Here, we report a rationally designed small molecule mimic of TRANCE-RANK causes osteopenic disorders such as osteoporosis and contributes to osteolytic metastases. Sustained osteoclast activation that occurs through TRANCE-RANK receptors of development and activation of osteoclasts in bone remodeling. The tumor necrosis factor family member, also known as osteoprotegerin (OPG), is known to be regulators of development and activation of osteoclasts in bone remodeling. Sustained osteoclast activation that occurs through TRANCE-RANK causes osteopenic disorders such as osteoporosis and contributes to osteolytic metastases.

The tumor necrosis factor family ligand, tumor necrosis factor-related activation-induced cytokine (TRANCE), and its receptors, receptor activator of nuclear factor-κB (RANK) and osteoprotegerin (OPG), are known to be regulators of development and activation of osteoclasts in bone remodeling. Sustained osteoclast activation that occurs through TRANCE-RANK causes osteopenic disorders such as osteoporosis and contributes to osteolytic metastases. Here, we report a rationally designed small molecule mimic of osteoprotegerin to inhibit osteoclast formation in vitro and limit bone loss in an animal model of osteoporosis. One of the mimetics, OP3-4, significantly inhibited osteoclast formation in vitro (IC_{50} = 10 μM) and effectively inhibited total bone loss in ovariectomized mice at a dosage of 2 mg/kg/day. Unlike soluble OPG receptors, which preclude TRANCE binding to RANK, OP3-4 shows the ability to modulate RANK-TRANCE signaling pathways and alters the biological functions of the RANK-TRANCE receptor complex by facilitating a defective receptor complex. These features suggest that OPG-derived small molecules can be used as a probe to understand complex biological functions of RANK-TRANCE-OPG receptors and also can be used as a platform to develop more useful therapeutic agents for inflammation and bone disease.

TRANCE, a TNF family member, also known as OPG, RANKL, ODF, and OCIF, plays a key role in the development of osteoclasts and in modulating their bone resorbing activity (1–4). Increased osteoclast activity has been reported in many osteoporotic disorders, including postmenopausal osteoporosis, Paget's disease, bone metastases, and rheumatoid arthritis (5–7). TRANCE interacts with two receptors: a secreted decoy receptor osteoprotegerin, OPG (8), and a transmembrane receptor, RANK (9–11). The interaction between TRANCE and RANK is essential for osteoclastogenesis because RANK is activated by TRANCE and then associates with TNF receptor-associated family members to trigger downstream signaling (11) events. OPG, on the other hand, plays an opposite role by preventing TRANCE from binding and activating RANK and thus is considered a “decoy” receptor. In this way, the interaction between TRANCE and OPG or TRANCE and RANK represents a complex network required for normal bone development, and any imbalance in the system potentially leads to bone disorders. Thus it is well recognized that this receptor complex network is an interesting drug development target for the treatment of bone disorders such as osteoporosis (12), and there is considerable interest in developing ways to modulate RANK functions which may prove beneficial for bone-related pathologies.

To date there is no three-dimensional structural information available for the RANK receptor complex. However, it is believed that the complex should resemble that of the TNF receptor as a member of TNF superfamily. The TNF-β-TNFFR1 co-crystal structure has been solved, thus facilitating rational drug design based on the receptor-ligand interaction sites coupled with rational ligand-receptor mutation studies (13). The crystal structure of the TNF receptor both in complex and uncomplexed forms provides a general structural framework for understanding the atomic surfaces these receptors use to bind to their ligands (14–16).

We have proposed that the modular organization of structures of biological proteins and the identification of localized ligand-receptor interaction sites permit the rational design of smaller, peptidomimetics that can interfere with these ligand-receptor interactions (17). The mimetics on their own may be therapeutically active but also can be used to guide authentic pharmaceutical designs. We have shown that a small peptide sequences derived from the TNFR1 surface which interacts with the ligand has reasonable activity in blocking the biological actions of the TNF receptor (18, 19).

In this study, we have identified several critical binding sites on TRANCE and OPG based on a three-dimensional structural model of the OPG-TRANCE receptor complex. The models were developed on the basis of co-crystal structures of TNF-β-TNFFR1 (13) and the deduced crystal structure of TRANCE (20, 21). The putative critical contacts were then used for the design of exocyclic peptidomimetics. In general the small molecules designed from OPG appear to inhibit TRANCE binding to RANK more effectively than mimetics designed from TRANCE.
EXPERIMENTAL PROCEDURES

Materials and Cell Cultures—A soluble form of recombinant TRANCE has been described previously (22). Recombinant human M-CSF, RANK, and OPG were obtained from PeproTECH (Rocky Hill, NJ). All other chemicals were from Sigma.

Cell Lines and Culture Systems—RAW264.7 cells were from American Type Culture Collection (Manassas, VA) and were cultured in RPMI (Invitrogen) containing 10% fetal bovine serum. Murine osteoclast precursors were from 6- to 8-week-old C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME). Murine bone marrow cells were cultured in α-minimal essential medium (Invitrogen) containing 10% fetal bovine serum and 5 ng/ml M-CSF for 12 h in 100-mm-diameter dishes at a density of 1 × 10⁶/dish. The nonadherent cells were then harvested and cultured with 30 ng/ml M-CSF in 100-mm dishes at the same density as before for an additional 48 h. Floating cells were removed, and attached cells were used as osteoclast precursors for generating osteoclasts (23).

Design of TRANCE and OPG Mimetics—Computer modeling was built using Modeleder 4.0 (24), and further refinement and analysis were performed using INSIGHT II (Molecular Simulation, Inc., San Diego). The OPG-TRANCE structure complex was initially modeled by molecular replacement using models constructed from crystallographic coordinates, Protein Data Bank (PDB) (25). The structure model of OPG was built using substructures of TNFR (1TNR), Fas (1BZI), and TRAIL (1DV and 1DG) receptors. Models were checked for consistency, and the ambiguous loop regions were reexamined and built using CONGEN (26). In the final consensus model the side chains involved in unfavorable interactions were adjusted manually as a side chain conformation search was carried out. The model was then optimized using energy minimization and molecular simulation calculations. The accuracy of the model was checked using Ramachandran plot (27) and by profile analyses (28). The binding sites involved in direct interaction between TRANCE and OPG were analyzed and used as the initial template for peptide design, as shown in Table 1. The model peptides designed were constructed from their sequences and folded using CHARMM. The folded peptides were minimized to convergence with a dielectric constant set to 80. Details of the design of receptor-based peptidomimetics have been described previously (18, 29).

Peptide Synthesis and Cyclization—Linear peptides were ordered from the Protein Chemistry Laboratory, University of Pennsylvania. The purity and identity of peptides were confirmed by reverse-phase high performance liquid chromatography (HPLC) and mass spectrometry. The peptides were cyclized by air oxidation in distilled water adjusted to pH 8.0–8.5 with (NH₄)₂CO₃ at 0.1 mg/ml, as described previously (18).

Kinetic Binding Studies by Surface Plasmon Resonance—Binding experiments were carried out on BioCORE 3000 (BioCORE, Uppsala, Sweden) at 25°C. Recombinant TRANCE, RANK, and OPG (PeproTECH, Inc.) were immobilized to the dextran hydrogel on the sensor surface (BioCORE CMS sensor chip) with a surface density of 2,000 resonance units. The surface was regenerated to remove all bound analyte among binding cycles using 0.2% SDS. The apparent rate constants (k_on and k_off) and the equilibrium binding constant (K_D) for the receptor-peptide/fusion-peptide binding interaction were estimated from the kinetic analysis of sensorgrams, using the BIA evaluation 3.0 software (BioCORE).

Immunoblotting—To evaluate the effect of the peptide on the regulation of downstream molecules of TRANCE signaling during osteoclastogenesis, RAW264.7 cells (5 × 10⁵/well) were cultured in 6-well plates for 12 h, treated with or without the designed peptide for 2 h, and then stimulated with TRANCE at 500 ng/ml for the indicated periods. Cells were then washed with ice-cold phosphate-buffered saline and lysed with lysis buffer. Cell lysates (15–30 µg) were separated by 12% SDS-PAGE, electroblotted onto nitrocellulose membranes (Osmonics, Westborough, MA), and probed with anti-phospho-ΙκBα, anti-ΙκBα, and anti-ERK2 antibodies (Cell Signaling Technology, Inc., Beverly, MA). The membranes were then developed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences).

Effect on TRANCE-induced Osteoclast Formation and Pit Formation in Vitro—To evaluate the effect of the peptide on TRANCE-mediated osteoclastogenesis, murine osteoclast precursors were cultured in 96-well plates (1 × 10⁵/ml, 200 µl/well) for 3 days in the presence of 30 ng/ml murine CSF-1 and 300 ng/ml sTRANCE with or without treatment of designed peptides at the indicated concentration. After 3 days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) as described previously (30). TRAP-positive multinucleated cells were counted as osteoclast-like multinucleated cells. For the pit formation assay, mouse osteoclast precursors (5 × 10⁵ cells/0.2 ml/well) were placed on dentin slices (4 mm in diameter) in 96-well culture plates and cultured for 1 h with 30 ng/ml M-CSF. Dentine slices were then transferred into 48-well culture plates (Corning Glass). Cells on dentin slices were cultured in the presence of 30 ng/ml M-CSF with or without 200 ng/ml TRANCE for 4 days, in the absence or presence of peptide OP3-4 at the indicated concentration. Medium was replaced on day 3. On day 4, cells were removed from the dentin slices with cotton, and the slices were then immersed in Mayer’s hematoxylin (Sigma) to stain the resorption pits formed by osteoclasts.

Effect on Bone Resorption in Ovariectomized (OVX) Mice—In 8-week-old female C57BL/6 mice, the dorsal skin was incised, and ovaries were excised under anesthesia. The sham operation group and ovariectomy group were treated with peptide or vehicle only. An Arzex® osmotic pump (DURECT Co., Cupertino, CA) was used to deliver OP3-4 subcutaneously after the ovariectomy. The pump was used for systemic administration at a consistent rate for up to 4 weeks without repeated dosing. The pump was filled up to 200 µl for the duration (4 weeks) and delivered at the rate of 0.25 µl/h. The OVX mice were given peptide OP3-4 subcutaneously at a daily dosage of 2 mg/kg/day for 28 days. The mice were euthanized, and the tibia and femur were removed, cleaned of soft tissue, and fixed in 10% formalin. Femur were scanned by peripheral quantitative computed tomography (pQCT) to examine the total bone density. Animals were maintained in accordance with guidelines of Institutional Animal Care and Use Committee of the University of Pennsylvania.

RESULTS

Molecular Modeling of TRANCE Binding to OPG and Development of OPG-like Mimetics—The overall topology of OPG is similar to that of the TNFR (Fig. 1A), although the homology in the primary structure is less than 50%. The three-dimensional model of OPG was built using modular fold of the TNFR superfamily (16, 31). The three-dimensional structure of OPG was built using substructures of TNFR (1TNR), Fas (1BZI), and TRAIL (1DV and 1DG) receptors. The structure of OPG consists of B2-A1-B2-A1-B1-B1-A1-B1 modular folds (16, 31, 32).
To study the fine details of the OPG-TRANCE complex, only the N-terminal region (22-185) was considered. The crystal structure of TRAIL-DR5 (33) was also used as template and used to obtain maximal receptor-ligand interaction. In the final analysis, the OPG-TRANCE complex was similar to the TRAIL-DR5 receptor complex (33).

Based on the structural analysis of TRANCE and OPG-TRANCE complex several contact sites have been identified in TRANCE and three sites on OPG (Fig. 1A). Sites on OPG were identified as following: TRANCE (Tyr74-His86) binds to OPG (Tyr70-Asp78); TRANCE (Ser61-Tyr68) binds to OPG (Tyr82-Glu96); and TRANCE (Tyr96-Phe103) binds to OPG (Leu113-Arg122). The sequences of the three binding sites from both ligand and receptor were then used as template for peptide design (Table I).

Exocyclic peptidomimetics were created from the conformational features of the critical sites on TRANCE and OPG with the addition of aromatic residues. Cyclization of linear peptides was confirmed by 5,5'-dithiobis(nitrobenzoic acid). Purity was evaluated by reverse-phase HPLC before and after cyclization. All of the cyclized peptides were enriched to a purity of >98% before use. The purified exocyclic peptides were soluble and used to make a stock solution at 1 mM in phosphate-buffered saline (pH 7.4).

**Inhibition of sTRANCE-induced Osteoclast Formation in Vitro**—We have designed several peptide mimetics from both OPG and TRANCE (Table I) and have screened them for biological activity. We have evaluated these mimetics at a 30 μM concentration on osteoclastogenesis in murine bone marrow cells co-cultured with TRANCE and CSF-1 (30). Several mimetics at a 30 μM concentration caused some reduction in the number of TRAP-positive multinucleated cells formed. Among all 11 peptides we designed from the three critical binding sites, 8 peptides exhibited 20–60% inhibitory activity (Fig. 1B). The peptides designed from OPG (OP series) showed a greater inhibitory effect than those designed from TRANCE (OL series). These data suggest that receptor-derived mimetics block ligand binding to its receptor differently than ligand mimetics. We have regarded the receptor as a better target in structure-based design and improved it further.
The peptides derived from binding site 3, from both ligands (OL3-1, OL3-2) and receptor (OP3-1, OP3-2, OP3-4), exhibited more inhibitory effects compared with other derived peptides. This observation implied that binding site 3 may be an important binding surface for the signal competence of the OPG/H18528 TRANCE complex. Similar results were obtained when we designed peptides derived from the TNFR based upon the TNF-TNFR complex, indicating that membrane proximal domains may frequently be used as a critical interaction surface between ligands and receptors in the TNF-TNFR superfamily (18). Earlier when we engineered a monoclonal antibody mimetic, AHNP, a HERCEPTIN mimetic (34), we found that residues in the exocyclic regions also play a critical role in stabilizing the conformation of amino acids within the cyclic ring. To see whether such an addition to OP3 would have any affect, we modified the sequence of OP3-1 by either removing the tail amino acid sequence of KHR as in the OP3-2 species or changing the amino acids KHR to LIR as in the OP3-4 forms. Subsequently we found that changing the residues in the exocyclic rings does affect the activity of the mimetic, indicating that the tail composed of KHR is critical for forming the binding site structure. The dissociation rate of mimetics has been shown to correlate with biological activity (35), and thus replacing KHR with LIR at the C terminus of OP3-4 actually enhanced the interaction between ligand and receptor, and consequently OP3-4 has become the most potent mimetic designed.

**Kinetic Binding Ability of OP3-4**

-Binding of OP3-4 to TRANCE was studied using surface plasmon resonance. OP3-4 bound to immobilized TRANCE in a dose-dependent manner. The measured binding affinity of OP3-4 ($K_d$) binding to TRANCE is $3.89 \times 10^{-6}$ M, and the apparent $k_a$ and $k_{off}$ rate constants were estimated to be $2.41 \times 10^{10}$ M$^{-1}$ s$^{-1}$ and $9.37 \times 10^{-4}$ s$^{-1}$, respectively. In a competition assay, OP3-4 blocked TRANCE (TR) binding to immobilized RANK in a dose-dependent manner. OP3-4 at a concentration of 50 $\mu$M significantly inhibited TRANCE binding to its receptor RANK.

![Fig. 2. Kinetic binding of OP3-4 to TRANCE. (a) OP3-4 binds to TRANCE in a dose-dependent manner. Binding of OP3-4 to immobilized TRANCE was evaluated at concentrations of 100, 50, 20, and 5 $\mu$M. The measured binding affinity of OP3-4 ($K_d$) binding to TRANCE is $3.89 \times 10^{-6}$ M, and the apparent $k_a$ and $k_{off}$ rate constants were estimated to be $2.41 \times 10^{10}$ M$^{-1}$ s$^{-1}$ and $9.37 \times 10^{-4}$ s$^{-1}$, respectively. (b) In a competition assay, OP3-4 blocked TRANCE (TR) binding to immobilized RANK in a dose-dependent manner. OP3-4 at a concentration of 50 $\mu$M significantly inhibited TRANCE binding to its receptor RANK.](image-url)
Fig. 3. Dose-dependent inhibition of OP3-4 in TRANCE-induced osteoclast formation in murine bone marrow cells. A, view of osteoclast formation in vitro. Purified marrow macrophages were isolated from the bone marrow of 6–10-week-old C57BL/6 mice and treated with 30 ng/ml M-CSF or 30 ng/ml M-CSF + 300 ng/ml TRANCE for 3 days, stained for TRAP, and photographed at ×10. B, cells were treated with TRANCE in the presence or absence of peptide OP3-4 at different concentrations from 0.4 to 50 μM. OP3-4 inhibited TRANCE-induced osteoclast formation in vitro effectively in a dose-dependent manner. C, in a pit formation assay, OP3-4 at 10 μM exhibited at least a 50% inhibition of TRANCE-induced pit formation.

In Vitro Analysis of OP3-4 Mimetic—The inhibitory activity of peptide OP3-4 was then examined in terms of its ability to inhibit TRANCE-induced osteoclast formation (Fig. 3). Peptide concentrations of 2 μM or more caused a dose-dependent decrease in the number of TRAP-positive (TRAP⁺) multinucleated cells formed (Fig. 3A). In the presence of 50 μM OP3-4, the number of TRAP⁺ multinucleated cells was less than 10% of the number formed in co-cultures performed without the peptide. The IC₅₀ was 10 μM. The decrease in the number of TRAP⁺ cells was apparently not the result of toxicity of the peptide because there was no change in the number of osteoblasts detected by staining for alkaline phosphatase (data not shown), nor was there a cytotoxic effect of 100 μM OP3-4 on RAW264.7 murine myeloid cells (data not shown).

TRANCE stimulates bone resorption by mature osteoclasts (35, 36). We therefore examined the effect of OP3-4 on TRANCE-induced bone resorption in vitro (Fig. 3C). Mature osteoclasts from neonatal mice were plated on dentin slices. The cells were cultured for 4 days with 200 ng/ml TRANCE alone or with 30 μM OP3-4 and TRANCE. As reported previously (35, 36), 200 ng/ml TRANCE stimulated bone resorption by nearly 3-fold. OP3-4 significantly blocked the increased bone resorption at 30 μM. The number of osteoclasts on the dentin slices were not changed by TRANCE alone or by OP3-4 plus TRANCE. Thus, interfering with TRANCE binding to RANK by the use of designed peptidomimetics was effective in inhibiting TRANCE-induced osteoclast formation in vitro.

Down-modulation of NF-κB by OP3-4—NF-κB activation in osteoclast precursors has been implicated as a signaling pathway involved in the successful differentiation of precursors to mature osteoclasts (10). Activation of NF-κB is controlled by an inhibitory subunit, IκB, which retains NF-κB in the cytoplasm. NF-κB activation requires the sequential activation, phosphorylation, ubiquitination, and degradation of IκB as well as consequent exposure of discreet nuclear localization signals on the NF-κB surface (37). As shown in Fig. 4, TRANCE markedly induced the phosphorylation of IκB-α by 5 min, and a concurrent degradation of IκBα. In Fig. 4, OP3-4 inhibited TRANCE-stimulated NF-κB activation as measured by lack of IκB-α phosphorylation and degradation. OP3-4 thus inhibited
TrANCE-induced osteoclast formation at least in part through suppressing the NF-κB pathway. Although TrANCE also activates c-Jun N-terminal kinase (38), OP3-4 did not exhibit an inhibitory or stimulatory effect on this pathway (data not shown).

**OP3-4 Protects Mice against Ovariectomy-associated Bone Loss**—It is well known that ovariectomy results in reduced bone mineral density associated with loss of estrogen (39). It was important to demonstrate that OP3-4 in vivo can protect against pathological decreases of bone density in O VX animals. The pQCT technique measures the true volumetric bone mineral density, including total, trabecular, and cortical bone density, respectively (40).

8-week-old female C57BL/6 mice underwent ovariectomy or were sham-operated. The O VX mice were then treated with OP3-4 for 28 days at a dosage of 2 mg/kg/day, which is less than the optimal dose (6 mg/kg/day) based on IC50. In vitro, OP3-4 actively inhibited osteoclast formation at low concentration 2 μM (0.015 mg/ml). Because of the limited solubility of OP3-4 and to avoid any effect resulting from peptide aggregation, which might occur at high doses, we chose to deliver OP3-4 using an Arzêlo® pump. The advantage of using the pump is that it can offer a consistent administration at low dosing.

The protection of bone density by OP3-4 provides evidence that OP3-4 can effectively inhibit postmenopausal osteoporosis in an in vivo animal model.

**DISCUSSION**

We have created a novel species to alter signaling of the RANK-TRANCE receptor complex based on the deduced structure of OPG. We have shown that a small molecule can effectively alter the functions of the RANK-TRANCE receptor complex and can reverse bone loss in vivo.

TNFR superfamily members play critical role in several immunological functions. Dysregulation of any member in the superfamily has profound effects on the overall immune system and leads to several immunological disorders. In recent years, specific roles played by several members in the super family have been elucidated (3). RANK-TRANCE-OPG are members of TNF superfamily and critical mediators and regulators of bone remodeling, T cell activation, dendritic cell survival, and lymph node formation (4, 5, 41). Although most of the knowledge about the function of RANK-TRANCE-OPG has been derived through genetic and biological assays (8, 42), there is little known about their structure-function relationship, perhaps because of a lack of structural details. Nevertheless, it is clear that altering the RANK-TRANCE receptor complex has beneficial effects in osteoporosis (43–45) and other bone-related pathologies. In fact, soluble OPG receptor is currently being evaluated for safety (Phase I) in breast cancer-related metastasis (46). Given the pleiotropic nature of TRANCE, it is not clear what the long-term effect of T cells and dendritic cell-mediated immunoregulatory pathways may be when the RANK receptor is disabled. TRANCE is not only involved in bone remodeling, but also participates in other processes including T cell activation (7, 47–50), dendritic cell survival (22, 51), and mamalian development (4, 52). TRANCE is also now regarded as a major participant in cytokine-regulated bone remodeling as well as immune inflammation. Recent studies have examined whether TRANCE is involved in human vascular diseases that link osteoporosis and arterial calcification (53, 54). Recently, it has been shown that the RANK-TRANCE system may play a critical role in preventing autoimmune pathologies. Green et al. (55) have shown that ablation of the RANK-TRANCE pathway results in decreased frequencies of CD4+CD25+ T regulatory cells in pancreas-associated tissue and, as a consequence, rapid progression to diabetes. These studies suggest that cautious approach is needed in blocking RANKTRANCE signaling. Design of small molecules capable of discreetly interfering with RANK-TRANCE signaling may be a better strategy when examining TRANCE activity in different biological systems. Also, it would be more beneficial therapeutically if the RANK-TRANCE receptor complex is modulated, rather than completely inhibited. To understand the molecular aspects of the RANK receptor complex, we have developed a novel species that specifically alters RANK-TRANCE signaling and exerts beneficial effects in vivo.

To date, little is known about the structural aspects of the RANK-TRANCE-OPG receptor system, except their overall topology. OPG is a member of the TNFR superfamily, and the secreted form of OPG is about 401 amino acids. The first half of the N-terminal domain (22–194) shares homology with TNFR superfamily members, and the C terminus is unique. The N-terminal domain (22–194) is found to be responsible for OPG function (8). Unlike other members of the TNFR superfamily, OPG does not have a signaling cytoplasmic domain. OPG is
synthesized as a monomer but exists predominantly as a dimer. The linker is mediated by a cysteine at the C terminus (8). It has been also shown that only dimeric OPG is active (8). It is hypothesized that the dimeric OPG binds to TRANCE and precludes it from binding to RANK (8). But the manner in which TRANCE is isolated from RANK is not clear. The crystal structure of TNF Rk has been determined with and without its ligand (13, 56). In the uncomplexed state, the soluble TNF receptor is observed as antiparallel dimers (56). It was proposed that TNF receptors might exist as dimers in the resting state but are trimerized in the presence of their ligands; it is hypothesized that soluble monomeric TNF receptors might form a homodimeric receptor complex and prevent ligand binding (56). On the other hand, soluble dimeric form receptors (Fc-fused receptors) might engulf the ligand and prevent binding to its receptor. In each case, the soluble receptors prevent TNF-α-induced signaling. To understand how OPG might interact with TRANCE, both dimeric and trimeric models were built, and the interaction patterns were studied. Unlike the TNF receptor, the OPG receptor does not contain transmembrane domain, but instead contains an unstructured domain at the C terminus. We hypothesized that dimeric OPG binding to TRANCE may be more stable than monomeric OPG binding to TRANCE because of the untethered or flexible C-terminal domain. This is evident from deletion mutant studies, where only Fe-fused OPG was active, and deleting the C-terminal regions from 195 did not affect the activity (8).

We designed peptides from both ligand and receptor; the peptides designed from OPG (OP series) showed a greater inhibitory effect than those derived from TRANCE (OL series). This may be related to the complexity of receptor-ligand association and the order of oligomerization. For example, TRANCE may first organize as a trimer and upon binding to the receptors may bring the signaling units in the cytoplasm to close proximity for signaling events. If ligand-derived mimetics cannot significantly interfere with the trimeric TRANCE, then the mimetics may consequently have less effect on ligand-receptor interactions. Thus, we hypothesized that ligand-derived mimetics may be less effective in disrupting the receptor complex and its signaling aspects.

Earlier, we have shown that small molecule mimetics derived from monoclonal antibody, and ErbB receptors, can mimic the macromolecule from which it was derived (34, 57). We have used a similar strategy to design mimetics that function like a soluble OPG, which is shown to be functional only when it is dimeric. Because OP3-4 is not dimeric as deduced from HPLC analysis, it was not clear whether it can effectively block TRANCE binding to RANK. We have used kinetic binding studies to check the mode of interaction of OP3-4 to RANK and TRANCE. OP3-4 bound to RANK ($K_d = 3.24 \times 10^{-5}$ M) and TRANCE ($K_d = 7.70 \times 10^{-6}$ M). This observation suggests that the mode of inhibition of receptor-derived mimetics altered the RANK-TRANCE receptor complex differently from the natural inhibitor, OPG. Mimetics derived from TRANCE (OL3-2 mimetics), on the other hand, bound only to RANK receptor ($K_d = 4.27 \times 10^{-5}$ M) and did not bind to TRANCE (data not shown). These observations suggest that mimetics derived from TRANCE function like a classical antagonist (i.e. competitive) such as an antibody, and the mimetics derived from receptor function differently.

OPG also serves as a receptor for the cytotoxic ligand TRAIL and inhibits TRAIL-induced apoptosis of Jurkat cells (58). The biological relevance of the OPG-TRAIL receptor complex is not fully understood in vitro (41). In prostate cancer cell lines, it has been shown that expression of OPG has an inverse correlation with TRAIL and is believed that OPG can as a survival protein (59). Nevertheless, OP3-4 was examined for association with TRAIL in binding studies. OP3-4 bound to both TRANCE at a $K_d$ of $3.89 \times 10^{-6}$ M and to TRAIL at a $K_d$ of $1.93 \times 10^{-5}$ M. Thus, OP3-4 binds to TRANCE with about a 5-fold higher affinity. OP3-4 binding to TRAIL was not unexpected because soluble OPG shows almost equal binding affinity to TRANCE and TRAIL in vitro, and these results only suggest that OP3-4 might behave like a soluble OPG.

Although the RANK-TRANCE-OPG receptor system is critical for osteoclast formation, it has been reported that TNF-α also can induce osteoclasts in both a TRANCE-dependent and independent manner (60, 61). The exact mechanism by which these two pathways affect osteoclast formation is not clear. However, it is seems that NF-κB, and downstream Akt/phosphatidylinositol 3-kinase activation may be critical signaling events and might be utilized by both TNF and RANK receptors (62–64). In separate studies, we found that peptide mimetics from TNF receptor inhibited TNF-α- and TRANCE-induced osteoclasts via NF-κB. Here we have shown that OPG-derived mimetic OP3-4 also inhibits NF-κB activation but has no effect on c-Jun N-terminal kinase pathways. These observations suggest that OP3-4 is able to alter RANK signaling exquisitely and limit osteoclast formation via NF-κB. Because OP3-4 is able to inhibit NF-κB activation, it is also possible that it may block TNF-α-induced osteoclasts.

One of the biological consequences of dysregulation in the axis of the RANK-TRANCE-OPG receptor complex is bone loss in a variety of pathologies such as postmenopausal osteoporosis in women, arthritis, and multiple myeloma (41, 65–67). We have shown that OP3-4 is able to limit bone loss in OVX mice, which is a prototypical animal model of osteoporosis. We have used OP3-4 to treat the animal at 2 mg/kg/day compared with OPG-Fc fusion protein, which was used at 5 mg/kg/day in a similar study (8). Despite the smaller dose, we observed significant ($p < 0.01$) and comparable improvement in restoring bone loss. These studies show that disabling the RANK-TRANCE receptor complex by OP3-4 has beneficial effects in vivo.

The molecular basis for altering RANK signaling by small OP3-4 mimetics may differ from protein therapeutics such as monoclonal antibody or soluble OPG or soluble RANK receptors. It is now well accepted that cell surface receptors transduce signals in response to conformational changes induced by oligomerization in a ligand-dependent or -independent manner (16, 68–71). However, it was not clear how much imperfection is tolerated in the receptor assembly and whether such imperfect oligomerization has any relevance to biological effects. It has been shown that only dimeric OPG (as Fe-fused) actively inhibited the RANK-TRANCE receptor complex, but OP3-4 did not form dimers as judged from HPLC analysis (data not shown). Nevertheless, it is possible that OP3-4 may still mediate ligand-receptor interaction similarly to erythropoietin-derived mimetics (72, 73). Thus, we see some evidence that OP3-4, like EPO antagonists (72, 73), may promote a defective receptor complex and lead to defective signaling. We hypothesized that receptor derived peptides such as OP3-4 which bind to both receptor and ligand, may form a defective complex by acting like a spacer between RANK and TRANCE. The overall scheme is illustrated in Fig. 6.

OP3-4 seems to promote a defective receptor complex as judged from surface plasmon resonance analysis. The small molecule mimetic, OP3-4, binds to both RANK and TRANCE, and it is not large enough (surface area) to prevent TRANCE associated with RANK completely. In a modified competition assay (57), TRANCE binding to the immobilized RANK recep-

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2 X. Cheng and R. Murali, unpublished data.
tor was inhibited in a dose-dependent manner when OP3-4 was allowed to prebind to the ligand (Fig. 2b). These observations suggest that OP3-4 may create a defective or unstable complex by increasing the overall dissociation rate of TRANCE binding to RANK. The unstable or defective receptor complex may lead to either weak or altered RANK signaling and therefore inhibition of osteoclast formation depending on the extent of distortion induced in the complex.

In summary, our results demonstrate that an exocyclic constrained peptide, OP3-4, derived from the deduced three-dimensional structure of OPG-TRANCE, is a novel species and inhibited TRANCE-induced osteoclast formation in vitro at an IC₅₀ of 10 μM as well as protected ovariectomy-associated bone loss in vivo at the dosage of 2 mg/kg/day through a mechanism different from that of OPG. These studies demonstrate that OP3-4 has the potential for developing therapeutics for bone loss in arthritis, periodontal disease, osteoporosis, and other TRANCE overexpression-related disease states.

Acknowledgments—We thank the Biosensor/Interaction Analysis and Structural Biology Cores Group, Department of Medicine, University of Pennsylvania, for assistance with BioCORE binding studies, and the Protein Chemistry Laboratory, University of Pennsylvania, for peptide synthesis and purification.

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