A rate-limiting role for DKK1 in bone formation and the remediation of bone loss in mouse and primate models of postmenopausal osteoporosis by an experimental therapeutic antibody

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Running title

Therapeutic effect of anti-DKK1 antibody in osteopenia

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Abstract

Genetic studies have linked both osteoporotic and high bone mass (HBM) phenotypes to LDL-receptor related proteins (LRP 4/5/6). LRP are receptors for inhibitory Dickkopf-1 (DKK1) protein and treatment modalities that modulate LRP/DKK1 binding may therefore act as stimulators of bone mass accrual. Here we report that RH2-18, a fully human monoclonal anti-DKK1 antibody elicits systemic pharmacologic bone efficacy and new bone formation at endosteal bone surfaces in vivo in a mouse model of estrogen deficiency induced osteopenia. This was paralleled by partial-to-complete resolution of osteopenia (bone mineral density, BMD) at all skeletal sites investigated in femur and lumbar-vertebral bodies and the restoration of trabecular bone micro-architecture. Importantly, testing of RH2-18 in adult, osteopenic rhesus macaques demonstrated a rate limiting role of DKK1 at multiple skeletal sites and responsiveness to treatment. In conclusion, this study provides pharmacologic evidence for modulation of DKK1 bioactivity in the adult osteopenic skeleton as a viable approach to resolve osteopenia in animal models. Thus, data described here suggest that targeting DKK1 through means such as a fully-human anti-DKK1-antibody provides a potential bone-anabolic treatment for postmenopausal osteoporosis.
Introduction

Bone mass and bone turnover in adults is maintained locally by the balance between osteoclastic bone resorption and osteoblastic bone formation, and multiple coupling 'factors' maintain this servo system at its physiological (homeostatic) steady state (Harada and Rodan, 2003). High bone turnover initiated during menopause and the succeeding progressive reduction in bone mass and microarchitecture result in an increase in the fragility of bone and its susceptibility to fractures, all characteristics of osteoporosis (Rodan & Martin, 2000).

The intermittent application of human parathyroid hormone fragment (hPTH 1-34) as a first bone-anabolic treatment has illustrated novel cues for effectively restoring lost bone mass, structure and strength in the clinic (Dobnig, 2004), however, primarily for severely osteoporotic patients.

Recent genetic evidence has substantiated a central role for the Wnt-signaling components low-density lipoprotein receptor-related proteins (LRP4, LRP5 and LRP6) in bone mass accrual during development (Gong et al., 2001; Kokubu et al., 2004; Mani et al., 2007; Choi et al., 2009). The keystone description of high bone mass (HBM) phenotypes in human triggered by single amino-acid substitutions in LRP5 (Boyden et al., 2002; Little et al., 2002; van Wesenbeeck et al., 2003) have heightened interest in the Wnt/LRP pathway in bone homeostasis extending into adulthood (Baron and Rawadi, 2007). An increasing number of negative regulatory mechanisms intersecting at Wnt-LRP5/6 have been identified in bone, among those most relevant are endogenous inhibitors of the Wnt-signaling pathway including the DKK proteins (Krupnik et al., 1999), Kremen-proteins (Mao et al., 2002) and Sclerostin (Semenov et al., 2005; Ellies et al., 2006). DKK1 binds with high affinity to LRP4/5/6 receptors and Kremen proteins (Choi et al., 2009; Mao et al., 2002; Bafico et al., 2001; Mao et al., 2001; Fleury et al.; 2010). Interestingly, LRP5 HBM is refractory to the inhibitory activities of DKK1 (Boyden et al., 2002; Ai et al., 2005) suggesting that this pathway promises enormous opportunities to identify novel treatment paradigms and to intervene pharmacologically with the course of low bone mass diseases (Khoshla et al., 2008). Although DKK1 is expressed and secreted within the bone
microenvironment (Robling et al., 2008), the protein is soluble and detectable in the peripheral
circulation (Kaiser et al., 2008). These cues have led us to develop monoclonal antibodies as
reagents that interfere functionally with the DKK1 role in Wnt-signaling and bone formation.
Recently DKK1 antibodies have been introduced into mice and have demonstrated efficacy in
disease models of rheumatoid arthritis (Diarra et al., 2007) and multiple myeloma (Yaccoby et al.,
2007; Fulcinitti et al., 2009; Heath et al., 2009) where DKK1 levels are elevated. These findings
corroborate a critical role for DKK1 in the bone microenvironment and point to the potential
clinical applicability of DKK1 neutralizing agents (Choi et al., 2009; Gavriatopoulou et al.,
2009). However, proof of principle in crucial animal models with systemic osteopenia through
estrogen-deficiency emulating post-menopausal osteoporosis in humans has not been
demonstrated.

Here we describe a pharmacologic approach applying a novel fully human monoclonal
anti-DKK1 antibody (Glantschnig et al., 2010). Our data support the hypothesis that systemic
restoration of bone mass can be achieved via pharmacologic modulation of DKK1 bioavailability
with concomitant resolution of osteopenia in murine animal models. More importantly, we
provide evidence for the inhibitory activity and the rate limiting role of DKK1 on bone mass
accrual in the aged ovariectomized primate. Thus we provide a first description of an
experimental therapeutic and putative clinically relevant treatment modality specifically targeting
DKK1 for the treatment of low systemic bone mass, a characteristic of post-menopausal
osteoporosis.
Materials and Methods

**Rhesus DKK1 protein.** Rhesus DKK1 fusion protein was expressed and purified as described previously (Glantschnig et al., 2010) and used in all biological assay.

**Generation of anti-DKK1 antibody RH2-18.** Cambridge Antibody Technology (Cambridgeshire, UK) phage displayed single chain Fv (scFv) antibody libraries were panned alternately against biotinylated rhesus or mouse DKK1 (R&D Systems, Minneapolis, MN) as described (Glantschnig et al., 2010). The scFv heavy chain variable regions were fused inframe with the IgG2m4 constant region (An et al., 2009) and resulting anti-DKK1 antibody structures were published previously (Glantschnig et al., 2010). Anti-DKK1 antibody was expressed in Chinese hamster ovary (CHO) cells using a mammalian expression vector and the secreted antibodies were purified from medium using standard protein A/G based affinity chromatography following manufacturers instruction (Pierce, Rockford, IL). Anti-DKK1 antibody RH2-18 binds to human DKK1 with high affinity, its binding epitope is located within the C-terminal domain of DKK1 and does not show detectable binding to DKK2 or DKK4 proteins (Glantschnig et al., 2010). This experimental antibody was used in all procedures below.

**Pharmacokinetic studies.** All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. These studies were carried out under the Institutional Animal Care and Use Committee (IACUC) of Merck Research Laboratories (West Point, PA). Female C57/BL6NTac mice (Taconic, Hudson, NY) were ovariectomized and housed for 7-weeks before use in pharmacokinetic (PK) studies and randomized by body weight (n=3-4/time point, 19-weeks of age; 28 grams). Animals were provided with water and chow ad libitum and maintained on a 12 hour light/dark cycle. Anti-DKK1 antibody RH2-18 dose solutions were prepared in a buffer consisting of 100 mM histidine, 100 mM arginine, and 6% sucrose (pH 6.0). Vehicle or RH2-18 at a dose of 2 mg/kg or 15 mg/kg was administered subcutaneously (s.c.) in a dose volume of 0.1 mL. Individual mice were sacrificed at each time point (n=3-4) and terminal blood samples were collected by cardiac puncture prior to dosing and on days 1, 4, 7, 11, 15 and 22 following dosing.
Male and female rhesus monkeys (n=3/group) from UL-Lafayette New Iberia Research
Center (New Iberia, LA) weighing 4.7-12.0 kg were used. RH2-18 dose solutions were prepared
in the same buffer as described above and the antibody was administered at a dose of 0.5 mg/kg
or 2.5 mg/kg (s.c., 0.16 mL/kg) or infused intravenously (i.v., 0.5 mg/kg). Serial blood samples
were collected from the saphenous or femoral vein pre-dose and subsequently blood samples
were drawn on day 2, 3, 4, 8, 15, 22, 29, 36, 43, 50, 56, 64, 71, 78, and 85 following antibody
administration. Serum was obtained after allowing blood samples to clot for 30 min in serum
separator tubes, and following centrifugation of coagulated blood (3,200 x g, 10 min, 4ºC). Serum
samples were stored at –70ºC and anti-DKK1 antibody RH2-18 levels determined as described in
detail below.

Pharmacokinetic analyses of anti-DKK1 antibody RH2-18 in mouse and rhesus
serum samples. Recombinant rhDKK1 (235 ng/well) in PBS was used to capture anti-DKK1
antibody in 96-well EIA plate (Fisher Scientific, Pittsburgh PA). Plates were washed once with
wash buffer PBST (PBS, 0.05% Tween-20), and 300 μL of blocking buffer (3% BSA, PBST) was
added to each well. Plates were then incubated at room temperature for 1.5 hours. After blocking,
each well was washed three times with PBST, then dried, sealed and stored at 4ºC. All serum
samples were diluted 1:20 with assay buffer (2% BSA, PBST containing NaN3), and
subsequently diluted with 5% serum assay buffer (5% control serum in assay buffer) to
concentrations of 1-100 ng/mL. Fifty μl aliquots of the previously diluted samples were added to
prepared rhDKK1 coated plates in duplicate, incubated at room temperature for 1 hour and then
washed three times with PBST. HRP-conjugated mouse anti-human IgG (1:3000;
SouthernBiotech, Birmingham, AL) was used as detection antibody. Turbo TMB (3, 3’, 5’,5’
tetramethylbenzidine; Pierce, Rockford, IL) was used as substrate with absorbance read at 450
nm following addition of stop solution (1 M sulfuric acid). Data were analyzed, using a 4-
parameter curve fit for data reduction, as defined by SoftMax® Pro (Molecular Devices,
Sunnyvale, CA). The concentration of human IgG in the test samples was determined by interpolation from the constructed standard curve.

**Determination of serum and urinary pharmacodynamic markers in mouse and rhesus monkey.** Biochemical markers of bone formation and resorption, including DKK1, were evaluated with commercially available kits following the manufacturer's instructions: Metra-BAP, Metra-Osteocalcin, Metra-C1CP, Metra-OPG (Quidel Corporation, San Diego, CA), UniQ-P1NP (Orion Diagnostica, Espoo, Finland), urinary NTx (uNTx, Wampole Laboratories, Princeton, NJ) and total human DKK1 (Assay Designs, Ann Arbor, MI). Serum parameters in mouse were determined by serum CTx (sCTx, Immunodiagnostic System Inc, Fountain Hills, AZ) and mouse P1NP (Immunodiagnostic System Inc, Fountain Hills, AZ) kits. Mouse serum DKK1 protein was determined in a competitive assay format, estimating free DKK1 in the presence of the treatment antibody RH2-18. Reacti-Bind White Opaque 96-well plate (Pierce) were coated with 200 ng RH2-18/well and blocked with StartingBlock-T20 Blocking Buffer in TBS (Pierce). Fifty microliters of each mouse serum samples was added per well. Assay standards were prepared using mouse DKK1 (R&D Systems, Minneapolis, MN) from 62.5-4000 pg/ml. Two mgs of N-terminal DKK1 detection antibody (RH1-28) was biotin labeled (1.4 mol biotin/mol IgG) with the EZ-link Sulfo NHS-LC Biotinylation Kit (Pierce, Rockford, IL) and used at 100 ng/well in the assays. Fifty µl of serum and Bt-RH1-28 were added and incubated in assay plates for an additional 2 hours. Following four washes (TBS+0.02% Tween 20), europium-labeled Streptavidin (Perkin Elmer, Wallac, Turku, Finland) was added to achieve a final concentration of 100 ng/ml and then incubated for additional two hours. After four washes (TBS+0.02% Tween 20) enhancement solution (Perkin Elmer) was added and time-resolved fluorescence (400 us, Ex/Em filter 340/615) was measured on a Victor 3 plate reader (Perkin Elmer).

**Efficacy study with anti-DKK1 antibody RH2-18 in ovariectomized mouse.** All mice (C57BL/6NTac) were received from Taconic (Hudson, NY) either ovariectomized (OVX) or sham operated at age 12-weeks and then allowed to lose bone for an additional twelve weeks.
Animals were provided with water and chow ad libitum and maintained on a 12 hour light/dark cycle. C57BL/6NTac mice were then randomized according to weight (n=10-14/group). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories. RH2-18 dose solutions were prepared in a buffer consisting of 100 mM histidine, 100 mM arginine, and 6% sucrose (pH 6.0). Weekly exposures of 2 mg or 20 mg RH2-18 were achieved by administering 1 mg or 10 mg/kg (s.c., dose volume 0.1 ml) twice weekly for eight weeks. Human (h) PTH (1-34) (Bachem Inc., Torrance, CA) 100 µg/kg (3x per week, s.c.) was included as a control group. Calcein (Sigma, St.Louis, MO; 8 mg/kg, s.c.) was injected on the tenth and third days before necropsy for evaluation of bone surfaces by dynamic histomorphometry.

The right femur and lumbar vertebra (LV1-4) were collected, cleaned of soft tissue and stored in 70% ethanol at necropsy. Bone mineral content and area were quantified using dual-energy X-ray absorptiometry (DEXA) by a Piximus II densitometer (GE/Lunar; Schenectady, NY) and used to calculate areal bone mineral density (aBMD). Femora were positioned with the distal condyles facing the source. Whole femur region of interest (ROI) encompasses the entire femur. Distal femur ROI represented 25% of the femoral length. The central left femur was prepared for histological analyses. A cross-sectional segment of the left femur located distal to the tapering end of the trochanter and 3 mm through the midshaft was excised, cleaned of marrow and then processed and embedded in 90% methylmetacrylate (Sigma, St. Louis, MO) using a Shandon Pathcenter processor (Therom, Cheshire, UK). Cross sections (100 µm) were obtained using a Leica SP1600 microtome (Leica Biosystems, Heidelberg, Germany) and mounted with Eukitt media (EMS, Fort Wasington, PA) onto glass slides. Unstained sections were quantitated for calcein label on periosteal and endosteal surface using an epi-fluorescence microscope (Nikon-Eclipse 80i; Nikon Instruments, Melville, NY) and the images analyzed by Bioquant Osteo (Ver. 7.20; Bioquant Image Analysis Corp., Nashville, TN). Analyses included bone volume per tissue volume BV/TV, %), mineralizing surface (MS/BS, %), mineral apposition rate
(MAR, μm/d) and bone formation rate (surface-based, BFR/BS, mm³/mm²/yr) and were calculated as described (Parfitt et al., 1987).

**Micro Computed Tomography.** Micro Computed Tomography (micro CT) was performed using a volumetric micro CT scanner (Explore Locus SP; GE Healthcare, London, Canada) with data collected by MicroView (GE Healthcare, Milwaukee, WI). Lumbar vertebrae (L3) were scanned with an isotropic voxel resolution of 8 µm with tube voltage and current at 80kV and 80 μA. Protocols were set to 500 projections per scan (180 deg) with 3000 ms integration time. A cylindrical ROI of 2.4 mm with a 0.7 mm diameter was centered along the long axis of the vertebrae about 0.2 mm distant and excluding the cranial and caudal growth plates. Thresholding was determined by the analysis software and averaged per treatment group. Bone mineral density (vBMD), bone volume fraction (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th.) and trabecular separation (Tb.Sp) were calculated using MicroView.

**Efficacy study with anti-DKK1 antibody RH2-18 in the ovariectomized rhesus macaque.** Female rhesus macaques, 14-20 years old (8-years post-ovariectomy) were used in this study. Animals were individually housed in the same temperature and humidity controlled room, with a 12 hour light/dark cycle. Water and standard laboratory chow (high protein monkey diet 5054; Purina, Gray Summit, MO) were provided *ad libitum*. RH2-18 (50 mg/ml, 1% sucrose, 10 mM histidine, 25 mM arginine, pH 6.0) at final dose level of 10 mg/kg (n=14) or respective control buffer (n=6) were administered as s.c. injections every two weeks (2x/month). All animals were fasted prior to collection of serum and urine samples. Serum and urine samples were collected up to day 71 of the study. Imaging of bone for density and morphological measures was performed in-life at baseline and at 3, 6 and 9 months of dosing with the following modalities at the indicated anatomical sites: Dual energy X-ray absorptiometry DXA (GE Lunar iDXA; GE Healthcare Piscataway, NJ) for whole body, spine (L2-4) and hip; High resolution-peripheral quantitative CT (hr-pQCT, Scanco XtremeCT, Scanco Medical, Zurich, Switzerland) at the distal radius and distal tibia; and Quantitative CT (GE-DST; GE Healthcare Piscataway, NJ) for spine, femoral neck and shaft. For QCT, animals were scanned in a head first supine position.
position with a phantom with varying mineral densities (Mindways) underneath the spine and hip of the animal for density calibration. Animals were scanned from L1 through mid-femur using the following CT protocol: 80 kVp, 0.625-mm slice thickness, 300 mA s, pitch 1, bed height of 95, 250 mm reconstruction field of view corresponding to a pixel size of .244um, and bone convolution kernel. All image analyses were carried out using Mindways 3-Dimensional Volumetric QCT Bone Densitometry of the Spine and Hip (QCT Pro software, Mindways Software, Inc.; Austin, TX). HR-pQCT was used to evaluate cortical and trabecular regions of bone below the growth plate in the ultradistal radius and tibia by positioning the imaged region at a fixed distance below a landmark line of the radius and tibia, respectively. The following scan parameters were used: 60 kVp, 900 mA; 1000 projections; 41 micron slice thickness; 3072 * 3072 pixels; Integration time 200 ms; Scan Distance/Dimension 9 mm ; Number of slices 220. All rhesus macaque were returned after completion of the studies.

**Statistical Analysis.** All data are expressed as a mean ± standard error (SE). Where appropriate a one-way ANOVA followed by Dunnett's post-hoc test to detect group differences were calculated using Graphpad Prism 5.0 (GraphPad Software, LaJolla, CA).
Results

Anti-DKK1 antibody (RH2-18) neutralizes DKK1 function in vitro

Fully-human anti-DKK1 antibody RH2-18 was identified from phage displayed scFv antibody libraries and selected by consecutive panning on the basis of binding to both rhesus and mouse DKK1 proteins. Analyses of RH2-18 showed high affinity for human DKK1 ($K_D$: 249 pM) at a discrete C-terminal epitope on DKK1 (Glantschnig et al., 2010), a domain necessary and sufficient for LRP5/6 interaction (Brott and Sokol, 2002) and thus the antibody interferes with receptor ligand interaction (Glantschnig et al., 2010). Interference of RH2-18 with DKK1 function was validated in cell based assay interrogating Wnt-signaling through β-catenin/TCF in HEK293 cells (Supplemental Figure. 1A) and in mouse pluripotent mesenchymal C3H10T1/2 cells (Supplemental Figures 1B and C). Wnt3A induced expression of luciferase-reporter, the selected markers alkaline phosphatase (AP), IGF-binding protein 2 (Igfbp-2), Troy (TNFRSF-19), and Axin-2 were markedly diminished by rhDKK1 (50 nM). Addition of anti-DKK1 antibody RH2-18 restored Wnt-induced activities and expression of the selected markers to or above control levels in vitro.

DKK1 neutralization results in a bone-pharmacodynamic response in ovariectomized mice

To evaluate pharmacokinetic properties in vivo, RH2-18 antibody (2 and 15 mg/kg) was administered to female ovariectomized (OVX) C57BL/6NTac mice by single dose subcutaneous (s.c.) injection. RH2-18 concentration in serum revealed a close to linear dose exposure relationship, with $C_{\text{max}}$ 138.9 ± 11.4 nM and 913 ± 122 nM, respectively. In general $T_{\text{max}}$ was noted at 1 to 4 days following subcutaneous administration and RH2-18 half-life ($t_{1/2}$) was 12 - 19 days (Fig. 1A). In parallel, serum mouse DKK1 levels determined by a competitive assay format designed to detect DKK1 protein not bound to RH2-18 dropped from baseline levels as expected at all doses tested. The suppression of free-DKK1 relative to the vehicle control group was nearly complete at 2.8 % (2 mg/kg), or below limit of quantification (15 mg/kg) on day 4 (Fig. 1B). Bioavailability of DKK1 increased in both groups up to 7.3% relative to vehicle treated mice on day 23 (Fig. 1B). Thus, RH2-18 demonstrated binding to mouse DKK1 in serum and...
prolonged stability in vivo. A pharmacodynamic response of the mechanistically relevant serum collagen marker of bone formation (P1NP) was evident on day 5 (+97% above baseline in 15 mg/kg group; p<0.05 vs. control) (Fig. 1C). No significant effects on serum levels of the collagen-I degradation product sCTx (CrossLaps) were detected (Fig. 1D). Importantly, ex vivo analyses of femora and lumbar vertebrae (L3-L4) on day 24 of the study showed significant increases (p<0.05 vs. vehicle control group) in femoral (Fig. 1E) and lumbar spine BMD (Fig. 1F) in both RH2-18 treatment groups. These results suggest that RH2-18 exposure mechanistically limited free DKK1 levels in parallel with increases in the bone formation marker P1NP and BMD in OVX mice.

**Pharmacologic suppression of DKK1 function increases bone formation and resolves low BMD in ovariectomized mice** To further evaluate the bone mass restoring potential of DKK1 inhibition in adult (age 23 weeks at study start) osteopenic mice, we studied RH2-18 effects in OVX mice with established estrogen deficiency induced osteopenia. Animals had OVX done at 12-weeks prior to study start to allow for bone loss (-6-7% LS-BMD vs. sham control) and were treated for 8-weeks with 2 mg/kg or 20 mg/kg RH2-18 per week. As a positive control, for a robust bone effect, hPTH (1-34) at a dose that is 140-fold over the equivalent weekly clinical exposure (6) resulted in expected changes in BMD and consistent effects on dynamic and static bone histomorphometry. Terminal RH2-18 serum concentrations following multiple dosing at week 8 were 113.8 ± 2.6 nM and 332.7 ± 38.9 nM, respectively. Bioavailable free DKK1 serum levels (baseline: 8.0 ± 3.4 ng/mL) were reduced on average by >90% in all RH2-18-treated groups.

Since bone formation biomarker and BMD responses in mouse are detectable after a single dose of RH2-18, we reasoned that bone efficacy might be detectable histologically after a relatively short treatment period. To test this hypothesis, we collected histological and BMD data from mouse femurs within a subset of treatment groups at day 24 (Table 1). Confirming this hypothesis, whole femur BMD was significantly increased in RH2-18 (2mg/kg) group versus
vehicle group by 4.5% (p<0.05) with trends towards increased BMD in the central (4.0%) and
distal (5.1%) femur. Interestingly, indices of new endosteal bone formation were significantly
increased (p<0.05) in the RH2-18 treatment versus the vehicle group. From a mechanistic
perspective, it is significant that endosteal bone formation rates were found to be increased in
RH2-18 treatment group on day 24 demonstrating the rapid onset of antibody mediated bone
anabolic activity in adult osteopenic mice.

At 8 weeks of treatment, RH2-18 resolved low BMD in the femora of OVX mice to
sham-OVX (Intact) vehicle levels. Overall mean-BMD in the whole femur was increased by
6.0% in the 20 mg/kg/week group (Fig. 2A). Similarly, BMD was higher in the distal femur (Fig.
2B) and central femur, representing cortical bone (Fig. 2C) with an increase of +7.2% and +6.6%,
respectively in the 20 mg/kg/wk dose group. Consistent with the initial findings after 24 days
distal endosteal mineralizing surface/bone surface (MS/BS) in the central femur was increased
significantly (p<0.05) at 8 weeks of treatment (Table 2). The increased MS/BS was primarily
responsible for a significant 2-fold rise in bone formation rate/bone surface (BFR/BS) at this
skeletal site. Periosteal MS/BS in the central femur rose modestly in response to treatment (Table
2), accounting for the modest increase in periosteal BFR/BS. Consequently, central femur mean
bone volume (BV/TV) was 5.8% greater than in vehicle OVX control group. The increase in
distal endosteal bone formation and the more modest effect on periosteal bone formation, were
paralleled by numerical increases in cortical area as well as reductions in endosteal perimeter
(Table 2).

Importantly, lumbar spine BMD (LV1-4) at 8-weeks was partially to completely restored
in RH2-18 treatment groups to levels seen in Sham-OVX (Intact) vehicle treated controls (Fig.
2D). This profound effect on lumbar spine aBMD was further characterized using ex vivo micro
CT analysis of trabecular bone (LV3) in vehicle and RH2-18 treatment groups (Table 3).
Volumetric analysis confirmed the significant effect on BMD by RH2-18 treatment and the
overall increased bone volume (up to 27%) within the trabecular compartment. RH2-18 treatment
(20 mg/kg) significantly augmented trabecular thickness (p<0.001 vs. OVX-vehicle; 13.6%) and
trabecular number (p=0.068; 15%) while reducing trabecular spacing (p<0.01; -25.7%). Thus, RH2-18 treatment produced pharmacological effects at multiple loci and improvement of bone micro-architectural parameters in the mature ovariectomized mouse.

Physical and biological properties of the anti-DKK1 antibody (RH2-18) enable the testing of DKK1’s role in non-human primates (rhesus macaque)

To address pharmacokinetic properties in non-human primates we first administered single doses of RH2-18 (0.5 or 2.5 mg/kg) via s.c. or intravenous (i.v.) routes to rhesus macaque and then drew blood samples at the indicated time points for analysis (Fig. 3). A comparison of exposure following administration of 0.5 mg/kg s.c. and i.v. doses suggests essentially complete absorption of RH2-18, an apparent T1/2 of approximately 2 weeks and a very low serum clearance of 2.7 μl/min/kg and a small volume of distribution (Vd) of 56 ml/kg revealing an exceptionally favorable pharmacokinetic profile of the antibody for further testing of the role of DKK1 in primates.

DKK1 functions as a rate-limiting suppressor of bone mineral density in aged and ovariectomized non-human primates (rhesus macaque)

We next introduced RH2-18 (10 mg/kg/2x month) into long-term ovariectomized rhesus macaques (14-20 years of age; >8-years post-OVX). Serum RH2-18 levels at day 7 and day 71 (the last time point tested) were 681 ± 160 nM and 690 ± 201 nM, respectively, indicating extremely stable exposure of the antibody over an extended time period and suggesting absence of substantial interfering anti-human antibody response. DKK1 levels were increased by treatment from baseline 0.15 ± 0.005 nM to 29.9 ± 7.7 nM (day 8) and remained essentially unchanged to the last time point tested (27.3 ± 6.6 nM; day 71). Within this time-frame P1NP a serum marker of bone formation was consistently elevated over levels in vehicle treated controls (Fig. 4A) while C1CP showed a significant albeit transient increase (Fig. 4B) in the RH2-18 treatment group. Other bone formation indices, BSAP and osteocalcin (Figs. 4, C and D) both
consistently trended upwards in the RH2-18 treatment group at later time points, without achieving statistical significance. There was no significant change in levels of the collagen-I degradation markers sCTX and uNTx or of osteoprotegerin (Figs 4, E, F and G) within the observation period.

Areal BMD (aBMD) was analyzed longitudinally by DXA in the vehicle and RH2-18 treatment groups at baseline and at 3-month intervals thereafter. The baseline lumbar spine aBMD of the ovariectomized animal cohort was 0.695 ± 0.075 g/cm², compared to historic in-house data of aged non-ovariectomized animals (0.814 ± 0.119 g/cm²). Several measures of bone density and morphology investigated by imaging showed statistically significant improvement from baseline and compared to the vehicle group. Whole body and spine aBMD increased longitudinally from baseline by 4.6% (p<0.05 vs control group) and by 5.0% (p<0.05 vs control group), respectively in RH2-18 treated animals at 9 months of treatment, while no significant changes were observed in the vehicle treated group (Figs. 5, A and B). These findings are supplemented by volumetric BMD (vBMD) at the lumbar spine (L1-L4) by QCT (Fig. 5C). Trabecular-vBMD (TbvBMD) of the spine showed an early treatment effect at 3-month, with a significant increase from baseline of 9.3 ± 1.8 % (mean ± SE; p=0.037), consistent with the concept that treatment with anti-DKK1 antibody RH2-18 primarily affected trabecular bone BMD. TbvBMD increased further at 6 months in most animals by 2.3 ± 0.6 % to 11.5 ± 1.6% (p<0.05) above baseline and reached a plateau at 9-month (11.5%; p<0.001). TbvBMD changes from baseline (4.6 ± 1.1%) in the vehicle group at 6-month were non–significant. Individual response size in lumbar spine TbvBMD at month 6 within the RH2-18 treatment group correlated well with early individual changes in levels of the bone formation marker P1NP from baseline at day 12 of the study (Supplemental Figure 2). In the aggregate, data presented here demonstrate significant increases in bone mass affecting whole body and regional lumbar spine BMD in adult primates. Furthermore, a trend to increased femoral neck total aBMD by DXA was observed in the RH2-18 treatment group (p=0.047 vs. Vehicle) at 9-month.
To further investigate fracture relevant sites, we extended the analyses to distal tibia and distal radius examining vBMD and trabecular micro-architecture by hr-pQCT. Integral vBMD (combined cortical/trabecular, represented by D100 in the Scanco analyses software) of the distal tibia was found to be increased by 3.7% (p<0.001) by treatment at 9-month (Fig. 5D), with detectable increases in trabecular thickness (TbTh) of 3.1% (p<0.01), 4.6% (p<0.005) and 5.6% (p<0.0001) at 3, 6 and 9 months, respectively (Supplemental Figure 3). Similar efficacies were observed in the distal radius of the treatment group at 6 and 9 months treatment, since integral vBMD showed increases of 3.6% (p<0.005) and 5.4% (p<0.005), respectively (Fig 5E), and trabecular thickness (TbTh) showed increases of 3.0% (p<0.01) and 3.3% (p<0.005), respectively (Fig. 5F). Thus, anti-DKK1 antibody RH2-18 treatment positively affected bone parameters at both axial and appendicular skeletal regions in aged and ovariectomized non-human primates.
Discussion

Bone anabolic treatments targeting the Wnt-signaling pathway offer novel therapeutic approaches for systemic diseases like postmenopausal or iatrogenic osteoporosis (Khoshla et al., 2008; Deal, 2009; McCarthy and Marshall, 2010) and for localized bone loss secondary to metastatic bone disease (Yaccoby et al., 2007; Gavriatopoulou et al., 2009; Fulcnitti et al., 2009, Heath et al., 2009) and rheumatoid arthritis (Diarra et al., 2007), or in fracture repair (Bajada et al., 2009). Though genetic models have provided overwhelming evidence for the role of DKK1 as a crucial regulator during bone growth and development (Li et al., 2006; Morvan et al., 2006), documentation of a role for DKK1 in the maintenance of the adult skeleton and the therapeutic potential for functional modulation of DKK1 is critical. Here we provide evidence for the inhibitory function of DKK1 in systemic low bone mass condition (osteopenia) in adult bone using an experimental therapeutic antibody. Furthermore, we show that targeting the DKK1 mechanism results in bone efficacy at multiple skeletal sites in both osteopenic murine and primate models.

The anti-DKK1 antibody described in this study displays several characteristics to address above paucity of data. First, the antibody identification strategy permitted cross-reactivity for testing in murine as well as primate species. Second, the antibody exhibits no measurable interaction with the closely related DKK2/DKK4 proteins assuring data interpretation that is tightly focused on DKK1 function (Krupnik et al., 1999; Glantschnig et al., 2010). High-bone mass (HBM) mutations of LRP5 diminish binding of DKK1 and confer resistance to the inhibitory actions of DKK1 on LRP5/6 and Wnt-signaling (Boyden et al., 2002; Fleury et al., 2010), thus providing a rationale for the mechanism of action of RH2-18 on bone and its efficacy via modulation of DKK1 function (Glantschnig et al., 2010). In addition DKK1 interaction with LRP4 (Choi et al., 2009) and Kremen receptors (Mao et al., 2002) may be affected by the pharmacological action of anti-DKK1 antibody, however, this was not experimentally tested.
We demonstrate here the profound systemic effects of anti-DKK1 antibody in adult osteopenic mice at multiple skeletal sites including endosteal surfaces comprised of trabecular and cortical compartments leading to partial to complete resolution of the osteopenic phenotype.

The most notable finding in this study is that when introduced de novo into estrogen-deficient osteopenic adult mice RH2-18 treatment elevated BMD to a level comparable with that of sham-operated controls within eight weeks of treatment. Wnt/β-catenin signaling has previously been reported to influence both bone formation and resorption (Baron and Rawadi, 2007; Pinzone et al., 2009). The anti-DKK1 antibody induced osteoanabolic activity was evidenced by increased circulating P1NP in treated mice and primates and confirmed by the observation of augmented bone formation at all endosteal surfaces examined in mouse. The approximate 2-fold increase in endosteal bone formation and detectable but non-significant effect on periosteal bone formation was paralleled by a progression towards an increase in the cortical area. The cellular process underlying this effect likely involves Wnt/β-catenin and thus a DKK1 inhibitable mechanism in the differentiation of mesenchymal progenitor cells to pre-osteoblast progenitors as well as in pre-osteoblast to osteoblast transitions (Pinzone et al., 2009). This interpretation is consistent with the genetic DKK1 loss of function phenotype in mice which includes both an increase in osteoblast number and elevated bone formation and paralleled by improved biomechanical strength (Morvan et al., 2006). Thus an augmenting effect on bone strength by a pharmacologic DKK1 inhibitor is inferred; however this awaits confirmation by future studies. Further validation of on-target efficacy by the experimental antibody RH2-18 could be gained using genetic models where DKK1 protein expression is strongly reduced or preferably completely absent in the adult mouse, and thus treatment effects by anti-DKK1 antibody are expected to diminish.

Evidence suggesting a role for local Wnt-pathway modulation through DKK1 in murine bone tissue has recently been provided by models of inflammatory joint disease (Diarra et al., 2007) and multiple myeloma in immune compromised mice (Yaccoby et al., 2007; Fulciniti et al.,
2009; Heath et al., 2009) in which DKK1 levels are mechanistically elevated. Anti-DKK1 antibodies have been shown to suppress bone resorption activity in these murine disease models (Diarra et al., 2007, Yaccoby et al., 2007), whereas in other studies the lack of an effect on bone resorption indices has been reported (Fulciniti et al., 2009; Heath et al., 2009). In general RH2-18 treatments elicited no long term effects on markers of bone-resorption but a transient suppressive effect on sCTx and uNTx levels in primates, leaving open the possibility of an associated though modest bone resorptive component in the current study. Studies in primates were non-invasive, and thus limited by the lack of information on bone histology, which could afford more detailed analyses of bone resorption processes at the tissue level. Diarra et al. have established that bone resorptive processes and the local apposition of bone (osteophyte) in joints involve particular regulatory cues controlled through DKK1, while neutralization of DKK1 also protects from systemic bone loss in inflammatory mouse models (Ruiz Heiland et al., 2010). Further studies will be needed to evaluate the potential effect of RH2-18 anti-DKK1 antibody on the local apposition or removal of bone in healthy and diseased joints during inflammation.

In addition to the loss in bone mass, deterioration in parameters of bone quality (e.g. trabecular architecture) can increase the risk of fracture in vertebral osteoporosis (McDonnell et al., 2007). DKK1 appears to play a rate limiting role in the adult osteopenic skeleton and during estrogen-deficiency, since RH2-18 treatment ameliorated the reductions in lumbar spine aBMD in mouse and primates and improved parameters of bone micro-architecture in the mouse vertebra. Likewise, lumbar spine TbBMD of adult non-human primates improved significantly with the treatment. Importantly this effect was not limited to the axial skeleton since RH2-18 treatment notably improved integral vBMD as well as trabecular thickness at the distal radius and tibia. Together, the findings strongly support DKK1’s role as a rate limiting factor in the adult skeleton. Moreover, this role is maintained in estrogen-deficiency where DKK1 neutralization ameliorates the reductions in BMD and improves micro-architectural integrity. In this context the investigation of potential bone efficacy by anti-DKK1 antibodies including RH2-18 in other
animal models of accelerated systemic bone loss unrelated to hormonal deprivation will be of interest (Ruiz Heiland et al., 2010).

Also consistent with a translational therapeutic effect is an early treatment response of the bone formation biomarker P1NP, which correlated moderately and positively with individual lumbar spine TbvBMD responses to RH2-18 treatment in non-human primates. Thus, the combined data provide strong evidence for an inhibitory role of DKK1 in bone homeostasis in adult ovariectomized primates and a bone anabolic response to a treatment with an experimental DKK1 neutralizing antibody resulted in bone mass accrual at multiple skeletal sites.

Given the known involvement of Wnt signaling in development and in certain cancer types, all molecules modulating the Wnt signaling pathway need to be investigated for their tumorigenicity potential and toxicity to other extra-skeletal tissues in particular in the treatment of chronic disorder such as osteoporosis (Baron and Rawadi, 2007). A wide range of DKK1 expression levels has been reported at various phases of tumorigenesis in multiple cancer phenotypes including prostate, breast, colorectal, esophageal, lung, and multiple myeloma (Pinzone et al., 2009). The role of DKK1 is not completely defined and may vary depending on the cellular context, and the potential risks through modulation of DKK1 function must be considered and further evaluated, although a DKK1-antibody has recently entered clinical trials for the treatment of multiple myeloma patients (Fulciniti et al., 2009).

In conclusion, we provide pharmacological evidence for modulation of DKK1 bioactivity resulting in partial to complete resolution of osteopenia in murine and primate models. Thus, modulation of DKK1 function through an experimental fully-human anti-DKK1 antibody exhibits translational potential as a bone-anabolic agent for the treatment of human low bone mass disease, a characteristic of postmenopausal osteoporosis.
Acknowledgments

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Authorship Contributions

Participated in research design: Glantschnig, Scott, McCracken, Fisher, Sandhu, Cook, Williams, Strohl, Flores, Kimmel, Wang and An.

Conducted experiments: Glantschnig, Scott, Wei, McCracken, Nantermet, Williams, Cook and Kimmel.

Contributed new reagents or analytic tools: Hampton, Wei, McCracken, Zhao, Vitelli, Huang, Wang and An.

Performed data analysis: Glantschnig, Scott, Wei, McCracken, Nantermet, Sandhu, and An.

Wrote or contributed to the writing of the manuscript: Glantschnig, Scott, Hampton, Fisher, Sandhu, Williams and An.
References


FIGURE LEGENDS

**Fig. 1.** Pharmacokinetics and pharmacodynamic responses of fully human anti-DKK1 antibody RH2-18 after single dose administration in ovariectomized C57BL/6-mouse *in vivo*. Vehicle (diamonds, dashed line) or RH2-18 (solid lines) at 2 mg/kg (triangles) or at 15 mg/kg (squares) was administered via s.c. injections and serum samples were collected at the time points indicated up to day 23. (A) Serum levels and pharmacokinetic profile of RH2-18 over 23 days after administration. (B) Reduction in bioavailable DKK1 in serum of mice treated with increasing RH2-18 doses. (C) P1NP serum levels show increase from baseline in RH2-18 treatment groups (p=0.02; day 5; 15 mg/kg) and numerical increased P1NP levels were observed up to 16 days. (D) Serum CTx levels were determined at day 2 and 16. (E) Whole femur aBMD and (F) lumbar spine aBMD (L1-4) increase in RH2-18 treatment groups at day 24. Data given as means ± SE. (n=3-4/group/time point). One-way ANOVA, Dunnetts’ test vs. vehicle (* p<0.05, ** p<0.01).

**Fig. 2.** Resolution of osteopenia (aBMD) in ovariectomized mice by 8 weeks treatment with fully human anti-DKK1 antibody RH2-18. Mice were dosed with vehicle or RH2-18 (2 or 20 mg/kg/wk) or hPTH (1-34), 80 µg/kg, 3x/week. Sham-OVX mice were dosed with vehicle. *Ex vivo* DXA analyses of aBMD. (A) Femoral aBMD acquired in the whole femur. (B) aBMD of the cancellous/cortical subregion of the distal femur. (C) Cortical aBMD determined in mid-shaft of the central femur. (D) aBMD in lumbar vertebral bodies (L1-4). Data given as means ± SE. (n=10-14/group). One-way ANOVA, Dunnetts’ test vs. Vehicle-OVX control group (* p<0.05, ** p<0.01, *** p< 0.001).

**Fig. 3.** Serum levels and pharmacokinetic profile of fully human anti-DKK1 antibody RH2-18 following administration of a single 0.5 mg/kg i.v. (closed triangles) or s.c. (open triangles) dose or a single 2.5 mg/kg s.c. (open squares) dose in rhesus macaque. Data given as means ± SE. (n=3/group).
Fig. 4. Serum bone formation and bone resorption marker profiles in aged ovariectomized rhesus macaques by anti-DKK1 antibody RH2-18 treatment (10 mg/kg/2-weeks). All serum and urinary analytes were determined at baseline and at time points indicated up to day 71 in vehicle and RH2-18 treatment groups. Data are given as percent change from baseline corrected for respective values in the vehicle control group (Δ) at the individual time points. (A) Collagen-I synthesis by product P1NP. (B) Collagen-I synthesis byproduct C1CP. (C) Bone-specific alkaline phosphatase, BSAP. (D) Osteocalcin. (E) Urinary collagen-I degradation product uNTx. (F) Serum collagen-I degradation product sCTx. (G) Osteoprotegerin, OPG. Data given as means ± SE. One-way ANOVA, Dunnetts’ test vs. baseline (* p<0.05, ** p<0.01).

Fig. 5. Bone-efficacy of fully human anti-DKK1 antibody RH2-18 reveals a rate limiting role for DKK1 in aged and ovariectomized rhesus macaque. Vehicle (open diamonds) or anti-DKK1 antibody RH2-18 (closed squares) was administered (s.c., 10mg/kg/2-weeks) up to 9-month and multiple bone endpoints were determined. X-axis indicates time points of data acquisition at baseline and at 3-month intervals. Y-axis indicates percent change from baseline. (A) Changes in whole body areal-BMD by DXA over treatment period. (B) Changes in Lumbar spine (LV2-4) areal-BMD over treatment period. (C) Increased trabecular-vBMD of the lumbar spine, quantitative-computed tomography (QCT). (D) Increase in integral vBMD (cortical and trabecular) of the ultradistal tibia, high-resolution peripheral qCT (hr-pQCT). (E) Change in integral vBMD at the distal radius (hr-pQCT). (F) Changes in trabecular thickness of the distal radius in rhesus macaque evaluated by hr-pQCT. All data given as percent change from baseline (mean ± SE). (n=6 in vehicle treatment group; n=14 in anti-DKK1 antibody treatment group). One-way ANOVA, Dunnetts’ test vs. vehicle control group (* p<0.05, ** p<0.01, *** p<0.001).
TABLE 1

Femoral BMD and histomorphometric indices of the central femur of ovariectomized mice after 24 days of treatment with vehicle or RH2-18 (2 mg/kg/wk) or hPTH (1-34)

BV/TV, bone volume/tissue volume; MAR, mineral apposition rate; MS/BS, mineralizing surface/bone surface; BFR/BS, bone formation rate/bone surface. Data given as means ± SE.

One-way ANOVA, Dunnetts’ test vs. vehicle (* p<0.05, ** p<0.01, *** p<0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>RH2-18</th>
<th>hPTH (1-34)</th>
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<tbody>
<tr>
<td><strong>Whole Femur</strong></td>
<td></td>
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<tr>
<td>BMD (mg/cm²)</td>
<td>48.28 ± 0.358</td>
<td>50.45 ± 0.759 *</td>
<td>51.81 ± 0.636 ***</td>
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<tr>
<td><strong>Distal Femur</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>51.71 ± 0.648</td>
<td>54.35 ± 1.159</td>
<td>55.93 ± 0.839 **</td>
</tr>
<tr>
<td><strong>Central Femur</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>44.81 ± 0.434</td>
<td>46.62 ± 0.784</td>
<td>48.06 ± 0.622 **</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>45.28 ± 0.637</td>
<td>45.87 ± 0.612</td>
<td>47.12 ± 0.965</td>
</tr>
<tr>
<td>MAR (μm/d)</td>
<td>1.21 ± 0.073</td>
<td>1.36 ± 0.052</td>
<td>1.36 ± 0.065</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>16.63 ± 3.140</td>
<td>27.76 ± 2.520 *</td>
<td>35.12 ± 3.680 ***</td>
</tr>
<tr>
<td>BFR/BS (μm³/μm²/yr)</td>
<td>73.17 ± 14.770</td>
<td>136.10 ± 12.180 *</td>
<td>175.30 ± 21.050 ***</td>
</tr>
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</table>
TABLE 2
Histomorphometric indices at the central femur of sham-OVX (intact) and OVX-mice after 56 days of treatment with either vehicle, RH2-18 or hPTH (1-34). BV/TV, bone volume/tissue volume; Ct.Ar., cortical area; Ec.Pm., endocortical perimeter; Ps.Pm., peristeal perimeter; MAR, mineral apposition rate; MS/BS, mineralizing surface/bone surface; BFR/BS, bone formation rate/bone surface. Data given as means ± SE. (n=9 -14/group). One-way ANOVA, Dunnetts’ test vs. OVX-Vehicle (* p<0.05, ** p<0.01, *** p<0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact Vehicle</th>
<th>O VX Vehicle</th>
<th>RH2-18 (2 mg/kg)</th>
<th>RH2-18 (20 mg/kg)</th>
<th>hPTH (1-34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>49.67 ± 0.597 ***</td>
<td>42.86 ± 0.602</td>
<td>45.79 ± 0.532 **</td>
<td>45.49 ± 0.725 *</td>
<td>48.37 ± 0.435 ***</td>
</tr>
<tr>
<td>Ct.Ar. (mm²)</td>
<td>0.81 ± 0.013 *</td>
<td>0.75 ± 0.021</td>
<td>0.78 ± 0.014</td>
<td>0.80 ± 0.018</td>
<td>0.82 ± 0.015 *</td>
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<tr>
<td>Ec.Pm. (mm)</td>
<td>3.40 ± 0.038 ***</td>
<td>3.74 ± 0.050</td>
<td>3.60 ± 0.025</td>
<td>3.67 ± 0.070</td>
<td>3.50 ± 0.026 **</td>
</tr>
<tr>
<td>MAR (μm/d)</td>
<td>1.33 ± 0.046</td>
<td>1.13 ± 0.497</td>
<td>1.22 ± 0.038</td>
<td>1.28 ± 0.065</td>
<td>1.30 ± 0.062</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>16.17 ± 2.766</td>
<td>13.34 ± 2.719</td>
<td>17.84 ± 2.288</td>
<td>22.85 ± 2.879 *</td>
<td>16.07 ± 2.247</td>
</tr>
<tr>
<td>periosteal</td>
<td></td>
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<tr>
<td>Ps.Pm. (mm)</td>
<td>4.67 ± 0.029 *</td>
<td>4.85 ± 0.065</td>
<td>4.78 ± 0.030</td>
<td>4.83 ± 0.062</td>
<td>4.76 ± 0.035</td>
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<tr>
<td>MAR (μm/d)</td>
<td>0.96 ± 0.055</td>
<td>1.09 ± 0.069</td>
<td>0.912 ± 0.042</td>
<td>1.04 ± 0.054</td>
<td>1.02 ± 0.049</td>
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<tr>
<td>MS/BS (%)</td>
<td>4.94 ± 1.415</td>
<td>1.23 ± 0.353</td>
<td>5.99 ± 1.791 *</td>
<td>2.69 ± 0.758</td>
<td>5.44 ± 1.466</td>
</tr>
<tr>
<td>BFR/BS (μm³/μm²/yr)</td>
<td>7.79 ± 3.094</td>
<td>2.38 ± 0.937</td>
<td>9.62 ± 4.051</td>
<td>7.32 ± 2.682</td>
<td>18.11 ± 5.413 **</td>
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</table>
TABLE 3
Histomorphometric indices at the lumbar vertebrae (L3) acquired by *ex vivo* micro-computed tomography (micro CT) in sham-OVX and OVX mice after 56 days of treatment with vehicle or anti-DKK1 antibody RH2-18. Representative micro CT slice images by vBMD from respective control and RH2-18 treatment groups. vBMD, volumetric BMD; BV/TV, Bone Volume/Tissue Volume; Tb.Th., trabecular thickness; Tb.N., trabecular number; Tb.Sp., trabecular spacing. Data given as means ± SE. One-way ANOVA, Dunnett’s test vs. OVX-Vehicle (* p<0.05, ** p<0.01, *** p<0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact</th>
<th>OXV</th>
<th>RH2-18 (2 mg/kg)</th>
<th>RH2-18 (20 mg/kg)</th>
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</thead>
<tbody>
<tr>
<td>vBMD (mg/cm³)</td>
<td>248.20 ± 0.7804 ***</td>
<td>187.20 ± 6.207</td>
<td>214.00 ± 8.023</td>
<td>258.70 ± 9.514 ***</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>0.161 ± 0.0088 *</td>
<td>0.134 ± 0.0064</td>
<td>0.152 ± 0.0058</td>
<td>0.184 ± 0.0097 ***</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.023 ± 0.0006 ***</td>
<td>0.019 ± 0.0005</td>
<td>0.023 ± 0.0004 ***</td>
<td>0.022 ± 0.0005 ***</td>
</tr>
<tr>
<td>Tb.N. (#/mm)</td>
<td>7.044 ± 0.3874</td>
<td>7.037 ± 0.3782</td>
<td>6.739 ± 0.2434</td>
<td>8.278 ± 0.3689</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.124 ± 0.0080</td>
<td>0.127 ± 0.0070</td>
<td>0.128 ± 0.0054</td>
<td>0.101 ± 0.0055 *</td>
</tr>
</tbody>
</table>
Figure 2

A

Whole femur BMD (g/cm²)

B

Distal femur BMD (g/cm²)

C

Central femur BMD (g/cm²)

D

Lumbar spine BMD (g/cm²)

Intact, Vehicle, 2 mg/kg/wk, 20 mg/kg/wk, hPTH (1-34)
Figure 4

A

$\Delta PINP$

B

$\Delta CTX$

C

$\Delta BSAP$

D

$\Delta Osteocalcin$

E

$\Delta uNTx$

F

$\Delta sCTX$

G

$\Delta OPG$