A Rate-Limiting Role for Dickkopf-1 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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ABSTRACT

Genetic studies have linked both osteoporotic and high bone mass phenotypes to low-density lipoprotein receptor-related proteins (LRP4, LRP5, and LRP6). LRPs are receptors for inhibitory Dickkopf-1 (DKK1) protein, and treatment modalities that modulate LRP/DKK1 binding therefore may 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) at all of the skeletal sites investigated in femur and lumbar-vertebral bodies and the restoration of trabecular bone microarchitecture. More 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 the 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 and Martin, 2000).

The intermittent application of human parathyroid hormone fragment [hPTH 1–34] as the first bone-anabolic treatment has illustrated novel cues for effectively restoring lost bone mass, structure, and strength in the clinic.
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., 2009a). 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) has 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–LRP pathway including the DKK proteins (Krupnik et al., 1999), Kremen proteins (Mao et al., 2002), and Sclerostin (Seménov et al., 2005; Ellies et al., 2006). DKK1 binds with high affinity to LRP4/5/6 receptors and Kremen proteins (Bafico et al., 2001; Mao et al., 2001, 2002; Choi et al., 2009a; Fleury et al., 2010). It is interesting to note that 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 (Khosla 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 role of DKK1 in Wnt signaling and bone formation. In recent studies, 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; Pulcini 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., 2009b; Gavriatopoulou et al., 2009). However, proof of principle in crucial animal models with systemic osteopenia through estrogen-deficiency emulating postmenopausal osteoporosis in humans has not been demonstrated.

In this study, 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 (OVX) primate. Thus, we provide the 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 postmenopausal 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 of the biological assays.

Generation of Anti-DKK1 Antibody RH2-18. Cambridge Anti-Body Technology (Cambridgeshire, UK) phage-displayed single-chain Fv antibody libraries were panned alternately against biotinylated rhesus or mouse DKK1 (R&D Systems, Minneapolis, MN) as described previously (Glantschnig et al., 2010). The single-chain Fv heavy chain variable regions were fused in-frame 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 cells using a mammalian expression vector, and the secreted antibodies were purified from the medium using standard protein A/G-based affinity chromatography following the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA). Anti-DKK1 antibody RH2-18 binds to human DKK1 with high affinity, and 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 of the procedures below.

Pharmacokinetic Studies. All of the 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 of Merck Research Laboratories (West Point, PA). Female C57BL/6NTac mice (Taconic Farms, Germantown, NY) were OVX and housed for 7 weeks before use in pharmacokinetic studies and randomized by body weight (n = 3–4 per point, 19 weeks of age, 28 g). Animals were provided with water and chow ad libitum and maintained on a 12-h 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 or 15 mg/kg was administered subcutaneously 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 before dosing and on days 1, 4, 7, 11, 15, and 22 after dosing.

Male and female rhesus monkeys (n = 3 per group) from the University of Louisiana at Lafayette New Iberia Research Center (New Iberia, LA) weighing 4.7 to 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 or 2.5 mg/kg (0.16 ml/kg s.c.) or infused intravenously (0.5 mg/kg i.v.). Serial blood samples were collected from the saphenous or femoral vein predose, and subsequently blood samples were drawn on days 2, 3, 4, 8, 15, 22, 29, 36, 43, 50, 56, 64, 71, 78, and 85 after antibody administration. Serum was obtained after allowing blood samples to clot for 30 min in serum separator tubes and after centrifugation of coagulated blood (3200g, 10 min, 4°C). Serum samples were stored at −70°C, and anti-DKK1 antibody RH2-18 levels were 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 phosphate-buffered saline was used to capture anti-DKK1 antibody in 96-well enzyme immunoassay plates (Thermo Fisher Scientific). Plates were washed once with wash buffer, phosphate-buffered saline with 0.05% Tween 20 (PBST), and 300 μl of blocking buffer (PBST containing 3% bovine serum albumin) was added to each well. Plates were then incubated at room temperature for 1.5 h. After being blocked, each well was washed three times with PBST, then dried, sealed, and stored at 4°C. All of the serum samples were diluted 1:20 with assay buffer (PBST containing 2% bovine serum albumin and NaN3) and subsequently diluted with 5% serum assay buffer (5% control serum in assay buffer) to concentrations of 1 to 100 ng/ml. Fifty-microliter aliquots of the previously diluted
samples were added to prepared rhDKK1-coated plates in duplicate, incubated at room temperature for 1 h, and then washed three times with PBST. Horseradish peroxidase-conjugated mouse anti-human IgG (1:3000; Southern Biotechnology Associates, Birmingham, AL) was used as the detection antibody. Turbo TMB (3,3',5,5'-tetramethylbenzidine; Thermo Fisher Scientific) was used as the substrate with absorbance read at 450 nm after the addition of stop solution (1 M sulfuric acid). Data were analyzed, using a four-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-Osteoprotegerin (Quidel Corporation, San Diego, CA), UniQ-P1NP (Orion Diagnostica, Espoo, Finland), and total human DKK1 (Assay Designs, Ann Arbor, MI). Serum parameters in mouse were determined with serum Ctx (aCtx) (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 plates (Thermo Fisher Scientific) were coated with 200 ng of RH2-18 per well and blocked with StartingBlock T20 blocking buffer in Tris-buffered saline (TBS) (Thermo Fisher Scientific). Fifty microtiter plates of each mouse serum sample was added per well. Assay standards were prepared using mouse DKK1 (R&D Systems) from 62.5 to 4000 pg/ml. Two milligrams 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 (Thermo Scientific) and used at 100 ng/well in the assays. Fifty microliters of the serum and biotinylated RH1–28 were added and incubated in assay plates for an additional 2 h. After four washes (TBS containing 0.02% Tween 20), europium-labeled streptavidin (PerkinElmer Life and Analytical Sciences–Wallac Oy, Turku, Finland) was added to achieve a final concentration of 100 ng/ml and then incubated for additional 2 h. After four washes (TBS containing 0.02% Tween 20), enhancement solution (PerkinElmer Life and Analytical Sciences) was added and time-resolved fluorescence (400 μs, excitation/emission filter 340/615) was measured on aVictor3 plate reader (PerkinElmer Life and Analytical Sciences).

**Efficacy Study with Anti-DKK1 Antibody RH2-18 in the O VX Rhesus Macaque.** Female rhesus macaques, 14 to 20 years old (8 years after ovariectomy), were used in this study. Animals were housed individually in the same temperature- and humidity-controlled room, with a 12-h light/dark cycle. Water and standard laboratory chow (high-protein monkey diet 5054; Purina, St. Louis, MO) were provided ad libitum. RH2-18 [50 mg/ml, 1% sucrose, 10 mM histidine, and 25 mM arginine (pH 6.0)] at a final dose level of 10 mg/kg (n = 14) or respective control buffer (n = 6) were administered as s.c. injections every 2 weeks (twice per month). All of the animals were fasted before the 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: DXA (GE Lunar iDXA; GE Healthcare) 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 (QCT) (GE-DST; GE Healthcare) for spine, femoral neck, and shaft. For QCT, animals were scanned in a head first supine position with a phantom with varying mineral densities (Mindways Software, Inc., Austin, TX) underneath the spine and hip of the animal for density calibration. Animals were scanned from L1 through midfemur 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 0.244 μm, and bone convolution kernel. All of the image analyses were carried out using Mindways Three-Dimensional Volumetric QCT Bone Densitometry of the Spine and Hip (QCT Pro software; Mindways Software, Inc.). 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. Whole femur region of interest encompasses the entire femur. Distal femur region of interest 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% methyl methacrylate (Sigma-Aldrich) using a Shandon Pathcentre processor (Therom, Cheshire, UK). Cross sections (100 μm) were obtained using a Leica SP1600 microscope (Leica Biosystems, Heidelberg, Germany) and mounted with Eukitt media (EMS, Fort Washington, PA) onto glass slides. Unstained sections were quantified for calcin label on periosteal and endosteal surfaces using an epifluorescence microscope (Nikon-Eclipse 80i; Nikon, Melville, NY), and the images were analyzed by Bioquant Osteo (version 7.20; Bioquant Image Analysis Corp., Nashville, TN). Analyses included bone volume per tissue volume (BV/TV, %), mineralizing surface/bone surface (MS/BS, %), mineral apposition rate (MAR; μm/day), and bone formation rate (BFR/BS, mm²/mm² per year, surface-based) and were calculated as described previously (Parfitt et al., 1987).

**Micro Computed Tomography.** Micro computed tomography (CT) was performed using a volumetric micro CT scanner (Explore Locus SP; GE Healthcare, London, ON, Canada) with data collected by MicroView (GE Healthcare Technologies, Waukesha, WI). Lumbar vertebrae (L3) were scanned with an isotropic voxel resolution of 8 μm with tube voltage and current at 80 kV and 80 μA, respectively. Protocols were set to 500 projections per scan (180°) with an integration time of 3000 ms. A cylindrical region of interest of 2.4 mm with a diameter of 0.7 mm was centered along the long axis of the vertebrae approximately 0.2 mm distant and excluding the cranial and caudal growth plates. Thresholding was determined by the analysis software and averaged per treatment group. Volumetric bone mineral density (vBMD), bone volume fraction (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), and trabecular separation (TbSp) were calculated using MicroView. Whole femur region of interest encompasses the entire femur. Distal femur region of interest 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% methyl methacrylate (Sigma-Aldrich) using a Shandon Pathcentre processor (Therom, Cheshire, UK). Cross sections (100 μm) were obtained using a Leica SP1600 microscope (Leica Biosystems, Heidelberg, Germany) and mounted with Eukitt media (EMS, Fort Washington, PA) onto glass slides. Unstained sections were quantified for calcin label on periosteal and endosteal surfaces using an epifluorescence microscope (Nikon-Eclipse 80i; Nikon, Melville, NY), and the images were analyzed by Bioquant Osteo (version 7.20; Bioquant Image Analysis Corp., Nashville, TN). Analyses included bone volume per tissue volume (BV/TV, %), mineralizing surface/bone surface (MS/BS, %), mineral apposition rate (MAR; μm/day), and bone formation rate (BFR/BS, mm²/mm² per year, surface-based) and were calculated as described previously (Parfitt et al., 1987).
The following scan parameters were used: 60 kVp, 900 mA, 1000 projections, 41-μm slice thickness; 3072 × 3072 pixels, integration time 200 ms, scan distance/dimension 9 mm, and number of slices 220. All rhesus macaques were returned after completion of the studies.

Statistical Analysis. All of the data are expressed as a mean ± S.E. Where appropriate, a one-way analysis of variance followed by Dunnett’s post hoc test to detect group differences was calculated using Prism 5.0 (GraphPad Software Inc., San Diego, CA).

Results

Anti-DKK1 Antibody (RH2-18) Neutralizes DKK1 Function In Vitro. Fully human anti-DKK1 antibody RH2-18 was identified from phage-displayed single-chain Fv 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 LRPS/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 a cell-based assay interrogating Wnt signaling through β-catenin/T cell factor in human embryonic kidney 293 cells (Supplemental Fig. 1A) and in mouse pluripotent mesenchymal C3H10T1/2 cells (Supplemental Fig. 1, B and C). Wnt3A induced expression of luciferase reporter, the selected markers alkaline phosphatase, insulin-like growth factor-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 OVX Mice. To evaluate pharmacokinetic properties in vivo, RH2-18 antibody (2 and 15 mg/kg) was administered to female OVX C57BL/6NTac mice by single-dose subcutaneous injection. RH2-18 concentration in serum revealed a close to linear dose-exposure relationship, with C_max 138.9 ± 11.4 and 913 ± 122 nM, respectively. In general, T_max was noted at 1 to 4 days after subcutaneous administration, and RH2-18 half-life (t1/2) was 12 to 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 of the doses tested. The suppression of free DKK1 relative to the vehicle control group was nearly complete at 12 to 19 days after multiple dosing at week 8 were 113.8 ± 2.6 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 of the RH2-18-treated groups.

Because 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 increased significantly in RH2-18 (2 mg/kg) group versus vehicle group by 4.5% (p < 0.05), with trends toward increased BMD in the central (4.0%) and distal (5.1%) femur. It is interesting to note that indices of new endosteal bone formation were increased significantly (p < 0.05) in the RH2-18 treatment group versus the vehicle group. From a mechanistic perspective, it is significant that endosteal bone formation rates were found to be increased in the RH2-18 treatment group on day 24, thus 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 femurs 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 per week group (Fig. 2A). Likewise, 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 per week dose group. Consistent with the initial findings after 24 days, endosteal 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 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 BV/TV was 5.8% greater than that in the vehicle OVX control group. The increase in endosteal bone formation and the more modest effect on periosteal bone formation were paralleled by numerical increases in the cortical area as well as reductions in endosteal perimeter (Table 2).

More importantly, lumbar spine BMD (L1–4) at 8 weeks was partially to completely restored in RH2-18 treatment groups to the levels seen in sham OVX (intact) vehicle-treated controls (Fig. 2D). This profound effect on lumbar spine aBMD was characterized further 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 TbTh ($p < 0.001$ versus OVX vehicle; 13.6%) and TbN ($p = 0.068; 15\%$) while reducing TbSp ($p < 0.01; -25\%$). Thus, RH2-18 treatment produced pharmacological effects at multiple loci and improvement of bone microarchitectural parameters in the mature OVX mouse.

Fig. 1. Pharmacokinetics and pharmacodynamic responses of fully human anti-DKK1 antibody RH2-18 after single dose administration in OVX 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 subcutaneous 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 increases from baseline in RH2-18 treatment groups ($p = 0.02; \text{day 5}; 15 \text{ mg/kg}$), and numerical increased P1NP levels were observed up to 16 days. D, serum CTx levels were determined at days 2 and 16. Whole femur aBMD (E) and lumbar spine aBMD (F) (L1–4) increase in RH2-18 treatment groups at day 24. Data are given as mean ± S.E. ($n = 3–4$ per group per time point). One-way analysis of variance, Dunnett’s test versus vehicle ($*, p < 0.05; **, p < 0.01$).

<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>Whole Femur BMD (mg/cm²)</td>
<td></td>
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<tr>
<td>Dorsal Femur BMD (mg/cm²)</td>
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<tr>
<td>Central Femur BMD (mg/cm²)</td>
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<tr>
<td>BV/TV (%)</td>
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<td></td>
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<tr>
<td>MAR (μm/day)</td>
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<tr>
<td>MS/BS (%)</td>
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<tr>
<td>BFR/BS (μm³/μm² per year)</td>
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</table>

Data are given as mean ± S.E. One-way analysis of variance, Dunnett’s test versus vehicle ($*, p < 0.05; **, p < 0.01; *** p < 0.001$).
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)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>RH2-18 2 mg/kg</th>
<th>RH2-18 20 mg/kg</th>
<th>hPTH (1–34) 2 mg/kg</th>
<th>hPTH (1–34) 20 mg/kg</th>
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<tr>
<td>Central femur</td>
<td>BV/TV (%)</td>
<td>49.67 ± 0.597***</td>
<td>45.79 ± 0.532**</td>
<td>45.49 ± 0.725*</td>
<td>47.38 ± 0.435**</td>
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<td></td>
<td>CtAr (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>
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<tr>
<td></td>
<td>Endosteal EcPm (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>
</tr>
<tr>
<td></td>
<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>
</tr>
<tr>
<td></td>
<td>Periosteal BFR/BS (µm²/µm² per year)</td>
<td>79.99 ± 14.620</td>
<td>56.19 ± 13.200</td>
<td>79.10 ± 9.828</td>
<td>109.60 ± 17.145*</td>
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<tr>
<td></td>
<td>PsPm (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>
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<tr>
<td></td>
<td>MAR (µm/day)</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>
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<td></td>
<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>
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<td>BFR/BS (µm²/µm² per year)</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>
</tr>
</tbody>
</table>

CtAr, cortical area; EcPm, endocortical perimeter; PsPm, periosteal perimeter.

Data are given as means ± S.E. (n = 9–14 per group). One-way analysis of variance, Dunnett’s test versus vehicle (*, p < 0.05; **, p < 0.01; ###, p < 0.001).

Physical and Biological Properties of the Anti-DKK1 Antibody (RH2-18) Enable the Testing of DKK1’s Role in Nonhuman Primates (Rhesus Macaque). To address pharmacokinetic properties in nonhuman primates, we first administered single doses of RH2-18 (0.5 or 2.5 mg/kg) via subcutaneous or intravenous routes to rhesus macaque and then drew blood samples at the indicated time points for analysis (Fig. 3). A comparison of exposure after administration of 0.5 mg/kg s.c. and i.v. doses suggests essentially complete absorption of RH2-18, an apparent t½ of approximately 2 weeks, and a very low serum clearance of 2.7 µl/min·kg⁻¹ and a small volume of distribution 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 BMD in Aged and OVX Nonhuman Primates (Rhesus Macaque). We next introduced RH2-18 (10 mg/kg, twice per month) into long-term OVX rhesus macaques (14–20 years of age, >8 years after OVX). Serum RH2-18 levels at days 7 and 71 (the last time point tested) were 681 ± 160 and 690 ± 201 nM, respectively, indicating extremely stable exposure of the antibody over an extended time period and suggesting the absence of substantial interfering anti-human antibody response. DKK1 levels were increased by treatment from base-
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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact</th>
<th>O VX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>RH2-18</td>
</tr>
<tr>
<td>Lumbar vertebra</td>
<td>vBMD (mg/cm²)</td>
<td>248.20 ± 0.70.804***</td>
</tr>
<tr>
<td></td>
<td>BV/TV (%)</td>
<td>0.161 ± 0.008%*</td>
</tr>
<tr>
<td></td>
<td>TbTh (mm)</td>
<td>0.023 ± 0.0006***</td>
</tr>
<tr>
<td></td>
<td>TbN (no./mm)</td>
<td>7.044 ± 0.3874</td>
</tr>
<tr>
<td></td>
<td>TbSp (mm)</td>
<td>0.124 ± 0.0080</td>
</tr>
</tbody>
</table>

TBn, trabecular number; TbSp, trabecular spacing.

Data are given as mean ± S.E. One-way analysis of variance, Dunnett’s test versus OVX vehicle (*, p < 0.05; ***, p < 0.001).

Fig. 3. Serum levels and pharmacokinetic profile of fully human anti-DKK1 antibody RH2-18 after 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 are given as mean ± S.E. (n = 3 per group).

Bone mass affecting the 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 versus vehicle) at 9 months.

To further investigate fracture relevant sites, we extended the analyses to distal tibia and distal radius, examining vBMD and trabecular microarchitecture by HR-pQCT. Integral vBMD (combined cortical/trabecular, represented by D100 in the Scanco analysis software) of the distal tibia was found to be increased by 3.7% (p < 0.001) by treatment at 9 months (Fig. 5D), with detectable increases in 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 Fig. 3). Similar efficacies were observed in the distal radius of the treatment group at 6 and 9 months treatment, because integral vBMD showed increases of 3.6% (p < 0.005) and 5.4% (p < 0.005), respectively (Fig. 5E), and TbTh showed increases of 3.0% (p < 0.01) and 3.3% (p < 0.005), respectively (Fig. 5F).
Discussion

Bone-anabolic treatments targeting the Wnt-signaling pathway offer novel therapeutic approaches for systemic diseases such as postmenopausal or iatrogenic osteoporosis (Khosla et al., 2008; Deal, 2009; McCarthy and Marshall, 2010) and for localized bone loss secondary to metastatic bone disease (Yaccoby et al., 2007; Fulciniti et al., 2009; Gavriatopoulou 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. In this study, we provide evidence for the inhibitory function of DKK1 in systemic low-bone-mass conditions (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 the 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, thus assuring data interpretation that is tightly focused on DKK1 function (Krupnik et al., 1999; Glantschnig et al., 2010). 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; Ai et al., 2005; Fleury et al., 2010), thus providing a rationale for the mechanism of action of RH2-18 on bone and its efficacy via the modulation of DKK1 function (Glantschnig et al., 2010). In addi-
tion, DKK1 interaction with LRP4 (Choi et al., 2009a) and Kremen receptors (Mao et al., 2002) may be affected by the pharmacological action of anti-DKK1 antibody; however, this was not tested experimentally.

We demonstrate here the profound systemic effects of anti-DKK1 antibody in adult osteopenic mice at multiple skeletal sites, including endosteal surfaces composed 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 8 weeks of treatment. Wnt/β-catenin signaling has been reported previously to influence both bone formation and resorption (Baron and Rawadi, 2007; Pinzone et al., 2009). The anti-DKK1 antibody-induced osteoanabolic activity was demonstrated by increased circulating P1NP in treated mice and primates and confirmed by the observation of augmented bone formation at all of the endosteal surfaces examined in mouse. The approximate 2-fold increase in endosteal bone formation and detectable but nonsignificant effects on periosteal bone formation were paralleled by a progression toward 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 preosteoblast progenitors as well as in preosteoblast to osteoblast transitions (Pinzone et al., 2009). This interpretation is consistent with the genetic DKK1 loss-of-function phenotype in mice that includes both an increase in osteoblast number and elevated bone formation 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 been provided recently 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 elevated mechanistically. 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 noninvasive 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. (2007) have established that bone resorptive processes and the local apposition of bone (osteophytes) in joints involve particular regulatory cues controlled through DKK1, whereas neutralization of DKK1 also protects from systemic bone loss in inflammatory mouse models (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, because RH2-18 treatment ameliorated the reductions in lumbar spine aBMD in mouse and primates and improved parameters of bone microarchitecture in the mouse vertebra. Likewise, lumbar spine TbBMD of adult nonhuman primates improved significantly with the treatment. More importantly, this effect was not limited to the axial skeleton, because RH2-18 treatment notably improved integral vBMD as well as TbTh at the distal radius and tibia. Taken 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 microarchitectural 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 (Heiland et al., 2010).

Also consistent with a translational therapeutic effect is an early response demonstrated by the bone formation biomarker P1NP, which correlated moderately and positively with individual lumbar spine TbBMD responses to RH2-18 treatment in nonhuman primates. Thus, the combined data provide strong evidence for an inhibitory role of DKK1 in bone homeostasis in adult O VX 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 of the molecules modulating the Wnt-signaling pathway need to be investigated for their tumorigenicity potential and toxicity to other extraskelatal tissues, in particular in the treatment of chronic disorders 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 defined completely and may vary depending on the cellular context, and the potential risks through the modulation of DKK1 function must be considered and further evaluated, although a DKK1 antibody has recently entered clinical trials for the treatment of patients with multiple myeloma (Fulciniti et al., 2009).

In conclusion, we provide pharmacological evidence for the 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.

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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, Cook, Williams, 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, Kimmel, and An.

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

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