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Halofuginone Prevents Estrogen-deficient Osteoporosis in Mice

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ABSTRACT

Osteoporosis is characterized by enhanced activity of osteoclasts relative to that of osteoblasts. Thus, the principal means of treating the most common form of osteoporosis, namely that attending menopause, is inhibition of osteoclast formation or function. We have demonstrated that the inflammatory cytokine, IL-17, mediates estrogen-deficient osteoporosis, in mice, and that genetic blockade of its function prevents ovariectomy-induced bone loss. We herein report that the febrifugine derivative, halofuginone, a small molecule drug, reduces abundance of Th-17 cells in mice and prevents estrogen-deficient osteoporosis by diminishing bone resorption without impacting osteogenesis. In keeping with IL-17 mediating its osteoclastogenic effects by promoting RANK ligand expression by osteoblasts, halofuginone does not directly inhibit the bone resorptive cell. Thus, halofuginone, which is presently FDA-approved for treatment of scleroderma, is a candidate therapeutic for post-menopausal osteoporosis. J. Cell. Biochem. 113: 3086–3092, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: OSTEOPOROSIS; OSTEOCLAST; HALOFUGINONE; ESTROGEN

Post-menopausal osteoporosis is endemic in Western society as approximately 50% of elderly women will fracture due to insufficient skeletal mass. Like all forms of osteoporosis, that due to estrogen insufficiency represents increased bone resorption relative to formation. As such, the osteoclast is the principal target when treating this disorder, largely with bisphosphonates. Because these drugs reside in the skeleton for decades, they often suppress bone remodeling which serves to replace effete bone with new [Bauer et al., 2006]. Despite enhanced bone mass, therefore, prolonged bisphosphonate administration is often associated with reduced skeletal replacement and in some, a predisposition to atypical, poorly healing fractures [Seeman and Delmas, 2006; Allen and Burr, 2007ab; Shane et al., 2010]. As such, shorter acting anti-resorptive agents are needed.

The osteoclast is a hematopoietic cell whose mononcyte/macrophage precursors differentiate into the mature polykaryon under the aegis of RANK ligand (RANKL) and M-CSF. These cytokines are produced by bone-residing stromal cell derivatives, particularly osteocytes and osteoblasts. RANKL expression is enhanced by inflammatory cytokines such as tumor necrosis factor (TNF) [Lam et al., 2000] and IL-1 [Wei et al., 2005] and, in the context of the present study, IL-17 [Sato et al., 2006].

Systemic IL-17 is primarily produced by Th17-cells, but mast cells, γδ T cells, a memory subset of cytotoxic T cells, invariant natural killer T cells, and neutrophils also express the cytokine [Shin et al., 1999; Ferretti et al., 2003; Happel et al., 2003; Kolls and Linden, 2004; Lockhart et al., 2006; Ivanov et al., 2007; Maek et al., 2007; Michel et al., 2007; Hueber et al., 2010]. Th17 cells arise when naïve CD4+ are stimulated with IL-6 and TGF-β, which induces both RORγt and Foxp3 transcription factors [de Jong et al., 2010].

For centuries the roots of *Dichroa febrifuga* have been used in ancient Chinese herbal medicine to treat malarial fevers. Its active ingredients include febrifugine and its stereoisomere, isofebrifugine. Halofuginone (7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone) is a febrifugine analog, most known for its beneficial effect on fibrotic conditions, such as scleroderma and graft versus host disease. It is FDA-approved to treat the former condition and has received orphan drug status. Importantly, halofuginone substantially reduces IL-17 abundance by inhibiting Th17 cell differentiation through activation of the

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nutrient-sensing amino acid response pathway [Sundrud et al., 2009].

We recently found that deletion of the IL-17 receptor or its effector molecule prevents experimental post-menopausal osteoporosis [DeSelm et al., 2012]. Given the capacity of halofuginone to dampen IL-17 abundance we hypothesized that this small molecule drug also spares estrogen-deficient bone loss and find such to be the case.

MATERIALS AND METHODS

ANIMAL CARE

All animal experimentation was approved by the Animal Studies Committee of the Washington University School of Medicine.

REAGENTS

Halofuginone was purchased from Mingdou Chemical Company Ltd. (Shanghai, China) and Santa Cruz (CA). Drug from both sources were used for in vitro osteoclastogenesis and in vivo experiments with similar results.

CELL CULTURE

Osteoclasts were grown in alpha10 media, containing alpha-MEM (Sigma–Aldrich, St. Louis, MO), 10% fetal calf serum (Hyclone, Waltham, MA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Osteoclasts were differentiated from bone marrow as described [Zhao et al., 2008]. Briefly, bone marrow was extracted from mice and cultured in the presence of 10% CMG 14-12 supernatant [Takeshita et al., 2000], an MCSF-containing cell supernatant. After 4 days, cells were lifted and replated on plastic in alpha10 media supplemented with 2% CMG and 100 ng/ml recombinant RANKL. Cells were fixed in 4% paraformaldehyde/PBS. TRAP staining on fixed cells was performed using a commercially available kit according to the manufacturer's instructions (Sigma, St. Louis, MO). All TRAP expressing cells with at least three nuclei were considered osteoclasts. Flow cytometry: spleen cells were prepared by gently crushing the tissue and filtering through a 40-μm cell strainer (BD Falcon). Cells were harvested, centrifuged, and incubated in red blood cell (RBC) lysis buffer. Cells were incubated in 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma–Aldrich) for 4 h at 37°C for stimulation. Brefeldin A (Sigma–Aldrich) was added during the last 2 h of culture at 10 μg/ml. Cells were stained with LIVE/DEAD Fixable Dead Cell Stain Kits (Molecular Probes, Invitrogen) followed by the appropriate surface antibody (anti-CD4) for 20 min at 4°C. Stained cells were fixed in 2% paraformaldehyde for 20 min at RT before permeabilization with 0.05% saponin. Intracellular staining for the cells was conducted at 4°C for 30 min using Alexa Fluor 488 anti-mouse IL-17A (1:1,000 dilution) (eBioscience). To determine background levels of cytokine staining, a set of cells was not stimulated, but was treated with brefeldin A and stained for intracellular cytokines, as was done for experimental cells. Samples were gated on live cells using the above LIVE/DEAD cell stain. All samples were analyzed on a FACSCalibur or FACSCanto (BD) and data were analyzed with FlowJo software (TreeStar). A total of 30,000 events were collected per sample.

MICROCOMPUTED TOMOGRAPHY AND OVARIECTOMY

Halofuginone was reconstituted in DMSO and 2 μg dissolved in PBS, daily, was injected intraperitoneally, beginning at the time of ovariectomizing (ovx) or sham procedure and lasting 28 days. Equivalent volumes of DMSO in PBS served as control injections. Mice were anesthetized with isofluorane and analyzed using in vivo microcomputed tomography (vivaCT 40, Scanco Medical, Brüttisellen, Switzerland) at day 0, 14, or 28. In every mouse, the right femora was positioned in the vivaCT in an identical orientation, assigned a random scan number, and analyzed in a blinded fashion. Thirty slices were analyzed in each bone, starting with the first slice in which condyles and primary spongiosa were no longer visible. A threshold linear attenuation coefficient of 1.2 cm^{-1} was used to differentiate bone from non-bone. A threshold of 160 was used for evaluation of all scans. Measurements included bone volume/total volume (BV/TV), connectivity density (Conn. Dens), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and trabecular number (Tb.N). For ovariectomies, all mice were aged 8 weeks at time of ovx, and ovaries were removed through two small dorsal incisions. Sham operated mice were anesthetized and opened equivalently, but ovaries were not removed. Serum carboxy-terminal collagen crosslinks (CTx) measurements. Blood was collected by cheek puncture after 6 h starvation. Plasma was obtained using plasma separator tubes with lithium heparin (Becton Dickinson). Serum CTx-I, a specific marker of osteoclastic bone resorption, was measured using a RatLaps ELISA kit from Nordic Bioscience Diagnostics A/S.

STATISTICS

All data was analyzed with Prism software (Graphpad, San Diego, CA), using two-tailed unpaired Student's *t* tests. Error bars represent standard deviation.

RESULTS

HALOFUGINONE PREVENTS ESTROGEN-DEFICIENT OSTEOPOROSIS

We first asked if halofuginone, at a dose which reduces the number of IL-17-producing cells (2 μg) [Sundrud et al., 2009], effects basal skeletal mass which we find not to be true (Fig. 1). To mirror the most common form of osteoporosis in humans, we produced an estrogen-deprived state by ovx mice. Beginning the day of ovx, the animals were treated with vehicle or halofuginone. After 2 weeks, BV/TV was reduced by approximately 23% in carrier-treated ovx mice whereas halofuginone completely protected the skeleton (Fig. 2). The changes at 4 weeks following ovx were even more dramatic (Fig. 3). Again sham operated and halifuginone (HL) treated ovx mice were indistinguishable whereas carrier-administered, estrogen deficient animals experienced a 32% reduction in BV/TV and significant alterations of all other uCT-determined indicators of systemic bone loss.

Protected bone mass in face of ovx may reflect enhanced formation or diminished resorption. To assess bone formation (BFR), we injected time-spaced doses of the bone fluorescent marker, calcein, into ovx mice. Following 2 weeks of halogfuginone or carrier administration, the animals were sacrificed and BFR histomorphometrically determined (Fig. 4). Both mineral apposition

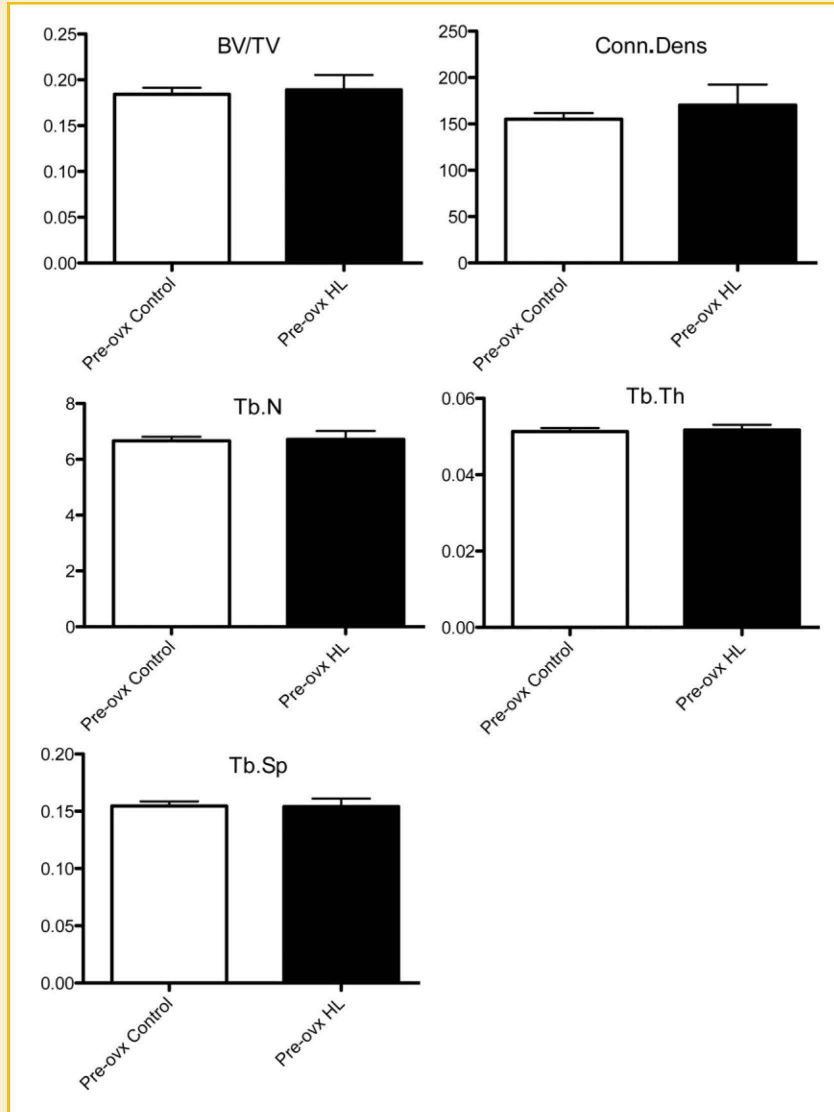

Fig. 1. μCT analysis of distal femurs of naïve mice treated with halifuginone (HL) or carrier for 4 weeks.

(MAR), which reflects mean osteoblast activity, and total BFR rate were unaltered by halogfuginone administration.

CTx are released from bone, by osteoclasts, and serum levels serve as a marker of skeletal degradation [Rosen et al., 2000]. Two weeks after ovx, vehicle-treated mice had significantly higher serum CTx, while those receiving halofuginone exhibited no statistically significant difference from sham (Fig. 5). Thus, halofuginone protects mice from post-menopausal osteoporosis.

HALOFUGINONE REDUCES Th17 CELL ABUNDANCE IN OVX MICE

We observed that inhibition of IL-17 signaling prevents ovx-induced osteoporosis in mice [DeSelm et al., 2012]. Since Halofuginone reduces the abundance of this cytokine by blunting Th17 cell maturation, we asked if the number of these lymphocytes is altered, by the drug, in the context of ovx. In fact, flow cytometry analysis of splenocytes demonstrates that 2 weeks treatment with halofuginone significantly reduces the percentage of CD4+ splenic T-cells producing IL-17 (Fig. 6).

These data suggest that halofuginone's protective effect on estrogen-deficient bone loss may reflect suppression of IL-17-mediated bone resorption. Since the cytokine exerts its anti-osteoclastogenic effects, indirectly, by promoting RANKL expression by osteoblast lineage cells [Sato et al., 2006], we would expect halofuginone, like IL-17, to have no direct effect on osteoclast formation. To determine if such is the case we generated osteoclasts from WT marrow macrophages in the presence of increasing amounts of the drug or equivalent vehicle. After 5 days the cells were stained for tartrate resistant acid phosphatase activity. No effect of halofuginone is evident on osteoclast abundance or morphology at doses less than 10 μg/ml which is toxic to the bone resorptive polykaryon (Fig. 7).

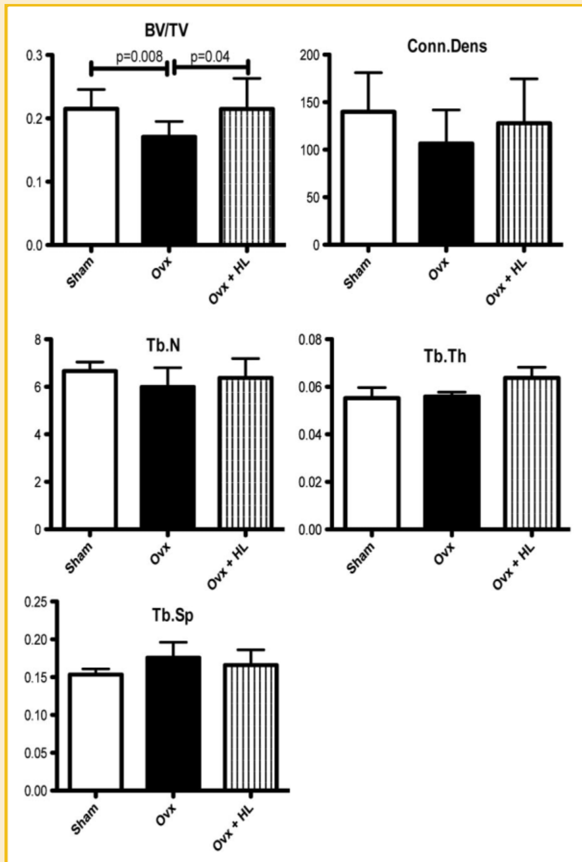

Fig. 2. μCT of ovx mice treated with carrier or HL for 2 weeks. Sham operated mice serve as control.

HALOFUGINONE TREATMENT DOES NOT CAUSE WEIGHT GAIN

IL-17 inhibits adipocyte differentiation from human mesenchymal stem cells [Shin et al., 2009]. IL17 receptor-deficient mice are heavier and have higher leptin levels, a marker of adopocyte mass [Goswami et al., 2009; Galic et al., 2010]. Leptin stimulates production [Liu et al., 2008] and increases circulating levels of IL-17 in obese women [Sumarac-Dumanovic et al., 2009]. These data suggest IL-17 inhibition may lead to the unfavorable side effect of weight gain. Magnetic resonance imaging (MRI), serving as a measure of body composition, revealed, however, that 12 weeks of treatment with halofuginone or vehicle control did not increase adiposity. In fact, halofuginone reduced total fat mass and percent body fat (Fig. 8). Additionally, halofuginone promoted a small but statistically significant increase in percent lean mass.

DISCUSSION

Estrogen-deficient osteoporosis represents an increase in osteoclast recruitment and accelerated bone resorption. Thus, understanding the mechanisms by which absence of the sex steroid promotes osteoclastogenesis is central to preventing and treating the most common form of systemic skeletal insufficiency.

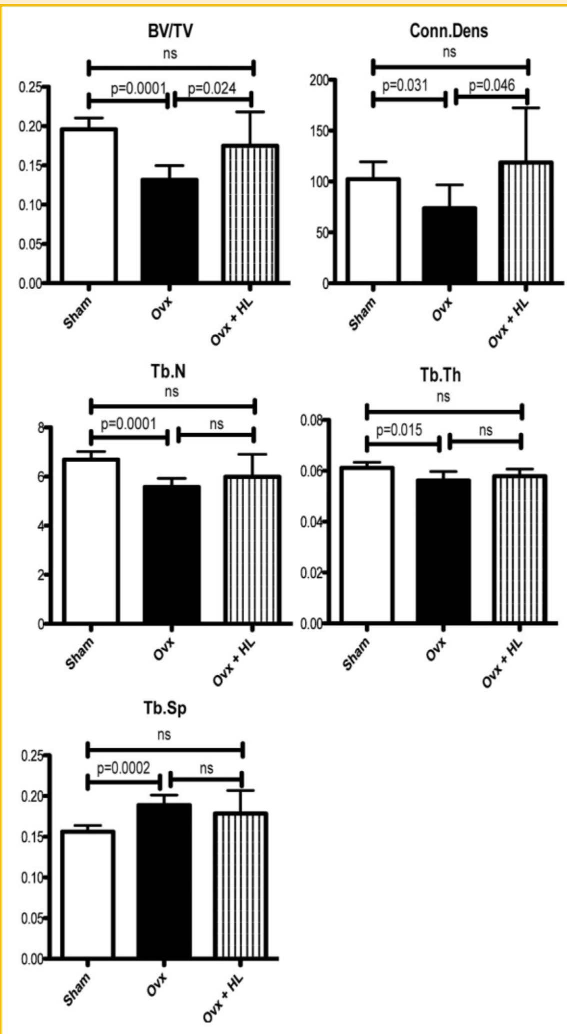

Fig. 3. μCT of ovx mice treated with carrier or HL for 4 weeks. Sham operated mice serve as control.

The discovery that the osteoclast precursor is of the monocyte/ macrophage lineage raised the possibility that inflammatory cytokines, produced by this family of cells, may mediate their maturation into bone resorptive polykaryons. In fact, IL-1 and TNF,

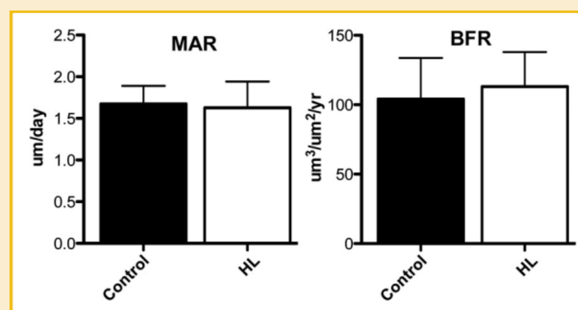

Fig. 4. Mice having undergone ovx were administered HL or vehicle for 14 days. Calcein was injected on days 7 and 12 and mineral apposition (MAR) and bone formation (BFR) rates determined by fluorescent histomorphometry.

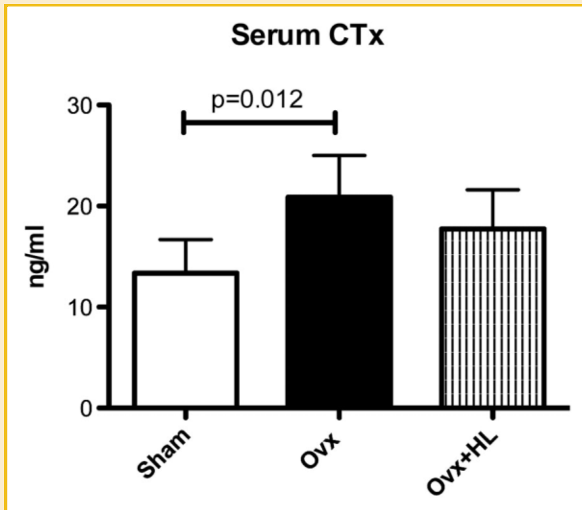

Fig. 5. Serum of sham operated, carrier- or HL-treated ovx mice was analyzed for CTx content 2 weeks after surgery.

among others, synergize with RANKL to differentiate precursors into osteoclasts [Lam et al., 2000; Wei et al., 2005]. As would be expected, this combination of molecules mediates the bone loss attending skeletal inflammation. More surprising, however, is the fact that these inflammatory cytokines participate in development of post-menopausal osteoporosis [Cenci et al., 2000; Roggia et al., 2001; Pacifici, 2010].

Inflammatory cytokines attending estrogen deprivation were initially thought to be produced by peripheral blood mononuclear cells but subsequent evidence indicated they likely derive from T-cells, particularly the Th1 CD4+ subset [Kotake et al., 2005]. In fact, almost 30 years ago, Fujita et al. [1984] noted altered distribution of T-cell subsets in patients with osteoporotic fractures. A current paradigm holds that estrogen deficiency enhances antigen presentation which increases the number of inflammatory cytokine-producing T-cells thereby stimulating RANKL production by stromal cells with consequent osteoclastogenesis [Pacifici, 2010]. This scenario, indicating that estrogen-deficient bone loss is an inflammatory condition, attended by altered immune function, is in keeping with a parallel model of rheumatoid arthritis wherein Th1 CD4+ cells are considered central to the osteolytic process and systemic bone loss. Sato et al. [2006] however, determined that this subset was unlikely to mediate periarticular osteoclastogenesis as it produced an abundance of interferon-γ which, in the context of severe inflammation, dampens differentiation of the bone resorptive polykaryon. The authors, instead, demonstrate that the newly described cytokine, IL-17, expressed by Th17 cells mediates inflammatory osteoclastogenesis and attendant joint destruction.

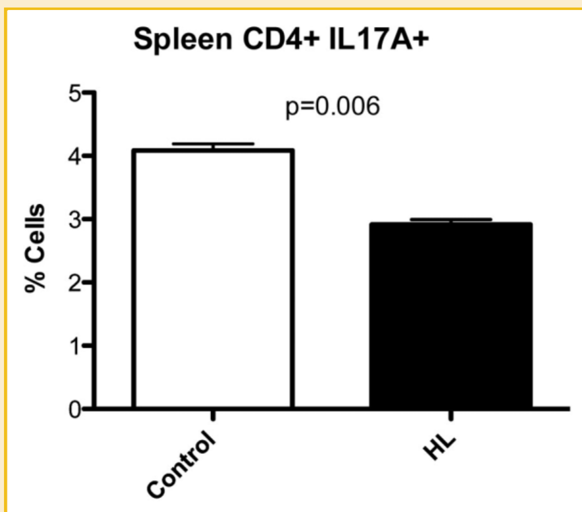

Fig. 6. Mice were treated with HL or vehicle daily for 2 weeks following ovx. Spleen cells were harvested, stimulated with ionomyocin, and analyzed by FACS gating on live cells.

Prompted by the hypothesis that estrogen-deficient osteoporosis is also an inflammatory disorder, we recently asked if IL-17 participates in its pathogenesis [DeSelm et al., 2012]. We found that arrest of IL-17 signaling, either by deletion of its principal receptor or its effector, Act-1, completely prevents ovariectomy-induced osteoporosis in mice by arresting RANKL expression via dampened RANKL expression. These observations prompted us to explore the possibility that pharmacological inhibition of IL-17 would also prevent the most common human form of systemic bone loss.

Halofuginone is a natural compound derivative with the capacity to retard Th17 cell development by activating the amino acid starvation response [Sundrud et al., 2009]. As such, halofuginone inhibits IL-17 expression and development of a Th-17-driven model of multiple sclerosis. Given our observation that IL-17 mediates murine, post-menopausal osteoporosis, we reasoned halofuginone may be effective in its prevention. In fact, we find that while the drug does not impact basal skeletal mass it completely prevents the bone loss induced by estrogen deprivation.

Because halofuginone significantly normalizes the increase in CTx induced by ovx it is likely the drug exerts its bone sparing properties by dampening IL-17-stimulated resorption. The means by which the cytokine enhances osteoclastogenesis are, however, controversial. Some note the cytokine directly promotes precursor differentiation [Adamopoulos et al., 2010] while others observe stimulation of RANKL expression by stromal lineage cells [Sato et al., 2006; DeSelm et al., 2012]. Our findings that halofuginone fails to directly impact osteoclast differentiation are in keeping with IL-17 mediating its resorptive effect by stimulating RANKL-expressing cells. Furthermore, Th17 cells, per se, produce RANKL and it is possible that the bone-sparing effects of halofuginone reflect, at least in part, its suppressive effect on their differentiation.

While halofuginone's IL-17-inhibitory properties suggest the drug may be of use in inflammatory disorders, its principal pharmacological success, to date, reflects its capacity to impair type I collagen synthesis, in fibrotic conditions such as scleroderma and cancer, without affecting collagen type II or III. The drug is effective whether administered orally, locally, or intraperitoneally [Granot et al., 1993; Choi et al., 1995]. The fact that type 1 collagen is the principal organic component of bone raised the possibility that in addition to inhibiting resorption, halofuginone may also dampen osteogenesis, thus compromising its anti-osteoporotic properties.

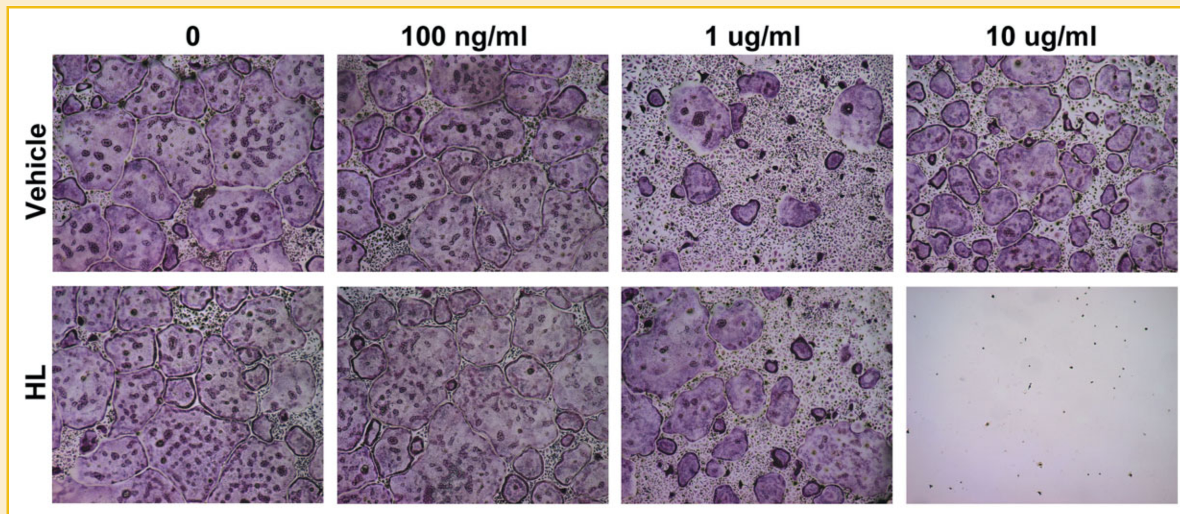

Fig. 7. Marrow macrophages were differentiated into osteoclasts by culture in M-CSF and RANKL. Increasing amounts of HL or equivalent vehicle were added. After 5 days the cultures were stained for TRAP activity.

However, we find this not to be the case, perhaps reflecting halofuginone's capacity to inhibit collagen synthesis in conditions of extravagant fibrosis but not physiological circumstances [McGaha et al., 2002]. Furthermore, the dose of halofuginone used in these studies reduces IL-17 production while not significantly impairing TGF-β signaling required for collagen synthesis [McGaha et al., 2002; Sundrud et al., 2009]. While additional mechanistic insights are necessary, our findings that halofuginone inhibits post-menopausal osteoporosis in mice without promoting obesity and its documented safety and toleration by patients, suggest it may be effective in treating its counterpart disease in humans.

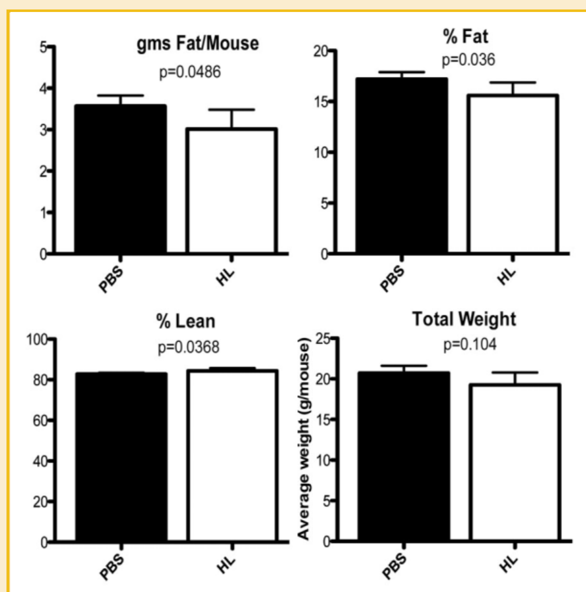

Fig. 8. Eight-week-old mice were administered HF of carrier, daily for 12 weeks. Whole body content was determined by MRI.

CONFLICT OF INTEREST DISCLOSURE

The first and senior author have filed a patent application through Washington University for a small molecule IL-17 inhibitor effective in preventing bone loss in ovariectomized mice.

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REFERENCES

Adamopoulos IE, Chao CC, Geissler R, Laface D, Blumenschein W, Iwakura Y, McClanahan T, Bowman EP. 2010. Interleukin-17A upregulates receptor activator of NF-kappaB on osteoclast precursors. Arthritis Res Ther 12:R29.

Allen MR, Burr DB. 2007a. Mineralization, microdamage, and matrix: How bisphosphonates influence material properties of bone. BoneKEy-Osteovision 4:49–60.

Allen MR, Burr DB. 2007b. Three years of alendronate treatment results in similar levels of vertebral microdamage as after one year of treatment. J Bone Miner Res 22:1759–1765.

Bauer DC, Garnero P, Hochberg MC, Santora A, Delmas P, Ewing SK, Black DM. 2006. Pretreatment levels of bone turnover and the antifracture efficacy of alendronate: The fracture intervention trial. J Bone Miner Res 21:292–299.

Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Pacifici R. 2000. Estrogen deficiency induces bone loss by enhancing T cell production of TNFα. J Clin Invest 106:1229–1237.

Choi ET, Callow AD, Sehgal NL, Brown DM, Ryan US. 1995. Halofuginone, a specific collagen type I inhibitor, reduces anastomotic intimal hyperplasia. Arch Surg 130:257–261.

de Jong E, Suddason T, Lord GM. 2010. Translational mini-review series on Th17 cells: Development of mouse and human T helper 17 cells. Clin Exp Immunol 159:148–158.

DeSelm CJ, Takahata Y, Warren J, Chappel JC, Khan T, Li X, Liu C, Choi Y, Kim YF, Zou W, Teitelbaum SL. 2012. IL-17 mediates estrogen-deficient

osteoporosis in an Act1-dependent manner. J Cell Biochem E Pub Ahead of Print.

Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. 2003. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J Immunol 170:2106–2112.

Fujita T, Matsui T, Nakao Y, Watanabe S. 1984. T lymphocyte subsets in osteoporosis. Effect of 1-alpha hydroxyvitamin D3. Miner Electrolyte Metab 10:375–378.

Galic S, Oakhill JS, Steinberg GR. 2010. Adipose tissue as an endocrine organ. Mol Cell Endocrinol 316:129–139.

Goswami J, Hernandez-Santos N, Zuniga LA, Gaffen SL. 2009. A bone-protective role for IL-17 receptor signaling in ovariectomy-induced bone loss. Eur J Immunol 39:2831–2839.

Granot I, Halevy O, Hurwitz S, Pines M. 1993. Halofuginone: An inhibitor of collagen type I synthesis. Biochim Biophys Acta 1156:107–112.

Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ, Shellito JE, Schurr JR, Bagby GJ, Nelson S, Kolls JK. 2003. Cutting edge: Roles of toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. J Immunol 170:4432–4436.

Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, Leipe J, Melendez AJ, McInnes IB. 2010. Mast cells express IL-17A in rheumatoid arthritis synovium. J Immunol 184:3336–3340.

Ivanov S, Bozinovski S, Bossios A, Valadi H, Vlahos R, Malmhall C, Sjostrand M, Kolls JK, Anderson GP, Linden A. 2007. Functional relevance of the IL-23-IL-17 axis in lungs in vivo. Am J Respir Cell Mol Biol 36:442–451.

Kolls JK, Linden A. 2004. Interleukin-17 family members and inflammation. Immunity 21:467–476.

Kotake S, Nanke Y, Mogi M, Kawamoto M, Furuya T, Yago T, Kobashigawa T, Togari A, Kamatani N. 2005. IFN-gamma-producing human T cells directly induce osteoclastogenesis from human monocytes via the expression of RANKL. Eur J Immunol 35:3353–3363.

Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. 2000. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488.

Liu L, Yang P, He H, Lin X, Jiang L, Chi W, Zhao C, Zhou H. 2008. Leptin increases in Vogt-Koyanagi-Harada (VKH) disease and promotes cell proliferation and inflammatory cytokine secretion. Br J Ophthalmol 92:557–561.

Lockhart E, Green AM, Flynn JL. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177:4662–4669.

Maek ANW, Buranapraditkun S, Klaewsongkram J, Ruxrungtham K. 2007. Increased interleukin-17 production both in helper T cell subset Th17 and CD4-negative T cells in human immunodeficiency virus infection. Viral Immunol 20:66–75.

McGaha TL, Phelps RG, Spiera H, Bona C. 2002. Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-beta-mediated Smad3 activation in fibroblasts. J Invest Dermatol 118:461–470.

Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, Wong CH, Schneider E, Dy M, Leite-de-Moraes MC. 2007. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. J Exp Med 204:995–1001.

Pacifici R. 2010. The immune system and bone. Arch Biochem Biophys 503:41–53.

Roggia C, Gao Y, Cenci S, Weitzmann MN, Toraldo G, Isaia G, Pacifici R. 2001. Up-regulation of TNF-producing T cells in the bone marrow: A key mechanism by which estrogen deficiency induces bone loss in vivo. Proc Natl Acad Sci USA 98:13960–13965.

Rosen HN, Moses AC, Garber J, Iloputaife ID, Ross DS, Lee SL, Greenspan SL. 2000. Serum CTX: A new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. Calcif Tissue Int 66:100–103.

Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, Tanaka S, Kodama T, Akira S, Iwakura Y, Cua DJ, Takayanagi H. 2006. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 203:2673–2682.

Seeman E, Delmas PD. 2006. Bone quality–The material and structural basis of bone strength and fragility. N Engl J Med 354:2250–2261.

Shane E, Burr D, Ebeling PR, Abrahamsen B, Adler RA, Brown TD, Cheung AM, Cosman F, Curtis JR, Dell R, Dempster D, Einhorn TA, Genant HK, Geusens P, Klaushofer K, Koval K, Lane JM, McKiernan F, McKinney R, Ng A, Nieves J, O'Keefe R, Papapoulos S, Sen HT, van der Meulen MC, Weinstein RS, Whyte M. 2010. Atypical subtrochanteric and diaphyseal femoral fractures: Report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res 25:2267–2294.

Shin HC, Benbernou N, Esnault S, Guenounou M. 1999. Expression of IL-17 in human memory CD45RO+ T lymphocytes and its regulation by protein kinase A pathway. Cytokine 11:257–266.

Shin JH, Shin DW, Noh M. 2009. Interleukin-17A inhibits adipocyte differentiation in human mesenchymal stem cells and regulates pro-inflammatory responses in adipocytes. Biochem Pharmacol 77:1835–1844.

Sumarac-Dumanovic M, Stevanovic D, Ljubic A, Jorga J, Simic M, Stamenkovic-Pejkovic D, Starcevic V, Trajkovic V, Micic D. 2009. Increased activity of interleukin-23/interleukin-17 proinflammatory axis in obese women. Int J Obes (Lond) 33:151–156.

Sundrud MS, Koralov SB, Feuerer M, Calado DP, Kozhaya AE, Rhule-Smith A, Lefebvre RE, Unutmaz D, Mazitschek R, Waldner H, Whitman M, Keller T, Rao A. 2009. Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. Science 324:1334–1338.

Takeshita S, Kaji K, Kudo A. 2000. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 15:1477–1488.

Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. 2005. IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest 115:282–290.

Zhao H, Ito Y, Chappel J, Andrews NW, Teitelbaum SL, Ross FP. 2008. Synaptotagmin VII regulates bone remodeling by modulating osteoclast and osteoblast secretion. Dev Cell 14:914–925.