiation.

To explore the effect of CNM on osteoclast differentiation, BMMs were cultured with M-CSF and RANKL and with several concentrations of CNM (2.5, 5 and 10µg/mL). In BMMs treated with M-CSF and RANKL, numerous large TRAP⁺ cells were formed after three days of culture (Figure 1B). However, when the stimulated BMMs were exposed to CNM, the number of multinucleated TRAP⁺ cells was substantially decreased in a concentration-dependent manner (Figure 1B, C) evidenced by the extensive prevention of multinucleated TRAP⁺ cells formation at the concentration of 10µg/mL. To exclude a possible cytotoxic effect of CNM on RANKL-induced osteoclasts, we performed a cell viability assay using MTT in the presence of M-CSF and RANKL alone or together with 2.5, 5, or 10 µg/mL of CNM. We found that CNM did not exhibit cytotoxic effects at any tested concentration, indicating that the inhibitory effect of CNM was not due to a cytotoxic effect on RANKL-induced osteoclast formation (Figure 1D).

CNM treatment affects the expression of osteoclastogenic markers in RANKL-stimulated cells.

To determine the effect of CNM on the expression of different osteoclastogenic marker genes we performed RT-qPCR analysis on cells. Treatment with M-CSF+RANKL upregulated the expression of *Nfya1*, *Trap/Acp5*, *Itgb3*, *V-ATPase*, *Ctsk* and *MMP-9*, (Figure 2A–F). We found a dramatic decrease in expression of all genes tested by CNM at 10µg/ml, although no clear differences in mRNA expression of osteoclast genes were noted at 2.5 and 5µg/ml concentrations. Additionally, we performed western-blot analysis to confirm the RT-qPCR data at the protein level (Figure 2G, H). The expression of NFATc1, a key transcription factor to osteoclast differentiation was down-regulated by CNM in a concentration dependent manner. On the other hand, the protein levels of integrin α V and CTSK were affected only at the highest concentration of CNM.

CNM treatment reduces both osteoclast gelatinolytic and resorption capacity.

Next, we performed two assays to examine osteoclast function and determine whether the inhibitory effect of CNM on osteoclast formation previously observed would also affect osteoclast function. First, we used a standard gelatinolytic assay to measure MMP-9 function. We observed a gradual inhibition of gelatinolytic activity in CNM-treated cells compared to control at 2.5 and 5µg/ml, as depicted in Figure 3A, while MMP-9 was almost completely inhibited at 10µg/ml, corroborating our RT-qPCR results. Second, in order to assess whether the quantitative change in osteoclast formation produced by CNM was associated with any change in their bone resorbing capacity, we evaluated the resorption in hydroxyapatite-coated plates. CNM significantly reduced the matrix resorption capacity at 5 and 10 µg/ml as shown in Figure 3B.

CNM attenuates alveolar bone loss in experimental murine periodontitis.

Given the clear effect of CNM on osteoclast differentiation and resorption capacity, we next investigated these parameters using a *P. gingivalis*-induced experimental model of periodontitis. The results showed reduced alveolar bone resorption in the *P. gingivalis* and CNM-treated group compared to the *P. gingivalis* alone group (Figure 4A). We also assessed *Trap/Acp5*, *Ctsk* and *Mmp9* mRNA levels in the maxillary tissue. The *P. gingivalis* and CNM-treated group showed a reduced mRNA level for *Trap/Acp5* and *Ctsk* compared to the *P. gingivalis* infected group, however, no differences were found in *Mmp9* mRNA expression with any treatment (Figure 4B).

Discussion

Bone loss diseases can be triggered by immune-inflammatory agents which have the ability to activate different pathways leading to induction of enzymes and transcription factor expression. Therefore, impairing the activity or expression of these targets could be an important therapeutic strategy for bone loss diseases (Wada et al., 2006). Among the available drugs for treating bone loss related conditions, bisphosphonate - an antiresorptive agent, increases the risk of osteonecrosis of the jaw in osteoporotic patients (Khosla et al., 2007). Therefore, alternatives to use of bisphosphonates have been sought in order to overcome these limitations.

In the present study, we show that CNM significantly inhibits RANKL-induced osteoclast differentiation in BMC, as evidenced by TRAP activity, multinucleation, and bone resorption by the cells. The resorptive capacity of mature osteoclasts is dependent on several components, such as transcription factors, structural proteins and enzymes. Therefore, once we verified the activity of CNM on osteoclast differentiation and activity, we investigated the influence of CNM on gene expression of important markers of osteoclast activity. In accordance to the results observed with osteoclast differentiation, CNM reduced the expression of the transcription factor NFATc1 and mRNA for TRAP and the transmembrane protein integrin β , which could explain the reduced M-CSF+RANKL-induced TRAP⁺ cell differentiation. Besides TRAP, CNM down regulated the gene expression of CtsK, Mmp9 and V-ATPase, enzymes responsible for matrix degradation (Helali et al., 2013, Qin et al., 2012). Thus, these results explain the reduced pit formation observed after pre-treatment with CNM.

Next, we explored the mechanisms underlying the inhibitory activity of CNM on osteoclast differentiation by evaluating protein expression levels of osteoclast activity markers. As shown Figure 3A, CNM attenuated protein expression of integrin α V, CtsK and NFATc 1 in a dose dependent manner in M-CSF+RANKL-stimulated BMC. Although the mode of action of integrins on osteoclast differentiation is not fully understood, it is known they play a key role in osteoclast migration and resorption activity by mediating cell-cell and cell-matrix interactions (Nakamura et al., 1999). The inhibition of these targets has been proposed as a new approach for the treatment of conditions characterized by uncontrolled osteoclast activity (An et al., 2016). Furthermore, in corroboration of previous data showing a reduced expression of CtsK mRNA, protein levels of this marker were also decreased after treatment with CNM. CtsK is a cysteine protease secreted by osteoclasts in the sealed zone and is responsible for degradation of type I collagen in hard tissues (Helali et al., 2013). Therefore, the down-regulation of both integrin α V and CtsK by CNM may explain the impaired activity of osteoclasts in the pit forming assay.

Following the RANKL/RANK interaction, important protein kinases and transducers are activated, such as MAPK and NF-Kb, followed by a complex cascade, that ends up with the expression of enzymes and structural proteins responsible for osteoclast differentiation and activity (Warren et al., 2015). It is well established that the NF-kB signaling pathway plays an important role in RANKL mediated osteoclast differentiation. The NF-kB activation promotes the expression of NFATc1, a transcription factor responsible for encoding important mediators of the osteoclastogenesis and inflammatory process (Jimi et al., 2004). Thus, selective inhibition of NF-kB represents a relevant target for preventing osteoclastogenesis as well as inflammatory bone destruction. As we observed in this study, CNM suppressed the expression of NFATc1 (Figure 3A), which can explain the consequent reduction of mRNA levels of the markers of osteoclast activity, as previously mentioned. The inhibitory activity of CNM on NFATc1 might be due to its regulatory effects on NF-kB and MAPK, as demonstrated previously (Franchin et al., 2016b).

Furthermore, we evaluated whether CNM affects MMP-9 activity. As depicted in Figure 3B, CNM reduced MMP-9 proteolytic activity at 5 and 10 μ M. MMP-9 belongs to the class of cysteine proteases and is an important agent of matrix degradation in conditions such as

periodontal disease, in which the overexpressed MMP-9 leads to alveolar bone and connective tissue loss (Sundaram et al., 2007). Both the reduced level of mRNA and enzyme activity evidenced on zymography suggest a possible inhibitory effect of CNM on inducers of MMP-9 expression upstream in the cascade.

Last, to confirm the *in vitro* activity of CNM on osteoclast formation and function, we evaluated the *in vivo* effects of CNM in a mouse bone loss model triggered by *P. gingivalis* infection. As shown in Figure 4, animals only infected with *P. gingivalis* exhibited a significantly greater bone loss when compared to a uninfected control group. However, the animals which simultaneously received the CNM treatment showed a reduction of bone resorption area, when compared to infected group. Further, we observed a significant down regulation in the expression of TRAP and CtsK mRNA upon treatment with CNM. These results suggest that CNM might have protective effects in *P. gingivalis*-induced periodontitis by attenuation of bone loss observed in the CNM-treated group. It is possible that CNM inhibited oral bone loss by targeting inflammatory pathways, such as MAPK and NF- κ B, and by damping pro-inflammatory cytokines as originally described using peritonitis and arthritis models of inflammation (Franchin et al., 2016b). Here, using an animal model, we suggest a possible novel therapeutic strategy for the treatment of disorders associated with bone loss such as periodontitis.

Conclusion

In summary, we have shown that CNM isolated from *M. scutellaris* geopropolis downregulates M-CSF+RANKL-induced osteoclast differentiation and resorption activity. Furthermore, the observed *in vitro* anti-osteoclastogenic efficacy of CNM was confirmed in an *in vivo* model of *P. gingivalis* infection induced periodontal disease. Taken together, these data suggest that CNM must be considered as a promising alternative to treat bone loss diseases, although further studies are necessary.

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Figure 1.

Effect of cinnamoyloxy-mammeisin (CNM) on osteoclast differentiation. (A) Chemical structure of CNM. (B, C) Mouse bone marrow cells (BMM) were cultured with or without the indicated concentration of CNM (2.5, 5 and 10µg/mL) in the presence of M-CSF (30ng/mL) and RANKL (10ng/mL). After three days, cells were fixed and stained for TRAP. Multinucleated TRAP+ cells containing more than three nuclei were considered mature osteoclasts and were measured (area) and counted (osteoclasts per well). (D) Cell viability was assessed using the MTT assay. All results are representative of at least three independent experiments. The bars represents mean $\pm$ SEM. One-way ANOVA with Bonferroni's multiple comparison tests was used to determine statistical significance. * $p < 0.05$.

Figure 2.

CNM affected expression of osteoclastogenic markers in M-CSF+RANKL induced osteoclastogenesis. (A-G) mouse bone marrow macrophages (BMM) were incubated in the presence of M-CSF (30ng/mL) and RANKL, (10ng/mL) and with or without CNM (2.5, 5 and 10 μg/mL). At day three, RNA was extracted and the mRNA expression of NFATc1(A), Trap/Acp5 (B), Itgb3 (C), V-ATPase (D), Ctsk(E) and Mmp9 (F) was analyzed by quantitative PCR (RT-qPCR). The graphs represents mean ± SEM. For protein levels, BMMs were cultured as describes above and the total cell lysates were subjected to Western blots with the indicate specific antibodies (G). β-Actin was used as a loading control. The graph represents the densitometric measurement normalized by β-Actin and bars represents mean ± SEM (H). All gels were run under the same experimental conditions. All results are representative of at least three independent experiments. One-way ANOVA with Bonferroni's multiple comparison tests was used to determine statistical significance.

*p<0.05

Figure 3.

Effect of CNM on osteoclast function. Mouse bone marrow macrophages (BMM) were incubated in the presence of M-CSF (30ng/mL) and RANKL, (10ng/mL) and with or without CNM (2.5, 5 and 10 μM). Gelatinolytic activity of total cell lysates with or without CNM was accessed by gelatin zymography (A). The graph represents the densitometric measurement relative to the M-CSF+RANKL group, and bars represents mean $\pm$ SEM.(B) BMM were cultured as described above and the resorptive activity was measured in hydroxyapatite coated plates at day four. The graph represents resorption area relative to RANKL. All results are representative of at least three independent experiments. One-way ANOVA with Bonferroni's multiple comparison tests was used to determine statistical significance. * $p < 0.05$.

Figure 4.

Effect of CNM on *P. gingivalis*-induced murine periodontitis. Representative images show maxillary bone loss in sham-infected, *P. gingivalis*-infected, and *P. gingivalis*-infected mice treated with 100 µg/kg i.d. of CNM (A). Effect of CNM in the quantitative analysis of bone loss between the cement-enamel junction (CEJ) and the alveolar bone crest (ABC) of the first and second molar of sham, *P. gingivalis*-infected and *P. gingivalis*-infected+CNM. Graphs represent mean ± SEM of mice pooled from two experiments (n=4). (B) mRNA levels of Trap/Acp5, Ctsk and Mmp9 in maxillary tissue. Bars represent mean ± SEM. One-way ANOVA with Bonferroni's multiple comparison tests was used to determine statistical significance. *p<0.05.