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Activating receptor activator of NF-κB (RANK) and TNF receptor (TNFR) promote osteoclast differentiation. A critical ligand contact site on the TNFR is partly conserved in RANK. Surface plasmon resonance studies showed that a peptide (WP9QY) that mimics this TNFR contact site and inhibits TNF-α–induced activity bound to RANK ligand (RANKL). Changing a single residue predicted to play an important role in the interaction reduced the binding significantly. WP9QY, but not the altered control peptide, inhibited the RANKL-induced activation of RANK-dependent signaling in RAW 264.7 cells but had no effect on M-CSF–induced activation of some of the same signaling events. WP9QY but not the control peptide also prevented RANKL-induced bone resorption and osteoclastogenesis, even when TNFRs were absent or blocked. In vivo, where both RANKL and TNF-α promote osteoclastogenesis, osteoclast activity, and bone loss, WP9QY prevented the increased osteoclastogenesis and bone loss induced in mice by ovariectomy or low dietary calcium, in the latter case in both wild-type and TNFR double-knockout mice. These results suggest that a peptide that mimics a TNFR ligand contact site blocks bone resorption by interfering with recruitment and activation of osteoclasts by both RANKL and TNF.

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A TNF receptor loop peptide mimic blocks RANK ligand–induced signaling, bone resorption, and bone loss

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Activating receptor activator of NF-κB (RANK) and TNF receptor (TNFR) promote osteoclast differentiation. A critical ligand contact site on the TNFR is partly conserved in RANK. Surface plasmon resonance studies showed that a peptide (WP9QY) that mimics this TNFR contact site and inhibits TNF-α–induced activity bound to RANK ligand (RANKL). Changing a single residue predicted to play an important role in the interaction reduced the binding significantly. WP9QY, but not the altered control peptide, inhibited the RANKL-induced activation of RANK-dependent signaling in RAW 264.7 cells but had no effect on M-CSF–induced activation of some of the same signaling events. WP9QY but not the control peptide also prevented RANKL-induced bone resorption and osteoclastogenesis, even when TNFRs were absent or blocked. In vivo, where both RANKL and TNF-α promote osteoclastogenesis, osteoclast activity, and bone loss, WP9QY prevented the increased osteoclastogenesis and bone loss induced in mice by ovariectomy or low dietary calcium, in the latter case in both wild-type and TNFR double-knockout mice. These results suggest that a peptide that mimics a TNFR ligand contact site blocks bone resorption by interfering with recruitment and activation of osteoclasts by both RANKL and TNF.

Nonstandard abbreviations used: BMD, bone mineral density; CRD, cysteine-rich domain; NFATC1, nuclear factor of activated T cells c1; OPGL, osteoprotegerin; O VX, ovariectomy; OVX; p55, peripheral quantitative computed tomography; RANK, receptor activator of NF-κB; RANKL, RANK ligand; sRANKL, soluble RANKL; TNFR, TNF receptor; TRAP, tartrate-resistant acid phosphatase; Tyr, tyrosine residue in position 6.

Conflict of interest: The research reported here was supported in part by ProStrakan Pharmaceuticals (previously ProSkelia Pharmaceuticals). P. Deprez, R. Blanque, P. Mollat, and R. Baron are currently or were previously employed by ProStrakan Pharmaceuticals and, as such, also own stock options in that company. M. Ishiguro received funding from Suntory Institute for Bioorganic Research.

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Introduction
The TNF receptor (TNFR) superfamily member receptor activator of NF-κB (RANK) (1) is expressed on osteoclasts and their precursors, hematopoietic precursors, dendritic cells, and mammary epithelial precursors. RANK ligand (RANKL [ref. 2], also known as OPGL, ODF, and TRANCE [refs. 3–5]) is a TNF-like protein that is expressed by osteoblasts, bone marrow stromal cells, and T cells. RANKL is synthesized as an integral membrane protein and is active both in its membrane-bound form and when released from its membrane anchor by specific proteases. Both RANK and RANKL are absolutely required for osteoclast differentiation in vitro and in vivo (refs. 4, 5; reviewed in refs. 2, 6, 7). Another TNF family member, TNF-α, enhances the osteoclastogenic response to low levels of RANKL (8) and contributes significantly to bone loss induced by ovariectomy (OVX) (9, 10) or by inflammation (11–13). RANKL also increases bone resorption by mature osteoclasts (14). The decoy receptor osteoprotegerin (OPG), a soluble homolog of RANK, prevents RANK activation, osteoclastogenesis, and bone resorption by binding to RANKL and preventing the RANKL-RANK interaction (15, 16). The relative levels of RANKL and OPG largely determine the number of active osteoclasts in bone and consequently, the amount of bone resorption that occurs (4, 15, 17).

Like other TNF superfamily members (18, 19), the active form of RANKL is a homotrimer (20). Conformations of several superfamily members have been determined, in some cases in complexes with their receptors (20–24). The core folding of family members is highly conserved, but the residues on the external faces of the subunits and the conformations of the loops that connect the β-strands show little conservation between family members (18, 19), consistent with the role of these regions in determining binding specificity. There is also little sequence homology among TNF superfamily ligand-binding domains other than disulfide-bonded cysteines and a few other residues that are critical for the folding of characteristic repeated cysteine-rich domains (CRDs) (25). Mutagenesis, molecular modeling studies, and crystal structures of p55 type I TNFR/TNFR-β [TNFR(β)/TNFR-β] (22) and TRAIL/death receptor 5 (23, 24) complexes indicate that TNFR members have 2 ligand contact surfaces at conserved locations (CRD2 and CRD3 in the TNFR). While the locations and general nature of the 2 contact surfaces are simi-
The activation of RANK by RANKL. Molecular modeling supported mimic of the TNFR(I) loop might bind to RANKL and inhibit the CRD3 loop in TNFR and death receptor 5 is conserved in RANK. Our results indicate that the ligand contact point function of the CRD3 loop in TNFR and death receptor 5 is conserved in RANK and suggest that interfering with the RANK CRD3 loop interaction with RANK in vivo can prevent normal RANKL-induced signaling and modulation of osteoclast differentiation and function. Indeed, interfering with both TNF- and RANKL-induced signaling would provide a potent therapeutic approach to hyperresorptive diseases, including rheumatoid arthritis and periodontal disease.

Results

Molecular modeling predicts that the WP9QY peptide binds to RANK and alters the conformation of RANKL-occupied RANK. The folding of members of the TNFR and TNF superfamilies and the locations of the receptor-ligand contact sites are highly conserved (27), with the specificity of the receptor-ligand interactions determined by specific differences in the sequences and conformations of the receptor and ligand loops that mediate the receptor-ligand interactions (20, 23, 24). The presence in RANK of key features of the TNFR(I) CRD3 contact point, particularly Trp107 and Ser108 (Figure 1A), suggested that the small cyclic WP9QY peptide modeled on the TNFR(I) CRD3 loop, which binds TNF-α and inhibits TNF-α-induced cytotoxicity (26), might also bind RANKL and alter RANKL-induced cell responses. The highly conserved folding of members of the TNFR and TNF superfamilies allows the structural modeling of receptor-ligand complexes that have not yet been experimentally characterized (28, 29). We therefore used computer modeling to initially examine the possibility that the WP9QY peptide could bind to the RANKL trimer and thereby affect the RANKL-RANK complex. A model of the RANKL-WP9QY complex created by homology modeling from the crystal structure of the TNFR/TNF-β complex (Figure 1B) indicated that the peptide would dock snugly into the cleft between RANKL subunits and that tyrosine residues in positions 6 and 9 of the peptide could play important roles in the binding of the peptide to RANK.

The model of the RANKL-RANK complex in the absence of the peptide showed RANK binding along the entire length of the lateral face of the RANKL trimer, with the C-terminal (membrane-proximal) portions of the 3 RANK molecules converging below the more narrow end of the RANKL trimer (Figure 1C, left), consistent with the known RANKL-induced clustering of the RANK...
cytoplasmic domains. Superimposing the RANKL moieties of the RANKL-WP9QY peptide complex and the RANKL-RANK complex (Figure 1C, right) suggested that the presence of the peptide in the cleft between the RANKL monomers would interfere with the binding of the C-terminal portion of the RANK extracellular domain involving the CRD3 loop region, but not with the second receptor-ligand contact site at the more distal point of the RANK extracellular domain. In the model, the presence of the peptide displaced the C-terminal portion of the extracellular domain, increasing the distance between the C termini of the ligand-bound RANK extracellular domains. (Compare the left and right images in Figure 1C.)

WP9QY binds to sRANKL but does not inhibit RANKL-RANK binding. We measured the binding of WP9QY to RANKL and its effect on the RANKL-RANK interaction using surface plasmon resonance. sRANKL bound to WP9QY immobilized on the biosensor surface ($K_d = 0.437 \pm 0.013 \mu M$). Changing the tyrosine residue in position 6 (Tyr$^6$) to asparagine (Y6N) reduced the binding to RANKL by more than 70% at concentrations up to 50 $\mu M$ (Figure 2A), consistent with the model’s prediction that this tyrosine is important for the binding. However, despite its ability to bind to RANKL, WP9QY failed to displace RANK from RANKL (Figure 2B), consistent with the model’s indication that the peptide would block the CRD3 contact site but have no effect on the CRD2 contact site. In contrast to the insensitivity of RANKL-RANK binding to WP9QY, the RANKL-RANK complex was completely disrupted by the decoy receptor OPG (Figure 2C).

WP9QY blocks RANKL-induced signaling. Since WP9QY bound to RANKL, we asked whether the peptide affects RANKL-induced signaling, notwithstanding the fact that it failed to displace RANK from RANKL. In the osteoclast precursor RAW 264.7 cell line, WP9QY acted in a dose-dependent manner to inhibit the RANKL-induced activation of NF-kB (Figure 3).
The effect of WP9QY was not due to a general reduction of cell signaling, since it had no effect on M-CSF–induced Erk or Akt phosphorylation, and JNK and Akt kinase activities (Figure 3, D and E). Furthermore, the Y6N control peptide had little or no inhibitory effect (Figure 3E). Thus, despite its failure to prevent RANKL-RANK binding, the WP9QY peptide effectively and specifically inhibited RANKL-induced signaling.

WP9QY inhibits soluble RANKL-induced osteoclastogenesis and bone resorption in vitro. We next examined the effect of the peptide on RANKL-induced osteoclastogenesis in vitro. Murine bone marrow cells were cultured for 5 days with 25 ng/ml M-CSF and 10, 30, or 100 ng/ml sRANKL alone or with increasing concentrations of the peptide, and tartrate-resistant acid phosphatase–positive (TRAP-positive) multinucleated cells were counted (Figure 4, A and B). WP9QY inhibited osteoclastogenesis with a clear dose response at each sRANKL concentration tested. The concentration of WP9QY needed to achieve 50% inhibition increased from 10 μM in the presence of 10 ng/ml sRANKL to more than 25 μM in the presence of 100 ng/ml sRANKL, strongly suggesting that WP9QY inhibited osteoclastogenesis by interfering with RANKL-induced signaling. In contrast to the effect of WP9QY, which reduced the number of TRAP-positive multinucleated cells by more than 90% at concentrations equal to or greater than 25 μM, the Y6N control peptide had little effect at concentrations as high as 50 μM (Figure 4C). As a positive control, 30 ng/ml OPG reduced the number of TRAP-positive multinucleated cells to about 25% of the numbers in untreated cultures.

sRANKL also stimulates bone resorption by mature osteoclasts (14). We therefore examined the effect of WP9QY and the control peptide on sRANKL-induced bone resorption in vitro (Figure 5). Mature osteoclasts from 3-day-old CD-1 mice were plated on dentin slices and cultured for 24 hours with no addition, with sRANKL (30 ng/ml) alone, or with sRANKL and WP9QY (50 μM), the control peptide (50 μM), or OPG (30 ng/ml). Consistent with previous reports, sRANKL potently stimulated bone resorption; the resorbed area (Figure 5B), resorbed volume (data not shown), and average depth (Figure 5C) of pits were increased by 24-fold, 50-fold, and 2.4-fold, respectively. WP9QY inhibited the increased bone resorption by more than 90%, while the control peptide inhibited the RANKL-induced increase in bone resorption by only 20–30%. In this assay, mature osteoclasts were plated, and the number of osteoclasts was not altered by RANKL alone or in combination with any other agent (data not shown).

WP9QY inhibits sRANKL-induced in vitro osteoclastogenesis in the absence of TNFRs. TNF synergizes with low levels of RANKL to induce osteoclast differentiation (8) and can itself induce low levels of osteoclastogenesis in M-CSF–treated bone marrow cultures (30–32). Thus, WP9QY, which inhibits TNFR(I) signaling (26), might affect osteoclastogenesis by inhibiting the effect of endogenous TNF rather than by preventing RANKL-induced RANK signaling. We therefore examined the effect of WP9QY on RANKL-induced osteoclastogenesis from precursors that lacked either the TNFR(I) or the p75 type II TNFR [TNFR(II)]. In the absence of WP9QY, RANKL-induced osteoclastogenesis was reduced by more than half when the osteoclast precursors lacked p55 but not when they lacked p75 (Figure 6A), suggesting that endogenous TNF enhances RANKL-induced osteoclastogenesis in the bone marrow culture system used here. Notwithstanding the reduced osteoclastogenesis seen in the p55-/- cultures, WP9QY
(5 μM) inhibited more than 80% of the sRANKL-induced formation of TRAP⁺ cells in both the p55⁻/⁻ and p75⁻/⁻ cultures (Figure 6A), even when antibodies that blocked p75 activation were present in the cultures of p55⁻/⁻ marrow cells or vice versa (Figure 6, B and C). Thus, WP9QY inhibited sRANKL-induced osteoclastogenesis even under the conditions where signaling by endogenous TNF is prevented, providing further evidence that the peptide inhibits osteoclastogenesis by blocking RANKL-induced RANK activation, in addition to its known effects on TNF-induced activity.

WP9QY prevents bone loss in vivo, even in the absence of TNF receptors. Given WP9QY’s ability to inhibit RANKL-induced osteoclast differentiation and bone resorption in vitro, as well as its previously reported ability to interfere with TNF-induced responses, we asked whether the peptide might prevent bone loss in vivo, examining WP9QY’s effect on OVX-induced bone loss, which requires both RANKL- and TNF-induced signaling (9, 15), and on bone loss caused by low dietary calcium, which may be less dependent on TNF. Histomorphometry and peripheral quantitative computed tomography (pQCT) analysis demonstrated that the low-calcium diet induced a rapid increase in bone resorption as a consequence of increased osteoclast numbers and activity in mice in a manner similar to what had been previously shown in rats (33) (data not shown). WP9QY had no effect on the body weight or the uterine weight in the OVX mice (data not shown). Treatment with WP9QY completely prevented the bone loss induced by either ovariec tomy (Figure 7, A and B, and Table 1) or low dietary calcium (Figure 7, C and D, and Table 2). In both models, WP9QY blocked the experimentally induced increase in deoxypyridinoline cross-links, a marker of bone resorption (data not shown). WP9QY inhibited the decrease in the cancellous bone volume and the increases in bone resorption parameters such as osteoclast surface and osteo-

![Figure 5](image_url)

**Figure 5**

WP9QY inhibits bone resorption by mature osteoclasts in vitro. Authentic osteoclasts were isolated from the long bones of 3-day-old CD-1 mice, plated on dentin slices, and cultured for 48 hours in the presence of the indicated combinations of sRANKL (30 ng/ml), WP9QY (50 μM), control peptide (50 μM), and OPG (30 ng/ml). (A) Typical image of pits formed in the absence (left panel) and presence (right panel) of WP9QY. The resorbed area (B) and resorbed volume (data not shown) were determined, normalized to the number of osteoclasts (OCs) on the dentin slices, and used to calculate the mean pit depth (C). WP9QY blocked the sRANKL-induced increase in bone resorption, while the control peptide did not. The values are the mean ± SD of 6 dentin slices and are representative of 2 independent experiments. *P < 0.001 versus untreated; **P < 0.001 versus sRANKL alone.

![Figure 6](image_url)

**Figure 6**

WP9QY inhibits osteoclastogenesis in bone marrow cultures when TNFRs are missing or blocked. (A) Bone marrow cells from mice that lacked either TNFR(I) (p55⁻/⁻) or TNFR(II) (p75⁻/⁻) or their wild-type littermates were cultured with M-CSF (30 ng/ml) and sRANKL (30 ng/ml), or without WP9QY (5 μM). In all cases, WP9QY reduced the number of TRAP⁺ multinucleated cells (MNCs) by more than 80% (*P < 0.001 relative to matched untreated culture). The results are representative of 2 independent experiments. (B) Bone marrow cells from mice that lacked p55 were cultured with an antibody that blocked p75 and increasing concentrations of WP9QY as indicated. The antibody had little additional effect on the number of TRAP⁺ MNCs produced. All concentrations of WP9QY efficiently inhibited the sRANKL-induced osteoclastogenesis (*P < 0.001 relative to culture with anti-p75 antibody). The results are representative of 2 independent experiments. (C) Bone marrow cells from mice that lacked p75 were cultured with an antibody that blocked p55 and increasing concentrations of WP9QY as indicated. In contrast to the lack of effect of anti-p75 in the p55⁻/⁻ marrow cultures, anti-p55 significantly reduced the TRAP⁺ MNC formation from p75⁻/⁻ bone marrow cells. WP9QY further inhibited TRAP⁺ MNC formation in a dose-dependent manner (*P < 0.001 relative to the cultures in the absence of anti-p55; **P < 0.02 relative to culture in the presence of anti-p55; ***P < 0.001 relative to culture in the presence of anti-p75). The results are representative of 2 independent experiments.
the absence of both TNFRs provides additional evidence that the peptide protects mice against bone loss in vivo, at least in part, by inhibiting a TNF-independent mechanism, most likely RANKL-induced osteoclastogenesis and bone resorption.

Discussion

The absolute requirement for RANKL-induced RANK signaling in osteoclastogenesis is well established and based on a variety of in vitro and in vivo studies, including the deletion of the genes for RANK (34, 35) and RANKL (17) and the overexpression of sRANKL-binding proteins (15, 36) in transgenic mice. Consistent with the key role of RANKL-RANK-dependent signaling in inducing osteoclast formation and promoting osteoclast bone-resorbing activity, antagonizing RANKL-induced stimulation of RANK is a potent therapeutic approach to treating conditions that are characterized by excessive bone resorption, such as osteoporosis (37). We report here that a cyclic peptide designed to mimic the CRD3 ligand contact surface of the TNFR that binds to TNF (26) also binds to RANKL and that it inhibits RANKL-induced signaling, RANKL-induced in vitro osteoclastogenesis, and RANKL-stimulated bone resorption by isolated osteoclasts in vitro. The ability to inhibit RANKL-induced cellular responses is relatively specific. The WP9QY peptide does not generally weaken the responsiveness of the cells, since it did not affect signaling induced by M-CSF. In addition, the inhibitory potency of the peptide was markedly reduced by changing a single tyrosine residue, predicted by molecular modeling to be important for binding, to asparagine. Most importantly, WP9QY is fully protective in 3 different strains of mice (CD-1, C57BL/6J, and 129/C57BL/6J) against bone loss induced using 2 different experimental protocols (low dietary calcium and ovariectomy).

Since TNF is known to enhance the response of osteoclast precursors to low concentrations of RANKL (8) and is involved in the bone loss induced by ovariectomy (9), this efficiency is probably linked to its ability to inhibit both RANKL- and TNF-induced responses. Although the ability of WP9QY to inhibit TNF-induced responses (26) complicates the demonstration that WP9QY directly antagonizes RANKL-induced osteoclastogenesis and bone resorption, our results demonstrate that the inhibitory effect of the peptide is at least in part a consequence of the peptide’s direct inhibition...
of RANKL-induced responses, since WP9QY was also effective in TNFR−/− cells and mice (Figures 6 and 8). In addition, the peptide inhibited RANKL-induced signaling in RAW 264.7 cell cultures (Figure 3). The peptide also inhibited osteoclastogenesis induced by higher concentrations of sRANKL (Figure 4B) that are insensitive to the presence of TNF (8). The reduced osteoclastogenesis in untreated cultures of marrow cells from p55−/− animals (Figure 6, A and B) is consistent with a TNF-dependent component in the osteoclastogenesis in these cultures and suggests that inhibition of both RANKL- and TNF-induced responses contributes to the peptide’s ability to inhibit osteoclastogenesis in cultures of wild-type cells.

Demonstrating that WP9QY inhibits RANKL-induced osteoclastogenesis and bone resorption in vivo is similarly difficult. Ovariectomy-induced bone loss is largely or completely dependent on both RANKL (15) and TNF (9), and it is therefore not possible to ascribe the bone-sparing effect of WP9QY in the ovariectomized mice solely to the inhibition of RANKL-induced signaling or of TNF-induced signaling, although the possibility remains that the prevention of osteoclast-mediated bone loss by WP9QY involves the inhibition of both RANKL- and TNF-induced responses. In contrast to ovariectomy-induced bone resorption, however, the bone loss induced by low dietary calcium is not completely dependent on TNF, since low dietary calcium induced a loss of bone in mice that lacked both TNFR(I) and TNFR(II) to a bone mineral density (BMD) similar to that seen in wild-type mice (Figure 8). WP9QY completely blocked the bone loss in the TNFR(II)−/− mice, just as it did in the wild-type mice, demonstrating that the peptide does act independently of TNF-TNF in vivo, as it does in vitro. The fact that the peptide is able to inhibit both TNF- and RANKL-induced signaling may increase its efficacy relative to agents that inhibit only one or the other mechanism.

Our binding studies indicate that WP9QY inhibits RANKL-induced signaling but not the binding of RANKL to RANK, in contrast to the ability of a peptide modeled on an OPG contact site for RANK to inhibit RANKL-RANK binding (38). The failure of WP9QY to displace RANK from RANKL raises the question of how the peptide affects the interaction of RANK and RANK at the molecular level and the RANKL-induced activation of RANK.

As noted above, members of the TNFR superfamily bind to their ligands via 2 distinct contact surfaces that are conserved in terms of the relative locations on the proteins but are highly specific in terms of sequence and conformation (20, 22–24, 39). By analogy with TNF-α and TNF-β (22, 40–42), the contact between the ligand surface and the receptor CRD2 appears to be the more important for binding and might be sufficient by itself to mediate the binding of the 2 proteins. Consistent with this possibility, mutations in the predicted CRD2-binding surface of RANKL markedly reduce or abolish RANKL’s osteoclastogenic activity (20). WP9QY’s failure to displace RANK from RANKL was consistent with our molecular modeling, which predicted that the binding of WP9QY to RANKL would have little or no effect on the overall RANK CRD2 interaction with RANKL. However, although by no means conclusive, the modeling predicted that the presence of the peptide in the binding site for CRD3 would displace the membrane-proximal portion of the RANK extracellular domain outward from the central axis of the RANKL trimer, possibly affecting the receptor’s signaling activity by interfering with the proposed ligand-induced clustering of the receptor cytoplasmic domains (27, 43, 44). However, this possibility remains hypothetical until it can be experimentally confirmed.

In conclusion, we have found that a cyclic peptide with sequence homology to a predicted ligand contact surface on RANK inhibits RANKL-induced signaling, osteoclastogenesis, and bone resorption in vitro, suggesting that the first loop of RANK’s third CRD is a critical ligand contact point, like the homologous loop in the TNFR. Furthermore, and notwithstanding the poor pharmacokinetics associated with peptide reagents, it acts in vivo to protect mice against experimentally induced bone loss, suggesting that development of agents that prevent the interaction of RANK CRD3 with RANKL may prove to be useful for the treatment of diseases where bone resorption is increased, such as osteoporosis, rheumatoid arthritis, and osteolytic bone metastases.

Methods

Materials. The WP9QY (YCWSQYLCY) and control (YCWSQNLCCY) peptides were synthesized, and the activity of WP9QY against the TNF-α-TNFFR interaction was assayed as described previously (26). Recombinant human M-CSF was from Calbiochem or R&D Systems. Recombinant soluble human RANKL was from ProStrakan and Wako Pure Chemical Industries Inc. Recombinant human OPG was from PeproTech. TNFR(II)-deficient mice (C57BL/6), TNFR(II)-deficient mice (C57BL/6), TNFR(II)/TNFR(II)-deficient mice (129/16), and genetically matched wild-type controls were produced by breeding mice obtained from Jackson Laboratory. Antibodies

### Table 1
Histomorphometric analysis of the protective effect of WP9QY on bone loss induced by ovariectomy (lumbar vertebra)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Ca/vehicle</th>
<th>Low Ca/vehicle</th>
<th>Low Ca/WP9QY (9 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>9.74 ± 1.05</td>
<td>4.44 ± 1.55a</td>
<td>10.28 ± 1.28b</td>
</tr>
<tr>
<td>TbTh (μm)</td>
<td>33.21 ± 3.34</td>
<td>28.19 ± 3.60b</td>
<td>31.57 ± 2.85</td>
</tr>
<tr>
<td>TbN (no./mm)</td>
<td>2.95 ± 0.39</td>
<td>1.58 ± 0.52b</td>
<td>3.26 ± 0.29</td>
</tr>
<tr>
<td>TbSp (μm)</td>
<td>310.2 ± 44.6</td>
<td>653.4 ± 193.1A</td>
<td>277.5 ± 25.70</td>
</tr>
<tr>
<td>Noc/BS (no./mm)</td>
<td>2.52 ± 0.48</td>
<td>4.06 ± 1.40c</td>
<td>1.98 ± 0.98b</td>
</tr>
<tr>
<td>OcS/BS (%)</td>
<td>8.53 ± 1.42</td>
<td>14.07 ± 4.18b</td>
<td>9.07 ± 5.57b</td>
</tr>
<tr>
<td>ObS/BS (%)</td>
<td>22.21 ± 5.41</td>
<td>31.47 ± 6.15c</td>
<td>24.15 ± 6.58</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.005 versus normal-calcium group. <sup>b</sup>P < 0.005 versus low-calcium group. <sup>c</sup>P < 0.05 versus normal-calcium group. <sup>d</sup>P < 0.0005 versus low-calcium group. <sup>e</sup>P < 0.05 versus low-calcium group.
and fusion proteins were from Cell Signaling Technology, except for the anti-Erk2 antibody, the anti–glycogen synthase kinase 3 antibody and the anti-p65 monoclonal antibody (all from Santa Cruz Biotechnology Inc.).

Osteoclast-like cell generation. Murine bone marrow cells were cultured in 48-well plates at a cell density of $1.5 \times 10^5$ cells/cm$^2$ in Minimal Essential Medium Eagle, Alpha Modification (Sigma-Aldrich), containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml M-CSF, and the desired concentration of RANKL for 4 days, with a change of culture medium on day 3. Cells were stained for TRAP and counted as described previously (45).

Modeling the RANKL-WP9QY and RANKL-WP9QY-RANK complexes. The RANKL–RANK complex model was built by homology modeling with Modeller module installed in Insight II (Accelrys Software Inc.), using the crystal structure of the TNF–TNF-β complex (Protein Data Bank code: 1TNR) as a template. The complex structure was energy minimized by use of the molecular mechanics program Discover 3 (Accelrys Software Inc.), until the root mean square deviation became 0.1 kcal/mol/Å$^2$. The WP9QY peptide structure was generated by separating the peptide fragment that corresponds to the WP9QY peptide from the model structure of RANK. After replacing the residues for the WP9QY peptide and forming the disulfide bond between cysteine residues in positions 2 and 8 of the peptide, the peptide structure in the complex form was energy minimized with Discover 3. Then the peptide and residues of RANK within 12 Å of the peptide were structure optimized with the molecular dynamics calculation. The optimized complex structure was selected from 100 energy-minimized structures sampled by the molecular dynamics calculations at 298 K for 100 ps.

The RANKL structure of the RANKL-RANK complex model was superimposed with the RANKL structure of the RANKL-WP9QY complex model. Examination of the pivoting points for the motion of the C-terminal moiety of RANK suggested that the rotation of the backbone at Ala114 best avoids the steric interference between the peptide and the C-terminal domain of RANK, so the C-terminal domain starting from Ala114 was moved outward by rotating the φ bond of the backbone at Ala114, and the final model of the RANKL-WP9QY-RANK complex was obtained from the initial complex structure by energy minimization with Discover 3.

Analysis of peptide-RANKL and RANKL-RANK binding by surface plasmon resonance. WP9QY binding to sRANKL was analyzed using an IAsys Manual System biosensor (Thermo Electron Corp.). WP9QY or the control peptide was coupled to the cuvette (approximately 0.33 ng/mm$^2$), and the binding of sRANKL to the immobilized peptides was performed according to the manufacturer’s protocol. The binding of RANKL to immobilized RANK was analyzed using a Biacore 3000.

RANK signaling assays. RAW 264.7 cells were maintained in DMEM (46). After overnight serum starvation, cells were treated with 2.0 μg/ml sRANKL together with various concentrations of WP9QY, the Y6N control peptide (50 μM), or OPG (0.5 μg/ml), as indicated, for 20 minutes. Total cell lysates were prepared and analyzed by Western blotting as described previously (47).

To analyze NF-κB nuclear translocation, RAW 264.7 cells were treated for 20 minutes with 2.0 μg/ml sRANKL, with or without 50 μM WP9QY, and were then fixed, permeabilized, and incubated with anti-p65 antibody followed by fluorescein-conjugated secondary antibody and examined using a confocal imaging system (LSM 510; Zeiss) as described previously (47).

To induce the activation of NFATc1 and c-Fos expression, bone marrow cells from 8-week-old C57BL/6J mice were cultured overnight in minimal essential medium, alpha modification, containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Nonadherent cells were recovered, replated in 6-well plates at a density of $2.5 \times 10^5$ cells/well, and cultured for 2 days in medium containing 10 ng/ml M-CSF. Cells were washed with medium and cultured for an additional 48 hours with 10 ng/ml M-CSF, 0.1 μg/ml RANKL, and the indicated concentrations of WP9QY or OPG. Cells were washed once in cold PBS, lysed with modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA) supplemented with 1 mM NaF, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 1 mM phenylmethanesulfonyl fluoride; and analyzed for NFATc1 and c-Fos protein by Western blotting.

Erk phosphorylation was assayed by Western blotting total cell lysates with anti–phospho-Erk antibody. JNK kinase activity and Akt kinase activity were assayed using SAPK/JNK Assay Kit and Akt Kinase Assay Kit (Cell Signaling Technology), respectively, according to the manufacturer’s instructions, except that the concentration of ATP was 200 μM.

EMSA was performed using nuclear extracts from treated RAW 264.7 cells and a [32P]-labeled double-stranded oligonucleotide containing an NF-κB binding site (5′-GAGTTGAGGGGACTTTCCAGGC-3′) as described previously (48).
Pit formation assay. Authentic murine osteoclasts were isolated and the in vitro bone resorption assay (pit assay) was performed as described previ- ously (45, 49). Briefly, slices of dentine (350 μm) were prepared using a low-speed diamond saw (EXACT) under water irrigation. One surface of these slices was lightly polished, washed in 70% ethanol, and sterilized by ultraviolet irradiation. Isolated osteoclasts from 3-day-old CD-1 mice were seeded on the polished surface of dentine slices placed in 48-well dishes. After 48 hours of culture, the dentine slices were fixed with 0.25% glutaral- dehyde solution and stained for TRAP. The area and volume of resorption pits were measured with a Laser Color 3D profile confocal microscope (VK-8510K; Keyence Corp.) using WinRoof image analyzing software (Mitani Corp.). Horizontal and vertical scanning was performed, and the resultant images were combined to reproduce the surface characteristics 3-dimen- sionally. The images were displayed at a resolution of 1,024 × 768 pixels.

Determination of the effect of WP9QY on in vivo bone resorption. The effect of WP9QY on ovariectomized-induced bone resorption was examined as described previously (50), with some modifications. Four groups (n = 5) of 12-week-old female C57BL/6J mice were housed singly in metabolic cages (Metablica; SugiyamaGen Corp.) to allow the collection of urine samples. After weighing, 3 groups underwent bilateral OVX, and the fourth group (vehicle, low-calcium diet plus vehicle, low-calcium diet plus WP9QY at 0.7 mg/kg/d; and O VX/WP9QY at 0.7 mg/kg/d) was weighed weekly, and 3 groups were sacrificed after the last injection), and lumbar vertebrae and tibiae were dissected free of soft tissue and fixed in cacodylate-buffered glutaraldehyde/formalin (2.5%/4%) fixative (pH 7.4) for 3 days, and washed with PBS. The BMD of tibiae and vertebrae was measured by dual energy X-ray absorptiometry (DEXA) (DCS-600R; Aloca) or pQCT (XCT Research Sa+; Stratec Medizintechnik GmbH) as noted. Specimens were embedded using standard procedures in methyl- methacrylate resin. Standard histomorphometric measurements (53) were performed on 5-μm toluidine blue-stained sections using the KS400 Image analyzing system (Zeiss) as described previously (45).

Statistical analysis. Data are presented as mean ± SD. Multiple intergroup compar- isons were performed by 1-way ANOVA. When a significant F ratio was identi- fied, groups were compared using Fisher’s protected least-significant differ- ence post-hoc test. The difference was considered significant when P < 0.05.

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