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RESEARCH PAPER

An endogenous regulator of inflammation, resolvin E1, modulates osteoclast differentiation and bone resorption

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Background and purpose: The inflammation-resolving lipid mediator resolvin E1 (RvE1) effectively stops inflammation-induced bone loss *in vivo* in experimental periodontitis. It was of interest to determine whether RvE1 has direct actions on osteoclast (OC) development and bone resorption.
Experimental approach: Primary OC cultures derived from mouse bone marrow were treated with RvE1 and analysed for OC differentiation, cell survival and bone substrate resorption. Receptor binding was measured using radiolabelled RvE1. Nuclear factor (NF)-κB activation and Akt phosphorylation were determined with western blotting. Lipid mediator production was assessed with liquid chromatography tandem mass spectrometry.
Key results: OC growth and resorption pit formation were markedly decreased in the presence of RvE1. OC differentiation was inhibited by RvE1 as demonstrated by decreased number of multinuclear OC, a delay in the time course of OC development and attenuation of receptor activator of NF-κB ligand-induced nuclear translocation of the *p50* subunit of NF-κB. OC survival and apoptosis were not altered by RvE1. Messenger RNA for both receptors of RvE1, ChemR23 and BLT_1 is expressed in OC cultures. Leukotriene B_4 (LTB_4) competed with [^{3}H]RvE1 binding on OC cell membrane preparations, and the LTB_4 antagonist U75302 prevented RvE1 inhibition of OC growth, indicating that BLT_1 mediates RvE1 actions on OC. Primary OC synthesized the RvE1 precursor 18*R*-hydroxy-eicosapentaenoic acid and LTB_4. Co-incubation of OC with peripheral blood neutrophils resulted in transcellular RvE1 biosynthesis.
Conclusions and implications: These results indicate that RvE1 inhibits OC growth and bone resorption by interfering with OC differentiation. The bone-sparing actions of RvE1 are in addition to inflammation resolution, a direct action in bone remodelling.
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Keywords: lipid mediators; inflammation resolution; osteoclasts; cell differentiation

Abbreviations: 18-HEPE, 18*R*-hydroxy-eicosapentaenoic acid; EPA, eicosapentaenoic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; LTB_4, leukotriene B_4; M-CSF, murine colony-stimulating factor; OC, osteoclast; PGE_2, prostaglandin E_2; PUFA, polyunsaturated fatty acid; RANKL, receptor activator of nuclear factor-κB ligand; RvE1, resolvin E1; TRAP, tartrate-resistant acid phosphatase

Introduction

The health benefits of polyunsaturated fatty acids (PUFAs) present in fish oil have been long known, but the cellular and molecular mediators of their actions are just being discovered (Serhan, 2007). Resolvin E1 (5*S*,12*R*,18*R*-trihydroxy-eicosapentaenoic acid, RvE1) is an oxygenated product of eicosapentaenoic acid (EPA), one of the main dietary essential ω-3 PUFAs (Serhan *et al.*, 2000; Arita *et al.*, 2005a). RvE1 was first described in inflammatory exudates of the murine dorsal pouch (Serhan *et al.*, 2000). RvE1 acts by reducing neutrophil infiltration and by promoting phagocyte removal, thus hastening inflammation resolution (Arita *et al.*, 2005a; Schwab *et al.*, 2007; Serhan, 2007). RvE1 reduces leukocyte infiltration into the peritoneum and protects from

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experimental colitis (Arita *et al*., 2005c). In experimental periodontal disease, topical RvE1 prevents *Porphyromonas gingivalis*-induced periodontal inflammation and alveolar bone loss (Hasturk *et al*., 2006). The question arising from the latter observation is whether the bone-sparing action is entirely mediated through RvE1's anti-inflammatory and pro-resolution actions, or whether RvE1 directly acts on bone cells. Other lipid PUFA derivatives such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) act directly on osteoclast (OC) in addition to their pro-inflammatory actions (Raisz, 2005).

Dietary intake of PUFA can also influence bone metabolism, as increasing the ratio of ω-6/ω-3 PUFA in the diet leads to increased arachidonic acid/EPA ratio in the bone, increased PGE_2 production and decreased bone formation in rats (Watkins *et al*., 2000). Treatment of bone marrow cultures with EPA, the precursor of RvE1, decreases OC formation *in vitro*, implying direct lipid action on OC (Sun *et al*., 2003). In the oral cavity, EPA-enriched diet reduces OC activity and alveolar bone resorption during orthodontic movement of rat teeth (Iwami-Morimoto *et al*., 1999). However, the molecular mechanisms of PUFA on bone cells are not known. In this study, we explore the potential of RvE1 to directly influence OC differentiation and bone resorption.

Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Boston University and were performed in conformance to the standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. C57BL/6 male mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All animals received standard laboratory chow diet and water *ad libitum*.

OC cultures

OC cultures were generated as described earlier (Gyurko *et al*., 2005). Briefly, bone marrow cells were flushed from freshly isolated femurs and tibias of 3- to 5-week-old mice. Bone marrow cells were cultured in 48-well tissue culture plates (5×10^5 cells each well) for tartrate-resistant acid phosphatase (TRAP) staining and for dentin disc resorption, or in 6-well plates (4×10^6 cells each well) for protein and RNA extraction. OC differentiation was induced with murine colony-stimulating factor (M-CSF, 50 ng mL^{-1}) and soluble receptor activator of nuclear factor-κB ligand (RANKL, 20 ng mL^{-1}) in a culture media, α-MEM (minimum essential medium) containing 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin. Culture media were replaced every other day. At the end of the incubation period, bone marrow cultures were fixed in 10% formalin and stained for TRAP for 15 min. Digital images of TRAP-stained OC were captured with an inverted microscope (Zeiss Axiovert 200) and a video camera. Images of TRAP-positive cells were digitally filtered to remove cells with fewer than three nuclei using image analysis software. OC data are expressed as the percentage of total culture dish area covered by TRAP-stained cells with three or more nuclei (OC covered area), or individual OC counts for nuclear count classification and for apoptotic OC.

In vitro *bone resorption*

Bone marrow cells were cultured as above in 48-well tissue culture plates containing one 5-mm dentin disc in each well. After 14 days of culture, dentine discs were washed with 0.2 M ammonium hydroxide and with water to remove cells, then dried. Discs were sputter-coated with gold and palladium and four rectangular images (total of 14 mm^2 surface area) of the disc were captured with a scanning electron microscope (Phillips XL-20) in sequential frames. Resorption pits were counted with Olympus MicroSuite image analysing software. For enumeration, a single pit was counted as any contiguous area of bone resorption, even if it contained more than one scalloped area.

Western blotting

Cells were collected using RIPA buffer and centrifuged at 10 000 *g* for 10 min. Protein content was measured by the Bradford method, and samples (50 μg) were resolved on a 10% sodium dodecyl sulphate-PAGE and electrophoretically transferred into polyvinylidene difluoride membranes (100 V; 60 min). The membranes were blocked with 5% non-fat dry milk for 60 min, and incubated overnight at −4 °C with polyclonal primary antibodies against p50 and Akt. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody in Tris-buffered saline for 1 h at room temperature. The band density was measured using an imaging densitometer.

Reverse transcription-PCR

Total RNA was isolated from OCs using TriZol reagent, and first strand cDNA was synthesized with High Capacity cDNA Archive kit according to the manufacturer's instructions. Amplification of mouse ChemR23, mouse BLT1 and β-actin was carried out with HotStartTaq DNA polymerase. The sets of specific primers were as follows: mouse ChemR23 (376 bp), 5′-CTGATCCCCGTCTTCATCAT-3′ for forward and 5′-TGGTGAAGCTCCTGTGACTG-3′ for reverse; mouse BLT1 (546 bp), 5′-GATCTGCGCTCCGAACTATC-3′ for forward and 5′-GACTCAGGAATGGTGGAGGA-3′ for reverse (β-actin (642 bp), 5′-ATGACCCAGATCATGTTTGAG-3′ for forward and 5′-AGGAGCAATGATCTTGATCTTCA-3′ for reverse (Omori *et al*., 2004). The thermal cycle (35 cycles) contained the following profile: denaturation, 95 °C for 1 min; annealing, 58 °C for mouse ChemR23 and BLT1 for 1.5 min and 60 °C for β-actin for 1 min; and extension, 72 °C for 1 min. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

Mediator lipidomics and pathway profiling

Liquid chromatography tandem mass spectrometry (LC-MS/MS)-based mediator lipidomic analyses were carried out as in Arita *et al*. (2005a). Here, 7-day-old OC cultures were treated

with acetylsalicylic acid (ASA, 500 μM) for 30 min, then treated with EPA (5 μM) for 30 min in 0.5 mL phosphate-buffered saline (pH 7.45) at 37 °C in 5% CO_2. In the OC and neutrophil co-culture experiments, OCs were treated with ASA and EPA as above, then freshly isolated human peripheral blood neutrophils (10^5 cells) were layered on the OC. Neutrophils were activated with zymosan (20 μg mL^{-1}) and the co-cultures were incubated at 37 °C for 30 min in 5% CO_2. Incubation was stopped by the addition of 1 mL methanol and cells were collected with a cell scraper. The resulting methanol fraction was centrifuged, and the supernatant was extracted using C-18 solid-phase extraction cartridge and subjected to analysis using a ABI Qstar XL Quatrupole TOF tandem mass spectrometer coupled with a Hewlett–Packard Series 1100 HPLC system, equipped with a UV diode array detector (G1315A) and a Phenomenex Luna C18(2) column (150 mm × 2 mm × 5 μm; part number: 00F-4252-B0) with mobile phase (methanol/water/acetic acid, 65:35:0.01) at a 0.2 mL min^{-1} flow rate, and UV spectra were recorded 0.1 min before samples entered MS/MS.

Radioligand-specific binding

Binding studies were conducted with tritiated RvE1 (6,7,14,15-[^{3}H]RvE1; 100 Ci mmol^{-1}) synthesized as in Arita *et al*. (2005a) using custom tritiation of acetylenic RvE1 followed by HPLC isolation. The binding mixture (100 μL) contained isolated cell membrane preparations (10 μg protein) and 5 nM of [^{3}H]RvE1 with or without unlabelled competitors in binding buffer (50 mM HEPES, 1 mM $CaCl_2$ and 5 mM $MgCl_2$) for 1 h at 4 °C. For determination of nonspecific binding, at least 1000 × concentration of unlabelled RvE1 (5 μM) was used. The bound and unbound radioligands were separated by filtration through Whatman GF/C glass microfibre filters and radioactivity was determined using a liquid scintillation counter (Packard Tri-Carb 2900TR).

Materials

M-CSF and RANKL were obtained from Peprotech (Rocky Hill, NJ, USA). RvE1 and 18*R*-hydroxy-eicosapentaenoic acid (18-HEPE) were prepared by total organic synthesis, as in Arita *et al*. (2005a), by N Petasis in Core C of the Specialized Center for Oral Inflammation and Resolution (P50 DE-16191). The structural integrity of RvE1 was monitored using UV tandem LC-MS/MS (Serhan *et al*., 2000; Arita *et al*., 2005a). Immediately before use, RvE1 was diluted in phosphate-buffered saline to final ethanol concentration of <1%. Culture media and TriZol reagent were obtained from Invitrogen (Carlsbad, CA, USA); the inverted microscope (Zeiss Axiovert 200) from Carl Zeiss Microimaging Inc. (Thornwood, NY, USA); the video camera (Sony DFW-X700) from Sony Corp. (Tokyo, Japan); image analysis software from Olympus MicroSuite (Olympus America Inc., Melville, NY, USA); 5-mm dentin disc, Immunodiagnostic Systems (Boldon, UK). The polyclonal primary antibodies against p50 and Akt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the High Capacity cDNA Archive kit was from Applied Biosystems (Foster City, CA, USA); HotStartTaq DNA polymerase from Qiagen (Valencia, CA, USA) and custom tritiation of acetylenic RvE1 from American Radiolabeled Chemicals (St Louis, MO, USA). The acetylenic RvE1 synthetic precursor for the radioligand-binding experiments was prepared by N Petasis in Core C of the Specialized Center for Oral Inflammation and Resolution (P50 DE-16191).

Statistical analysis

Differences between treatment groups were analysed using Student's *t*-test or ANOVA followed by Neuman–Keuls test for pairwise comparisons.

Results

RvE1 treatment decreased OC growth and diminished dentin resorption in vitro

Mouse bone marrow cells were cultured in the presence of M-CSF and RANKL for 7 days. Culture media were supplemented with RvE1 throughout the 7-day period at doses similar to those found effective in resolution of inflammation (Arita *et al*., 2005a). TRAP-positive cells having three or more nuclei were considered OC and presented as the proportion of the culture dish area covered by OC (OC covered area). RvE1 treatment significantly decreased OC covered area in the culture dish at concentrations of 1–10 ng mL^{-1}, with the peak inhibition of 60% at 3 ng mL^{-1} (equivalent to 8.7 nM) (Figure 1a). In comparison, the RvE1 precursor EPA at identical doses significantly increased OC covered area when administered in a similar manner at the same doses (Figure 1b). EPA doses that are higher by an order of magnitude (60 ng mL^{-1} and above) inhibited OC growth (data not shown). Moreover, when the first intermediate of the RvE1 synthetic pathway, 18-HEPE was administered for 7 days at 3 ng mL^{-1}, no change in OC formation was observed (Figure 1a). To confirm that RvE1 induces changes in the development of functionally active OC, bone marrow cells were differentiated on dentine discs in the presence or absence of RvE1 (3 ng mL^{-1}) for 14 days. At the end of the incubation period, the surfaces of dentine discs were processed for scanning electron microscopy and resorption pits were counted. RvE1 decreased resorption pit counts to a comparable degree with decreased OC growth (Figures 1c and d).

RvE1 interfered with OC differentiation

To further characterize RvE1 actions on OC growth, OCs were counted in 7-day-old cultures and classified into three groups: (1) TRAP-positive cells with one or two nuclei, (2) TRAP-positive cells with three to five nuclei and (3) TRAP-positive cells with more than five nuclei. RvE1 (3 ng mL^{-1}) treatment significantly decreased the number of mature OC (>5 nuclei), whereas the number of TRAP-positive cells with 5 or fewer nuclei was increased under RvE1 treatment (Figure 2a). Time course experiments were performed by terminating OC cultures growing in the presence or absence of RvE1 after 1, 3, 5 and 7 days. In 1- and 3-day-old cultures, very few multinuclear TRAP-positive cells could be found

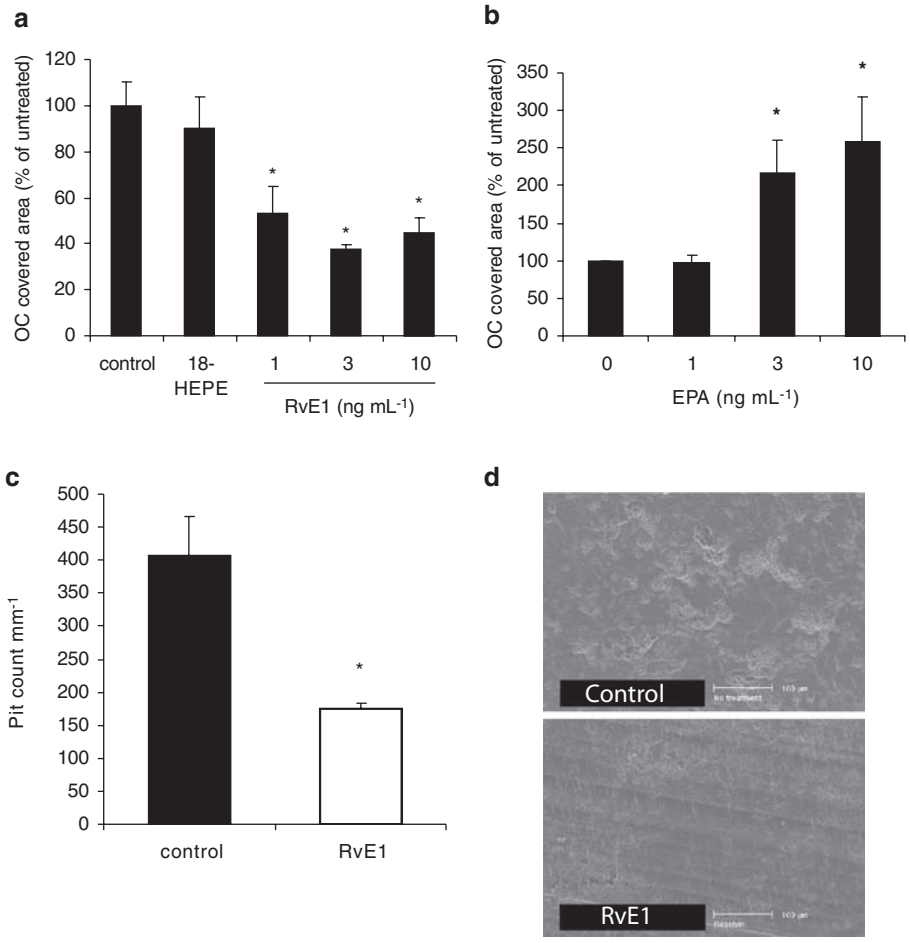

Figure 1 Resolvin E1 (RvE1) decreased osteoclast (OC) growth and dentin resorption *in vitro*. (**a**) RvE1 decreased OC growth in bone marrow cultures treated with murine colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). RvE1 was administered throughout the 7-day culture period in doses indicated. The RvE1 precursor 18*R*-hydroxy-eicosapentaenoic acid (18-HEPE) was administered in a similar manner at 3 ng mL^{-1}. The cumulative surface area of tartrate-resistant acid phosphatase (TRAP)-positive cells with >3 nuclei are compared with that of control cells. (**b**) Eicosapentaenoic acid (EPA) at similar doses increased OC covered area when administered for 7 days. (**c**) RvE1 decreases dentin disc resorption. Bone marrow cultures were seeded on dentine discs and OC differentiation was induced with M-CSF and RANKL for 14 days in the presence or absence of RvE1 (3 ng mL^{-1}). Resorption pits were counted on scanning electron microscopy (SEM) images of discs. In (**a**–**c**), means and s.e.mean are shown, $n=4$ (**a**, **b**), $n=5$ (**c**); *$P<0.05$. (**d**) SEM images of resorption pits of dentin discs from control and RvE1-treated cultures (scale bar: 100 μm).

in either control or RvE1-treated (3 ng mL^{-1}) BM cultures. In 5-day-old cultures, significant OC growth was observed in the control group, whereas with RvE1-treated cultures of the same age OC covered area was significantly smaller (Figures 2b and c). In 7-day-old cultures, RvE1-mediated decrease in OC growth was even more pronounced. To assess RANKL-induced NF-κB activation, we monitored the nuclear translocation of the *p50* subunit of NF-κB. In mature OC cultures grown for 7 days in the presence of RvE1, nuclear translocation of *p50* was decreased when compared with control OC cultures (Figure 2d).

OC death and apoptosis was not altered by RvE1

To explore whether RvE1 influences OC death, bone marrow cells were cultured in the presence of M-CSF and RANKL for 7 days to induce OC differentiation. At day 7, M-CSF and RANKL were withdrawn and RvE1 (3 ng mL^{-1}) or vehicle control in α-MEM and 10% fetal bovine serum was added. OC cultures were stopped at regular intervals (0, 2, 4, 6 and 8 h after M-CSF and RANKL withdrawal) and stained with TRAP and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) to monitor OC survival and nuclear morphology at each time point. M-CSF and RANKL withdrawal induced OC cell death within hours as demonstrated by the rapidly declining OC covered area (Figure 3a). RvE1 treatment did not alter the rate or extent of OC cell death (Figure 3b). OC apoptosis was assessed on TRAP and DAPI double-stained OC under simultaneous transmission and epifluorescence microscopy. In each culture well, 200 TRAP-positive multinuclear cells were evaluated for chromatin condensation and nuclear fragmentation, characteristic of apoptosis and the proportion of apoptotic OC was expressed as a percentage of all OC examined (Figure 3c). The proportion of apoptotic OC increased over time after M-CSF and RANKL withdrawal; however, RvE1 treatment did not significantly alter apoptosis in these cultures. Phosphorylation of Akt was determined in 7-day-old bone marrow cultures grown in the presence of

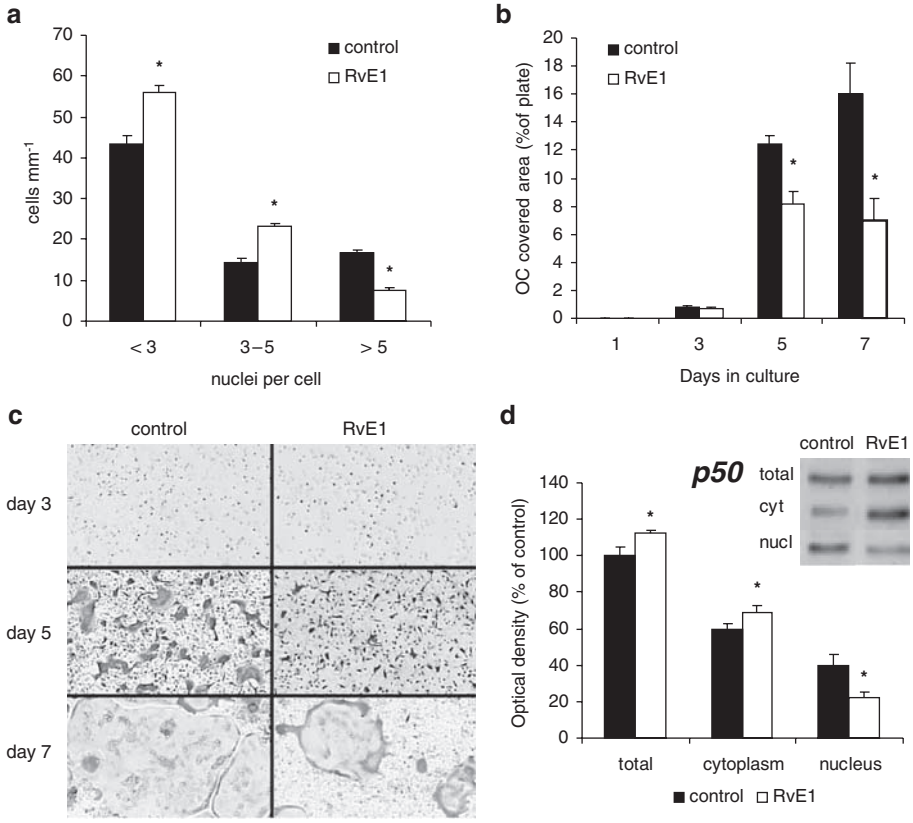

Figure 2 Resolvin E1 (RvE1) interfered with osteoclast (OC) differentiation. (**a**) OC counts in 7-day-old cultures stratified according to nuclear count. RvE1 (3 ng mL^{-1}) treatment decreased the number of mature OC (>5 nuclei), whereas the numbers of smaller OC were increased. (**b**) Time course of OC growth expressed as OC covered area. OC differentiation was initiated in bone marrow cells with murine colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) in the presence or absence of RvE1 (3 ng mL^{-1}) and cultures were stopped and tartrate-resistant acid phosphatase (TRAP)-stained at time points indicated. (**c**) Representative images of TRAP-stained OC cultures in the time course study. Original magnification: ×100. (**d**) RvE1 interfered with nuclear translocation of the *p50* subunit of nuclear factor (NF)-κB. OC cultures were grown with M-CSF and RANKL in the presence and absence of RvE1 (3 ng mL^{-1}). In 7-day-old cultures, cytoplasmic and nuclear protein extracts were analysed with western blotting (insert) for the presence of *p50* and compared between treatment groups in a semiquantitative manner based on densitometric readings of band intensity. In (**a**, **b**, **d**) means and s.e.mean are shown, $n=6$ (**a**) and (**d**), $n=5$ (**b**); *$P<0.05$.

M-CSF and RANKL and in the presence or absence of RvE1 (3 ng mL^{-1}). RvE1 treatment significantly attenuated Akt phosphorylation in OC at amino-acid residues Ser 473 and Thr 308, whereas it did not influence the total amount of Akt (Figure 3d).

RvE1 bound to BLT1 but not to ChemR23

Two cell surface receptors, ChemR23, a seven-transmembrane domain cell surface receptor originally identified as the receptor for chemerin, and BLT_1, an LTB_4 receptor, have been identified as receptors for RvE1 (Arita *et al*., 2005a). Reverse transcription-PCR performed on RNA isolated from OC cultures indicated that both ChemR23 and BLT_1 are expressed in OC cultures (Figure 4a). To determine the relevant RvE1-binding site in OC cultures, [^{3}H]RvE1 was utilized in receptor-binding studies. [^{3}H]RvE1 (5 nM) was incubated with membrane preparations from mature OC cultures in the presence or absence of the BLT_1 ligand LTB_4 (5 μM), the ChemR23 ligand chemerin peptide (5 μM) or unlabelled RvE1 (5 μM). LTB_4 displaced [^{3}H]RvE1 binding as effectively as unlabelled RvE1, whereas chemerin did not displace [^{3}H]RvE1 (Figure 4b). Co-incubation of RvE1 with the BLT_1 antagonist U75302 (1 nM) rescued OC cultures from RvE1-induced growth inhibition, indicating that RvE1's actions on OC differentiation are mediated by BLT_1 (Figure 4c).

Primary OC cultures produce 18-HEPE and LTB_4

To explore whether local molecular circuits contribute to RvE1 production, we have investigated the capacity of cultured OC to generate oxygenated derivatives of EPA. Here, 7-day-old mature OC cultures pretreated with ASA (500 μM in phosphate-buffered saline for 30 min) were incubated with the RvE1 precursor EPA (5 μM) for an additional 30 min and the cells, including the supernatant, were collected in two volumes of methanol for lipid extraction and LC-MS/MS analysis. In ASA and EPA-treated OC cultures, 18-HEPE, but not RvE1, was detected (Figure 5a). Control groups receiving no treatment, or ASA or EPA treatment alone generated no 18-HEPE. Conversely, LTB_4 was found in OC cultures without EPA and ASA treatment only (2.23 ± 1.77 ng mL^{-1}).

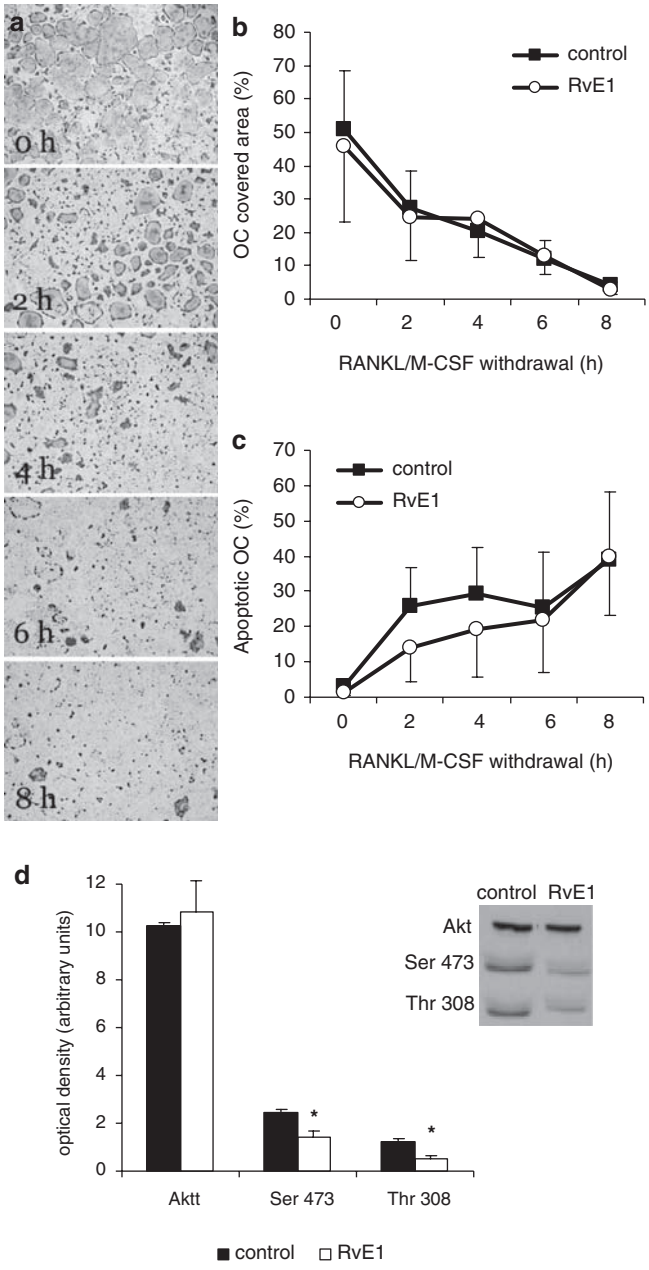

Figure 3 Osteoclast (OC) death and apoptosis were not altered by resolvin E1 (RvE1). (**a**) Withdrawal of murine colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) induced rapid OC death. OC cultures were grown in the presence of M-CSF and RANKL for 7 days. Culture media were removed on the seventh day and substituted with culture media containing 10% fetal bovine serum (FBS) but no M-CSF and RANKL (0 h time point). Cultures were stopped at 2-h intervals and analysed after combined tartrate-resistant acid phosphatase (TRAP) and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) staining. Representative photographs of control OC cultures at each time point after M-CSF and RANKL withdrawal are shown. (**b**) OC covered area decreased over time following M-CSF and RANKL withdrawal similarly in the presence and absence of RvE1 ($3\,ng\,mL^{-1}$). (**c**) The proportion of OC showing nuclear changes indicative of apoptosis gradually increased over time after M-CSF and RANKL withdrawal. No significant differences were detected between control and RvE1-treated cultures. (**d**) Akt phosphorylation was downregulated by RvE1. In separate experiments, OCs were cultured for 7 days in the presence and absence of RvE1 ($3\,ng\,mL^{-1}$) and protein extracts were analysed using western blotting (insert) with specific antibodies against Akt as well as phospho(Ser 473)-Akt and phospho(Thr 308)-Akt. Results are presented as means and s.e.mean, $n=6$, $^*P<0.05$.

OC and neutrophil co-incubations synthesize RvE1

To determine whether co-incubations of leukocytes and OC can biosynthesize RvE1, peripheral blood neutrophils (10^5 cells) were overlaid onto OC pretreated with ASA (500 μM) and EPA (5 μM). Neutrophil-OC incubations were activated with zymosan ($20\,\mu g\,mL^{-1}$) for 30 min at 37 °C. Lipid extraction and LC-MS/MS analysis were performed as described above. RvE1 ($10\,pg\,mL^{-1}$) was identified in the OC and neutrophil co-incubations (Figure 5b), although no evidence for RvE1 production was found in control groups (OC alone, neutrophils alone and cell-free media) undergoing similar treatment protocol.

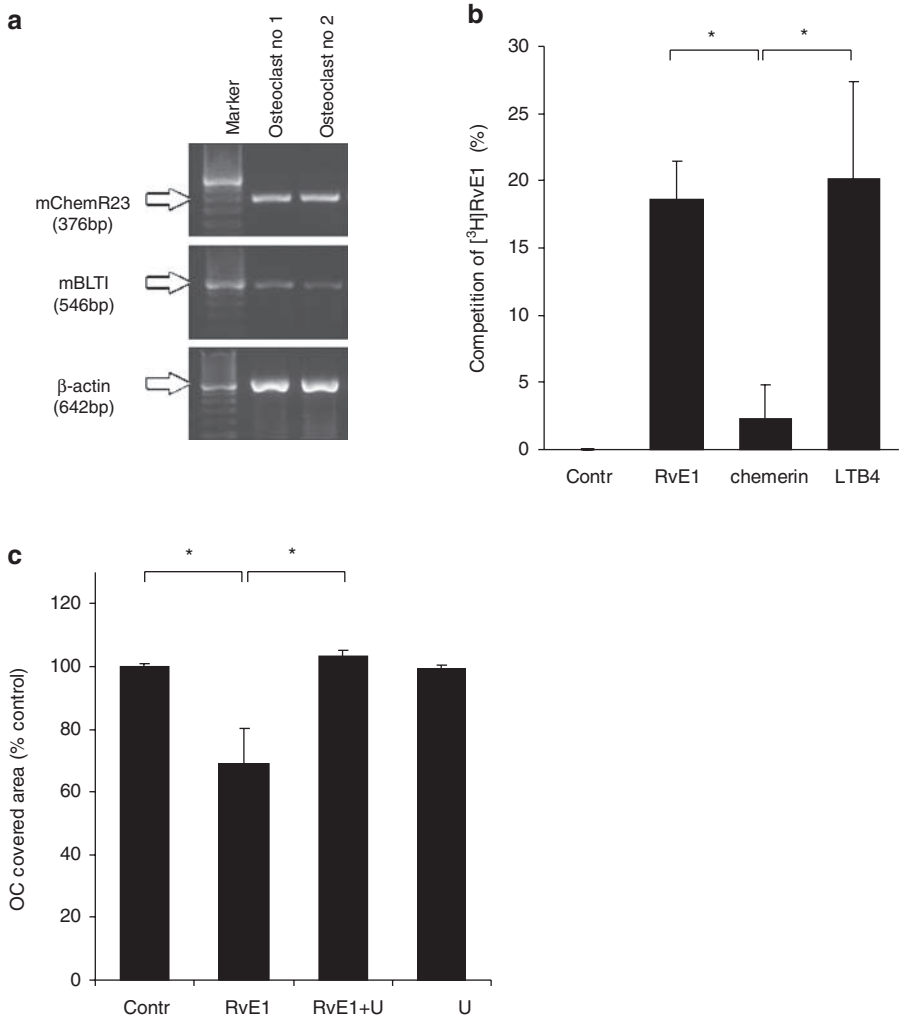

Figure 4 Resolvin E1 (RvE1) bound to BLT_1 but not to ChemR23. (**a**) Osteoclast (OC) mRNA expression of RvE1 receptors ChemR23 and BLT_1. Reverse transcription (RT)-PCR was used to detect mouse ChemR23 and mouse BLT_1 transcripts in RNA isolated from two separate OC cultures. (**b**) Ligand competition with [^{3}H]RvE1-specific binding in mouse OC. The BLT_1 agonist leukotriene B_4 (LTB_4) competed with [^{3}H]RvE1 to the same degree as unlabelled RvE1, whereas the peptide ChemR23 receptor agonist chemerin did not compete effectively with [^{3}H]RvE1. (**c**) Inhibition of the BLT_1 receptor with U75302 (1 nM) prevented RvE1-mediated OC growth inhibition. U75302 (U) alone did not influence OC growth. Results are presented as means and s.e.mean, $n=3$, $^*P<0.05$.

Discussion

RvE1 was initially discovered in resolving inflammatory exudates and identified as a potent regulator of resolution of acute inflammation (Serhan *et al*., 2000; Arita *et al*., 2005a). RvE1 acts by reducing neutrophil infiltration and by promoting phagocyte removal, thus hastening inflammation resolution (Arita *et al*., 2005a; Schwab *et al*., 2007; Serhan, 2007). OC-mediated bone resorption is often a consequence of inflammatory disease. Results presented here support a novel function for RvE1 in directly modulating OC differentiation and consequently bone resorption.

Several lipid mediators have been shown to regulate bone metabolism, including prostaglandins and leukotrienes. PGE_2 stimulates OC differentiation through prostanoid receptors EP_4 and EP_2 by inducing RANKL production and inhibiting osteoprotegerin expression (Suzawa *et al*., 2000; Nukaga *et al*., 2004; Liu *et al*., 2005). Direct binding of PGE_2 to OC has been proposed, but remains controversial, as differentiated OC do not express EP_4 and EP_2 receptors (Sakuma *et al*., 2000; Kobayashi *et al*., 2005). LTB_4 increases bone resorption *in vivo* as well as *in vitro* in calvarial organ cultures and isolated OCs (Garcia *et al*., 1996). High- and low-affinity LTB_4 binding sites have been identified on avian OCs, suggesting a direct cellular action (Flynn *et al*., 1999). On the other hand, bone protection in pathological inflammation has been ascribed to EPA (Sun *et al*., 2003). However, the molecular mechanism of the action of EPA and its metabolites on OC remains unclear. Data presented here show that direct administration of EPA to OC cultures in nanomolar doses results in increased OC formation, whereas administration of RvE1 in similar doses inhibits OC differentiation, suggesting that the bone sparing by EPA observed *in vivo* may not be a direct action of EPA on OC, but rather the action of a metabolic derivative of EPA such as RvE1. Significantly higher EPA doses were required for direct

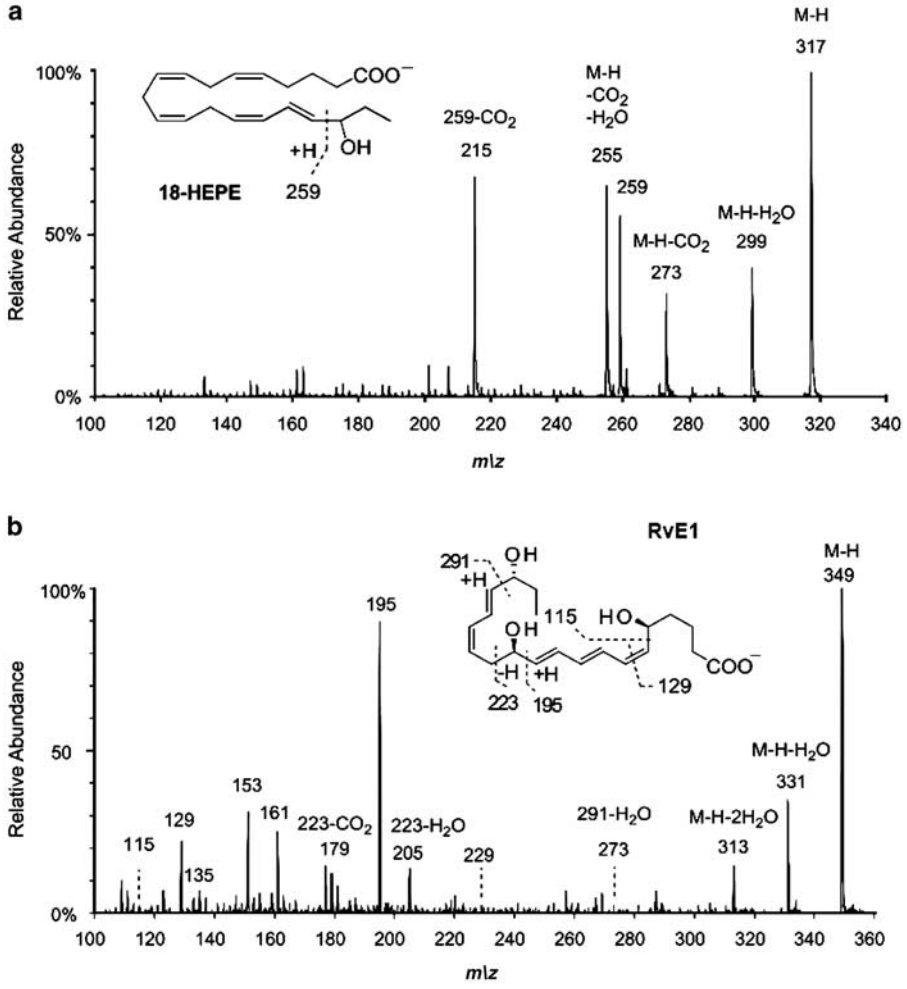

Figure 5 Lipid mediator transcellular biosynthesis by osteoclast (OC). (**a**) OC cultures produced 18*R*-hydroxy-eicosapentaenoic acid (18-HEPE) but not resolvin E1 (RvE1). MS/MS spectrum of 18-HEPE obtained from primary OC cultures treated with acetylsalicylic acid (ASA) (500 μM) and eicosapentaenoic acid (EPA) (5 μM). 18-HEPE was identified by the presence of its molecular anion ([M–H]- = *m/z* 317), retention time matching in liquid chromatography (LC) and its diagnostic product ion (see inset). Results are representative of three separate OC cultures ($n = 3$). (**b**) OC and neutrophil co-cultures synthesized RvE1. OCs were treated with ASA and EPA as above, followed by the addition of neutrophils to the OC and activation with zymosan (20 μg mL^{-1}). RvE1 was identified by the presence of its molecular anion ([M–H]- = *m/z* 349), retention time matching in LC and its diagnostic product ion (see inset).

inhibition of OC growth, an observation consistent with EPA cytotoxicity demonstrated in other cell types such as breast cancer cells (Schley *et al*., 2005). In acute inflammation, RvE1 is produced from EPA in the presence of ASA through oxygenation by acetylated cyclooxygenase in endothelial cells, resulting in the production of 18-HEPE. 18-HEPE is further oxygenated by 5-lipoxygenase-like activity found in leukocytes to generate RvE1 (Serhan *et al*., 2000; Arita *et al*., 2005a). An alternative pathway also exists in bacteria-rich organs such as the gut or the oral cavity where microbial cytochrome *P*450 monooxygenase can generate 18-HEPE and initiate RvE1 production in the absence of acetylated cyclooxygenase (Arita *et al*., 2005b). LC-MS/MS analysis presented here shows that in the presence of EPA and ASA primary OC cultures are capable of generating the first intermediate, 18-HEPE. For further processing of 18-HEPE into RvE1, 5-LO activity is necessary, supplied here by activated neutrophils. The observation that co-incubation of OC with neutrophils results in RvE1 generation is the first evidence that transcellular biosynthetic pathways between OC and neutrophils exist *in vitro* and that locally produced RvE1 may have a significant function *in vivo* in moderating inflammation-induced bone resorption (Serhan *et al*., 2000; Hasturk *et al*., 2006).

OC differentiation from myelomonocytic progenitor cells is induced by the coordinated actions of M-CSF and RANKL (Boyle *et al*., 2003). M-CSF is a permissive factor necessary in early cell-fate determination, whereas RANKL is the final mediator of many physiological regulators of bone resorption (such as parathyroid hormone, calcitonin and calcitriol) and is an obligatory factor for OC differentiation and survival (Boyle *et al*., 2003). RANKL induces the fusion of mononucleated OC precursors to form a giant polykarion, which consequently forms an actin ring to seal off the ruffled membrane active in bone resorption. RvE1 decreases RANKL-induced OC growth and dentin disc resorption to a similar

degree, suggesting that RvE1 downregulates the development of functional OC, but OC that do develop can function normally. Several lines of evidence suggest that RvE1 primarily affects OC differentiation: (1) the number of small OC precursors increase, whereas the number of larger OC decrease in the presence of RvE1, suggesting a partial blockade of OC fusion, (2) time course experiments show decreased OC growth at the earliest observable stages of polykarion development and (3) the nuclear translocation of the *p50* subunit of NF-κB, a cardinal transcription factor in OC differentiation, is decreased. Interestingly, a primary action of RvE1 on leukocytes is inhibition of chemotaxis (Arita *et al*., 2005c; Schwab *et al*., 2007). OCs are also highly motile (Chellaiah *et al*., 2000); however, molecular mechanisms regulating OC mobility are just beginning to be discovered (Yu *et al*., 2004). As both RvE1 and LTB_4 are powerful modulators of chemotaxis in leukocytes, further studies are warranted to evaluate the potential of RvE1 and LTB_4 in regulating mononuclear OC precursor migration and fusion.

OC cell death does not appear to be influenced by RvE1. OC die by apoptosis upon RANKL withdrawal (Boyle *et al*., 2003). OC covered area rapidly declined *in vitro* in the absence of RANKL and in 8 h, most OC disappeared from the culture dish in our experiments. RvE1 did not alter the course of cell death or the rate of apoptosis as determined by OC integrity and nuclear morphology. However, RvE1 treatment markedly decreased the phosphorylation of Akt, an intracellular mediator generally associated with cell survival. Phosphorylated Akt (the active form of the kinase) promotes cell survival in many cell types by phosphorylating BAD, caspase-9 and AFX (Datta *et al*., 1999). In OC, however, the function of Akt is debatable. siRNA-induced gene silencing experiments indicate that Akt is not required for OC survival, but it is critical for OC differentiation as it mediates RANKL-induced NF-κB activation (Sugatani and Hruska, 2005). Moreover, pharmacological inhibition of the Akt phosphorylating mediator PI3 kinase results in suppression of OC fusion (Hotokezaka *et al*., 2006). Thus, downregulation of Akt phosphorylation by RvE1 may mediate its inhibitory actions on OC growth.

Two cell surface receptors denoted ChemR23 and BLT_1 bind and mediate the intracellular actions of RvE1 in leukocytes (Arita *et al*., 2005a, 2007). In this study, we found that both ChemR23 and BLT_1 mRNA are expressed by cultured OC. Receptor-binding studies using radiolabelled RvE1 demonstrated specific RvE1 binding on cell membrane preparations of OC that could be specifically displaced with unlabelled RvE1 as well as with the BLT_1 ligand LTB_4. In contrast, the ChemR23 peptide ligand chemerin was unable to compete with [^{3}H]RvE1-specific binding on OC. Chemerin competes with RvE1-specific binding to ChemR23 in monocytes (Arita *et al*., 2005a); however, as this competition was not observed in OC, it is likely that the BLT_1 is the main site of RvE1 actions in OC culture. Inhibition of the BLT_1 receptor with U75302 reversed RvE1-mediated inhibition of OC growth, providing functional evidence for BLT_1's critical function in mediating Rv1's action on OC. BLT_1 is also a receptor for LTB_4, a pro-inflammatory arachidonic acid derivative that is known to enhance bone resorption *in vitro* and *in vivo* (Garcia *et al*., 1996). LTB_4 is specifically implicated in promoting OC fusion (Flynn *et al*., 1999). As RvE1 decreases only the number of large OC, it is plausible that RvE1 and LTB_4 have counter-regulatory functions on the BLT_1 receptor in determining OC growth and function.

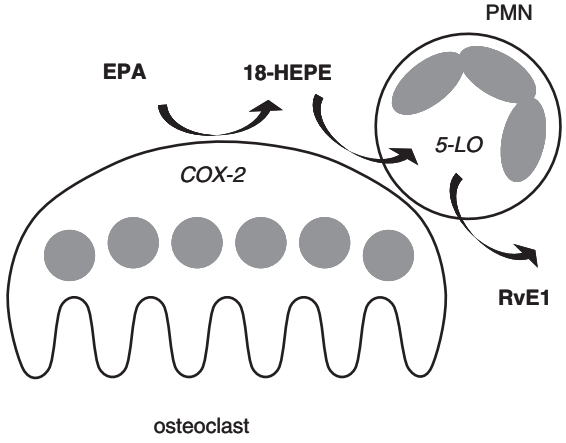

Figure 6 Proposed transcellular biosynthetic pathway of resolvin E1 (RvE1) generation by osteoclast (OC) and neutrophils. Acetylsalicylic acid (ASA) acetylates COX-2, which in turn converts eicosapentaenoic acid (EPA) into 18*R*-hydroxy-eicosapentaenoic acid (18-HEPE), the first metabolic intermediate of RvE1. 18-HEPE is further oxygenated by activated leukocytes through 5-lipoxygenase and subsequent reactions resulting in bioactive RvE1. In turn, the locally generated RvE1 may act on OC to inhibit OC growth, as well as on leukocytes to mediate resolution of inflammation.

In summary, the results presented here indicate that RvE1 blocks OC differentiation and bone resorption *in vitro*, suggesting a bone-sparing action distinct from RvE1's known anti-inflammatory and pro-resolution actions. RvE1 interfered with the late phase of OC differentiation corresponding to the formation of the polykarion. RvE1 bound to the BLT_1 receptor and attenuated the nuclear translocation or the *p50* subunit of NF-κB as well as the phosphorylation of Akt. OC can generate RvE1 by transcellular biosynthesis through interactions with leukocyte lipoxygenases, providing a local mechanism for RvE1 production in sites of inflammatory bone resorption (Figure 6). RvE1 attenuated OC growth at nanomolar concentrations, therefore, in conditions where inflammation is present along with potential bone resorption, such as rheumatoid arthritis (Walsh *et al*., 2005), post-menopausal osteoporosis (Raisz, 2005) or periodontitis (Hasturk *et al*., 2007), RvE1 may be beneficial not only in resolving local inflammation but also as a novel bone-preserving factor.

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Conflict of interest

The resolvins as biotemplates for stable analogues are US patents assigned to Brigham and Women's Hospital (CNS).

CNS and TEVD are co-inventors on use patents assigned to Boston University. These patents are licensed for clinical development and are the subject of consultant agreements for CNS and TEVD.

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