Novel and Selective Small Molecule Stimulators of Osteoprotegerin Expression Inhibit Bone Resorption

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ABSTRACT

Osteoprotegerin (OPG), a secreted member of the tumor necrosis factor receptor superfamily, is a potent inhibitor of osteoclast formation and bone resorption. Because OPG functions physiologically as a locally generated (paracrine) factor, we used high-throughput screening to identify small molecules that enhance the activity of the promoter of the human OPG gene. We found three structurally unrelated compounds that selectively increased OPG gene transcription, OPG mRNA levels, and OPG protein production and release by osteoblastic cells. Structural analysis of one compound, a benzamide derivative, led to the identification of four related molecules, which are also OPG inducers. The most potent of these compounds, Cmpd 5 inhibited osteoclast formation and parathyroid hormone-induced calvarial bone resorption. In vivo, Cmpd 5 completely blocked resorptive activity (serum calcium, osteoclast number) in parathyroid hormone-treated rats. Furthermore, Cmpd 5 reduced the ability of a rat breast cancer to metastasize to bone. Finally, the compound also prevented bone loss in a rat adjuvant arthritis model. These results provide proof of the concept that low molecular weight compounds can enhance OPG production in ways that can result in effective therapies.

Osteoprotegerin (OPG), a soluble member of the tumor necrosis factor (TNF) receptor superfamily, inhibits osteoclast formation during the late stage of their development (Simonet et al., 1997; Yasuda et al., 1998). OPG functions as a decoy receptor and blocks the binding of the receptor activator of NF-κB (RANK) ligand (RANKL, a TNF family member) to its receptor RANK, thereby inhibiting both osteoclast formation and activity. Transgenic mice overexpressing OPG develop osteopetrosis due to the failure of osteoclast formation and bone resorption comes from studies using bone organ and cell cultures from mice rendered null for the OPG gene (Udagawa et al., 2000). An engineered form of OPG, which has a very prolonged half-life (OPG-Fc), has been used in pharmacological studies in human subjects and experimental animals (Hofbauer and Heufelder, 2000; Bekker et al., 2001; Kostenuik and Shalhoub, 2001). Treatment with OPG-Fc inhibits osteoclastogenesis and the bone resorption response to ovarectomy, parathyroid hormone (PTH)-related protein (PTHrP), 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], interleukin-1α, TNFα, low calcium, tumor-induced humoral hypercalcemia, bone metastasis, periodontal disease, and adjuvant arthritis (Simonet et al., 1997; Kong et al., 1999; Morony et al., 1999; Capparelli et al., 2000; Min et al., 2000; Teng et al., 2000). Consistent with its important regulatory role in bone resorption, OPG expression by stromal/osteoblastic cells is altered both in vitro and in vivo in response to the many osteotropic factors, which either enhance or inhibit oste-
oclase formation and bone resorption associated with either show these to be effective in preventing the increased oste-
ify the OPG gene promoter and study, we developed a high-throughput, cell-based screen to agents could be useful for the treatment of clinical conditions that is not orally bioavailable, and thus requires parenteral administration. Furthermore, prolonged use of such a modified version of OPG raises the possibility of an immune response. The likelihood that the physiological function of OPG is primarily a paracrine regulator (Udagawa et al., 2001). Currently OPG-Fc is in early phase evaluation in humans as a potential therapeutic (Bekker et al., 2001), but one limitation for its widespread use is the fact that it is a large protein that is not orally bioavailable, and thus requires parenteral administration. Furthermore, prolonged use of such a modified version of OPG raises the possibility of an immune response. The likelihood that the physiological function of OPG is primarily a paracrine regulator (Udagawa et al., 2000) provides a basis for the development of small molecules that could selectively stimulate endogenous OPG expression to levels sufficient to result in a therapeutic benefit similar to that obtained with parenteral administration of OPG. Such agents could be useful for the treatment of clinical conditions characterized by enhanced bone resorption. In the present study, we developed a high-throughput, cell-based screen to identify compounds that activate the OPG gene promoter and show these to be effective in preventing the increased osteoclase formation and bone resorption associated with either pharmacological treatment or models of disease.

Materials and Methods

**Reporter Constructs.** Cloning of the 5.9-kb fragment of the human OPG promoter (pOPG5.9-gal), fused with β-galactosidase (β-gal) reporter gene of the p6-gal-Basic reporter vector (BD Biosciences Clontech, Palo Alto, CA), was performed using standard cloning procedures as previously described (Thirunavukkarasu et al., 2000). All constructs were verified by restriction mapping and DNA sequencing using the dyeoxy-chain termination method.

**Cell Culture, Transfection Assays, and Analyses.** The rat osteoblast-like osteosarcoma cell line, UMR 106, and the human osteoblast-like osteosarcoma cell line, SaOS-2 were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (3:1) (In-vitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) plus 2 mM glutamine (In-vitrogen). All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. Experiments were initiated when cells were approximately 70 to 80% confluent.

For stable transfection experiments, the OPG promoter construct pOPG5.9β-gal (−5917 to +1) or SV40 promoter β-gal was cotransfected with a second plasmid pBLCMV (In-vitrogen) encoding the neomycin gene into UMR 106 cells using Fugene 6 (Roche Applied Science, Indianapolis, IN) as previously described (Thirunavukkarasu et al., 2000). Details of the selection and characterization of the stable clones used in this study for OPG promoter analysis have been described (Thirunavukkarasu et al., 2001). Stable clones of UMR 106 cells expressing Runx2 (an osteoblast-specific promoter) or 6XOSE (contains six copies of the Runx2 binding site) fused to luciferase reporter gene were prepared as previously described (Ducy and Karsenty, 1995; Krishnan et al., 2003). The stable clones were stimulated with recombinant TGF-β1 (10 nM, R&D Systems, Minneapolis, MN), trichostatin A (1 × 10−4 to 1 × 10−5 μM, Calbiochem, San Diego, CA), sodium butyrate (5 × 10−3 to 5 × 10−2 μM, Sigma-Aldrich, St. Louis, MO), or compounds (0–30 μM) for 24 h. Cell extracts were assayed for β-gal activity using the β-Gal reporter gene assay kit (Roche Applied Science) as recommended by the manufacturer. The luciferase reporter gene assay, high sensitivity (Roche Applied Science) was used for analysis of luciferase in the cell extracts. Assays were done in white, opaque Microtite 96-well plates (Dynex, Chantilly, VA). Luminescence was measured in a MLX microtiter plate luminometer (Dynex) and light integration measured at 2 s (RLU summed).

**High-Throughput Screening of Compounds.** A stable UMR 106 clone containing the β-gal reporter gene under the control of a 5.9-kb human OPG promoter (OPG-β-gal) was used for screening. Each compound of the library (Research Triangle Park Laboratories, Eli Lilly and Company, Research Triangle Park, NC) was dissolved in DMSO at 1 mg/mL. The UMR 106 cells containing OPG-β-gal were plated in 96-well plates (Corning, Cambridge, MA) and incubated for 24 h with each compound (final concentration was 10 μM in medium), following serum withdrawal for 12 to 16 h. Cell lysates were then assayed for β-gal activity as described. All results from each experiment were compared with control wells (DMSO only) and TGF-β1-treated positive control. Compounds that showed ≥50% of the activity of TGF-β1 were studied further.

**OPG ELISA Assay.** To quantify the amount of OPG secreted into the cell culture medium, SaOS-2 cells (50,000 cells in a 96-well plate) were treated with TGF-β1 (5 ng/ml), trichostatin A (5 × 10−8 to 5 × 10−4 μM), sodium butyrate (5 × 10−8 to 5 × 10−4 μM), or compounds (0–30 μM) for 48 h. OPG secreted into the culture medium was analyzed using a sandwich ELISA, utilizing rabbit polyclonal antiserum directed against recombinant human OPG, as described previously (Onyia et al., 2000; Ma et al., 2001; Yang et al., 2002). A standard curve was generated using recombinant human OPG. Similarly, serum circulating OPG levels were determined by sandwich ELISA with rabbit polyclonal anti-human OPG IgG. Briefly, 96-well plates (IMMUNO 4 flat bottom; Dynatech Labs, Chantilly, VA) were coated with 0.5 μg/well purified rabbit anti-OPG antibody (IgG) (diluted in 100 μl of carbonate/bicarbonate buffer, Sigma-Aldrich), incubated at 4°C overnight, and blocked for 1 h at room temperature with 200 μl/well Bovine casein in PBS (Pierce Endogen, Rockford, IL). The plate was incubated with 100 μl of serum diluted in 1% bovine serum albumin PBS with 0.05% Tween 20 (TPBS) for 1 h and followed by three washes with TPBS. To each well was added 100 μl of diluted biotinylated anti-OPG IgG for 1 h and followed by 100 μl/well streptavidin-HRP conjugate (Zymed Laboratories, South San Francisco, CA) for 30 min with TPBS washing. ELAST ELISA amplification system (PerkinElmer Life Sciences, Boston, MA) was applied before color development according to the manufacturer’s protocol. Color was developed with 100 μl/well TMB Microwell Peroxidase Substrate System (KPL, Inc., Gaithersburg, MD) for 15 min, stopped with 100 μl/well 1 N phosphoric acid, and analyzed by reading the absorbance at 450 nm.

**Isolation of Poly A+ RNA and Northern Blotting.** In UMR 106 cells, the effects of TGF-β1 (10 ng/ml) on OPG mRNA expression were evaluated by Northern blot analysis following 24 h of treatment. Time-dependent effects of Cmpds 1, 2, and 3 on OPG and RANKL mRNA expression were evaluated in human SaOS-2 cells treated with 10 μM compound for 2, 24, or 48 h. Cultures (four T150 flasks/group) of cells were grown (as described above) to 80 to 90% confluence and then changed into medium containing 0.1% FBS overnight. The cells were then treated with the indicated concentrations of compound for the specified time periods. Total RNA was extracted from the osteoblast cultures by adding Ultraspec-II RNA (Biotec Laboratories, Inc., Houston, TX) directly to the culture.
flasks. The resulting cell lysates were passed several times through a 10-ml pipette before collection. Poly A⁺ RNA was isolated from total RNA using Oligotex (Qiagen, Valencia, CA) according to the manufacturer’s protocol and quantitated by spectrophotometry. The absorbance at 260 nm was determined and the 260/280-nm absorbance ratio was calculated to ensure the absence of protein contamination. Samples of poly A⁺ RNA (2 μg) were denatured in 0.04 M MOPS, pH 7.0, 10 mM sodium acetate, 2.2 M formaldehyde, and 50% formamide at 60°C for 10 min and size fractionated by electrophoresis through 1% agarose gels in 2.1 M formaldehyde and 1× MOPS and transferred to nylon membranes (Brightstar-Plus; Ambion Inc., Austin, TX). The nylon membranes were air-dried, and the RNA samples were cross-linked to the membranes by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). Migration of 28 S and 18 S ribosomal RNA was determined by ethidium bromide staining. DNA probes were labeled by the random primer method (Invitrogen) using [α-32P]dCTP. Prehybridization and hybridization were carried out at 48°C in NorthernMax buffers (Ambion Inc.). After hybridization, membranes were washed for 30 min at room temperature in buffer containing 2× SSC and 0.1% SDS and then 30 min at 48°C in 0.2× SSC and exposed to Biomax MS X-ray film (Eastman Kodak, Rochester, NY) at −70°C. Autoradiograms were quantitated by scanning laser densitometry (2400 Gel Scan XL; Amersham Biosciences Inc., Piscataway, NJ). Labeled bands were quantitated as densitometric units and normalized to that of the GAPDH signals to correct for variations in RNA transfer and gel loading. The data were expressed as -fold change versus untreated control samples.

**Generation of Radiolabeled Probes for Northern Analysis.**

To generate probes for Northern analysis, the inserts containing OPG or RANKL cDNA were released from the plasmid by restriction digest. Rat GAPDH cDNA probes were cloned using polymerase chain reaction with specific primer pairs as published previously (Onyia et al., 1995, 2000). Twenty-five nanograms of cDNA were labeled by the random primer method (Invitrogen) using [α-32P]dCTP (Amersham Biosciences Inc.). Free nucleotides were removed by centrifugation through a Centricon-50 column (Millipore Corporation, Bedford, MA).

**Coculture Assay of Bone Marrow Cells and BALC Cells.** The effects of recombinant human OPG or compounds on osteoclast differentiation were studied in cocultures of bone marrow cells and BALC cells (a murine calvarial-derived cell line) as previously described (John et al., 1996). Briefly, bone marrow cells from the femur of 8-10-week-old C57BL/6 mice (aged 8–9 weeks; The Jackson Laboratory, Bar Harbor, ME) were seeded into 24-well cluster dishes (5× 10⁴ nonmononuclear cells/cm²) in growth media [RPMI 1640 media (Invitrogen) containing 5% heat-inactivated FBS (Hyclone) and 1% antibiotic/antimycotic solution (Invitrogen)]. BALC cells (1.5× 10⁴ cells/cm²) were cocultured with the bone marrow cells. The cultures were treated with 1.25(OH)₂D₃ (10⁻⁶ M, BIOMOL Research Laboratories, Plymouth Meeting, PA) in the presence or absence of 0.01 to 30 μM compound or OPG for 6 days with fresh medium and reagents added on day 3. The cultures were fixed with formalin, stained for tartrate-resistant acid phosphatase (TRAP), and the number of TRAP-positive cells with three or more nuclei were quantitated per well as previously described (John et al., 1996).

**Calvarial Organ Culture.** Calvariae were dissected from mouse pups (aged 3–5 days; Taconic Farms Inc., Germantown, NY), cleaned of soft tissue, and the calvariae were cut in half along the sagittal suture (Fukayama et al., 1988). Half-calvariae were placed in 17× 100 mm propylene round bottom tubes that contained 1 ml of Dulbecco’s modified Eagle’s medium (Invitrogen) with 15% heat-inactivated horse serum (Hyclone), 2.8 mM l-glutamine (Invitrogen), 10 units/ml sodium heparin (Elkin-Sinn, Cherry Hill, NJ), and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively; Invitrogen) in the presence or absence of trichostatin A (0.5–50 nM), sodium butyrate (0.5–50 μM), Cmpd 1 (0.1–30 μM), or Cmpd 5 (0.1–30 μM). The tubes were gassed with 50% oxygen, 5% carbon dioxide, and 45% nitrogen, sealed, and incubated for 24 h at 37°C on a roller drum (New Brunswick Scientific Co., Inc., Edison, NJ). Following the 24-h preincubation, the media were changed and fresh compound in media or control media alone was added, which did or did not contain 1 nM human PTH 1-38 (Zeneca Inc., Wilmington, DE). After 72 h in culture, the medium was collected and ionized calcium was analyzed using a Ciba-Corning 634 ISE Ca²⁺/pH analyzer (Chiron Diagnostics Corp., East Walpole, MA).

**Parathyroidectomized Rat Model.** Weaning, Sprague-Dawley female rats (Taconic Farms, Inc.) weighing 60 to 70 g were used as previously described (Ma et al., 2001). Animals were parathyroidectomized by vendor and delivered to our facility 2 to 3 days posturgery. They were maintained on a 12 h light/dark cycle at 22°C with access to tap water ad libitum. To minimize the gut and kidney effects on serum calcium, rats were fed a calcium-free diet containing 0.02% calcium, and 0.3% phosphorus (TD 99171; Teklad, Madison, WI) during the experimental period. Rats were pretreated with vehicle or Cmpd 5 (0.5, 5, and 50 mg/kg) daily by subcutaneous (s.c.) injection for 3 days with 4 rats in each group. At 2 h after the last dosing, synthetic human PTH 1-38 (1 μg/100 g/s, Zeneca Inc.) was given s.c. via Alzet pump (Durect Corp., Palo Alto, CA) for 6 h. Cmpd 5 was first dissolved in 5% DMSO, then 10% castor oil, and finally water was added to the final solution. PTH was prepared in a vehicle of acidified saline containing 2% heat-inactivated rat serum. Blood was collected under isoflurane anesthesia at indicated time points for the measurement of blood ionized calcium (Ciba-Corning 634 Ca²⁺/pH analyzer) and serum OPG. Bones were collected for histologic analyses as described (Ma et al., 1995). All animal studies were approved by the Eli Lilly and Company Animal Care and Use Committee.

**Rat Mammary Carcinoma Tumor Bone Metastasis Model.** Cells of the rat mammary carcinoma tumor 13762 (2× 10⁶ cells/rat in a 200-μl volume) were injected into the left ventricle of 7- to 8-week-old Fischer-344 female rats (Harlan, Indianapolis, IN) (Alvarez et al., 2003). Cmpd 5 (0.1 and 1 mg/kg/day) was administered for 4 weeks by s.c. injection starting at 1 week after cell inoculation. After 3 and 4 weeks of treatment (4 and 5 weeks postinoculation), tumor progression was evaluated using a high resolution Faxitron X-ray system (model MX-20; Faxitron X-ray Corp., Buffalo Grove, IL).

**Image Analysis of Osteolytic Lesions.** Radiographic images were analyzed for tumor-induced osteolysis. Lesions visible in tibiae, femurs, and fibulae were counted, and the margins were traced to determine lesion number and area using an Image-Pro Plus System (version 3.0.1, Media Cybernetic, Silver Spring, MD).

**Radiographic Analysis of Osteolytic Lesions.** Paraffin sections were obtained from the tibiae, and osteoclast number was determined using a Sangset system (version 3.0.1, Media Cybernetic, Silver Spring, MD).

**Quantitation of Osteoclast Number.** Bones were fixed in 10% buffered formalin for 2 days, decalcified in Decalcifier I (Surgipath, Richmond, IL), and processed for embedding in paraffin. Longitudinal 5-μm-thick sections were stained with 0.1% toluidine blue for proximal tibial metaphysis or hematoxylin-eosin staining (ankle joint). Osteoclast number was measured on the entire marrow region within the cortical shell between 0.67 and 2 mm proximal tibial metaphysis or 1 mm (distal tibial metaphysis) distal to the growth plate metaphyseal junction under 20× magnification. Trabecular bone surface was measured using a digitizing image analyzing system named Osteomeasure (OsteoMetrics, Inc. Atlanta GA). The osteoclast numbers were then normalized to trabecular bone surface (Ma et al., 1995).

**Statistical Analysis.** Blood ionized calcium, osteoclast number, serum OPG, bone lesion number, total bone lesion area, paw and spleen weight, and trabecular area and osteoclast number in the calcaenum were presented as mean ± S.E.M. Differences in group means were
assessed by analysis of variance using Fisher's protected least significant difference for which the significance level was $P < 0.05$.

**Results**

**Identification of Small Molecule Inducers of OPG Promoter Activity.** From among several clones generated, a stable clone harboring the OPG-$\beta$-gal was selected, isolated, and characterized for responsiveness to TGF-$\beta_1$, an agent known to induce OPG transcription (Takai et al., 1998; Thirunavukkarasu et al., 2001). This clone demonstrated a 5-fold increase in OPG promoter activity in response to TGF-$\beta_1$, a pattern reminiscent of its effect on endogenous OPG mRNA and protein expression (Fig. 1A) (Thirunavukkarasu et al., 2001). For screening, OPG-$\beta$-gal-containing cells were treated with individual synthetic organic compounds at a concentration of 10 $\mu$M for 24 h, and compounds that induced OPG-$\beta$-gal activity to $\geq 50\%$ of the levels seen with TGF-$\beta_1$ were then analyzed further. Compounds that were found to stimulate OPG-$\beta$-gal in a concentration-dependent manner were further evaluated in a second UMR 106 stable cell clone, which contained a SV40 promoter-$\beta$-gal reporter (SV40-$\beta$-gal) and produced $\beta$-gal constitutively. Only those compounds, which selectively induced OPG-$\beta$-gal and had no detectable activity on SV40-$\beta$-gal (up to 30 $\mu$M tested), were further studied. Based on this approach, after screening approximately 387,000 compounds, 3 structurally unrelated compounds Cmpds 1, 2, and 3 that selectively induced the OPG promoter (Table 1) and not the SV40 promoter (Fig. 1B) were chosen for further studies.

**Effects on OPG mRNA and Protein.** To confirm that the increase in OPG transcription/promoter was reflected in endogenous mRNA and protein, we evaluated the effects of the compounds on OPG production by rat UMR 106 cells and human SaOS-2 osteoblast-like osteosarcoma cells. An OPG ELISA was used to evaluate secreted OPG in the cell culture media whereas the changes in mRNA were quantitated by Northern blotting of Poly A$^+$ RNA. Cmpds 1, 2, and 3 increased OPG protein levels in both SaOS-2 (Fig. 1C) and

![Fig. 1. TGF-$\beta_1$ and small molecules induced OPG promoter activity and OPG secretion but not SV40 promoter activity. A, TGF-$\beta_1$-induced OPG promoter activity in UMR 106 cells harboring the OPG-$\beta$-gal construct as indicated by relative light units (RLU). Symbols represent the mean ± standard error of four to eight separate treatments. The inset shows endogenous OPG mRNA expression in response to TGF-$\beta_1$ treatment in UMR 106 cells. B, Cmpds 1, 2, and 3 had no effect on SV40 promoter activity in UMR 106 cells harboring the SV40-$\beta$-gal construct as indicated by RLU. FBS (25%) and isobutylmethylxanthine (IBMX, 10 $^{-3}$M) served as the positive controls. Symbols represent the mean ± standard error of four to eight separate treatments. C, in human SaOS-2 cells, effect of treatment with Cmpds 1, 2, and 3 on secretion of OPG into the culture medium was evaluated. D, Northern blots showing effects of treatment with compounds (10 $\mu$M) on mRNA for OPG, RANKL, and GAPDH. The OPG values are expressed as $\text{-fold}$ induction, relative to GAPDH levels at each time point.]
UMR 106 cells (data not shown). In SaOS-2 cells, the increase in protein expression was also associated with a time-dependent increase in OPG mRNA expression (Fig. 1D). At the time points of peak OPG mRNA expression, no effects were observed on the levels of mRNA for RANKL, the OPG cognate ligand, or GAPDH (Fig. 1D). Up-regulation of GAPDH expression was observed at time points following peak OPG mRNA expression (Fig. 1D), and the reason for this increase is unclear. The time course for onset of OPG activation (6–24 h) was compound-dependent. Together these results provide evidence that these small molecules induce OPG expression at least in part via activation of transcription.

Selectivity and Structure-Activity Relationships. Of the three compounds, we focused further analysis on Cmpd 1 (a benzamide derivative), which we believed would be a more likely candidate for drug development based on its chemical structure (stability and structural simplicity). Four additional active compounds (Table 2 and Fig. 2A) were identified by analysis in the OPG promoter and secretion assays of 115 compounds that are substructurally related to Cmpd 1. The structural similarities and activity of the five active (Cmpds 1, 4, 5, 6, and 7) and two structurally related inactive compounds were studied (Table 2 and Fig. 2A). All five active compounds stimulated OPG promoter and protein expression but had no effect on the SV40 promoter activity (Table 2, Fig. 2, A–B). In the OPG promoter assay, Cmpds 1, 4, and 5 had similar efficacies and potencies whereas Cmpds 6 and 7 were less potent and efficacious. In the OPG secretion assay all five active compounds had a comparable efficacy but Cmpd 5 was 16- to 100-fold more potent (Table 2). Structure activity analysis demonstrated that activity was retained when the position occupied by the methoxy group in Cmpd 1 was either replaced with a tertbutyl (Cmpd 4), a nitrile (Cmpd 7), or left unoccupied (Cmpd 6). Addition of a 3 pyridylmethenyl carbonate group (Cmpd 5) resulted in increased potency. An addition of a pyrimidine ring (compare Cmpds 6 and 8) or the replacement of the position 2 carbon in the pyrimidine ring of Cmpd 4 (compare Cmpds 4 and 9) resulted in loss of activity. To demonstrate further the selectivity of this series for the OPG gene, we compared the effect of Cmpd 5 (the most potent of this series) on the OPG promoter with activity on other promoters, SV40 (a generic constitutive promoter), Runx2 (a bone osteoblast specific promoter), and 6XOSE (which contains 6 copies of the Runx2 binding site, Fig. 2B). Cmpd 5 had no significant effect on transcription of any of the other promoters examined suggesting that the compound activity is specific for OPG and not likely a generalized, nonspecific effect on transcription. These results are consistent with a structure activity relationship and confirm the effect of this compound class on OPG expression.

The benzamide derivative, Cmpd 5, has been shown to inhibit histone deacetylase (HDAC) activity (Suzuki et al., 1999). Therefore, we evaluated the effects of trichostatin A and sodium butyrate, both of which are broad spectrum HDAC inhibitors, on OPG promoter activity and secretion. Although we were able to confirm the HDAC inhibitory effects of Cmpd 5 (data not shown), neither trichostatin A nor
Table 2. Chemical structure, MW and OPG stimulatory activity of Cmpd 5 and related compounds.

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<th>Compound</th>
<th>Molecular Structure</th>
<th>MW</th>
<th>OPG Promoter EC₅₀ (µM)</th>
<th>OPG Promoter (% of untreated control)</th>
<th>OPG Secretion EC₅₀ (µM)</th>
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Fig. 2. Cmpd 5 induces OPG promoter activity, but has no effect on 6XOSE, Runx2, and SV40 promoter activity. A, in UMR 106 cells, OPG promoter activity in response to Cmpds 1, 4, 5, 6, 7, 8, or 9 was determined. The TGF-β1 control for this experiment was 212.5 ± 32.0% of untreated control. B, the effects of Cmpd 5 on OPG, 6XOSE, Runx2, and SV40 promoter activity were evaluated. Stable clones of UMR 106 cells expressing the indicated promoter fused to either β-gal (OPG and SV40) or luciferase reporter genes (Runx2 and 6XOSE) were treated for 24 h and activity was indicated by relative light units. Data are expressed as percentage of untreated (vehicle) control. Each point represents the mean ± standard error of four to eight separate treatments.
sodium butyrate stimulated OPG promoter activity or protein secretion (Fig. 3, A–B) in contrast to the results with Cmpd 5 (Table 2, Fig. 2A). In the calvarial bone resorption model, Cmpd 5 but not trichostatin A or sodium butyrate, inhibited PTH-induced bone resorption (Fig. 3C). Based on these results, we concluded that the HDAC inhibitory activity of Cmpd 5 is unrelated to its activity on OPG expression and bone resorption.

**Small Molecule Inducers of OPG Secretion Inhibit Osteoclast Differentiation and Bone Resorption in Vitro.** Organ and cell cultures have been used to show that, in the bone microenvironment, osteoblast-derived OPG inhibits osteoclast formation (from hematopoietic precursors) and decreases bone resorption (Udagawa et al., 2000). Using the same strategy, we evaluated the ability of Cmpds 1 and 5 to inhibit osteoclast formation in vitro (coculture of mouse bone marrow cells and BALB/c 3T3 cells). In these studies, Cmpd 5 inhibited osteoclast differentiation and bone resorption in a concentration-dependent manner (Fig. 4A–B). The inhibitory effect of Cmpd 5 was concentration-dependent and similar to that of the positive control, sodium butyrate. In contrast, trichostatin A was less effective, with a lower IC50 value for osteoclast inhibition. These results suggest that Cmpd 5 is a potent inhibitor of osteoclast differentiation and bone resorption.

**Fig. 3.** Trichostatin A and sodium butyrate do not induce OPG promoter activity or protein secretion and do not inhibit calvarial bone resorption. A, in UMR 106 cells expressing the OPG-β-gal construct, OPG promoter activity in response to trichostatin A and sodium butyrate was determined. B, in human SaOS-2 cells, effect of treatment with trichostatin A and sodium butyrate on secretion of OPG into the culture medium was evaluated. C, effects of trichostatin A, sodium butyrate, and Cmpd 5 on PTH-induced bone resorption (ionized calcium released to the medium) in the mouse calvarial organ culture model. Cmpd 5: a = 0.3 μM, b = 3 μM, c = 30 μM; trichostatin A: a = 0.5 nM, b = 5 nM, c = 50 nM; sodium butyrate: a = 0.5 mM, b = 5 mM, c = 50 mM. Each point represents the mean and standard error of results from five separate half-calvariae.

**Fig. 4.** Effects of Cmpds 1 and 5 on osteoclast differentiation and calvarial bone resorption. A, in cocultures of murine bone marrow cells and BALB/c 3T3 cells, effects of Cmpds 1 and 5 on osteoclast differentiation (TRAP-positive multinucleated cell formation; untreated control, 335.7 ± 11.4 osteoclasts/well) was determined. The insert demonstrates OPG effects on osteoclast differentiation in this model. Each point represents the mean and standard error of results from five or six replicate wells. B, effects of Cmpds 1 and 5 on PTH-induced bone resorption (ionized calcium released to the medium) in the mouse calvarial organ culture model. Each point represents the mean and standard error of results from five separate half-calvariae.
Fig. 5. In vivo antiresorptive activity of Cmpd 5 in PTX rats. A and B, effect of Cmpd 5 (0.5–50 mg/kg/day) on PTH-induced rise in blood ionized calcium in two independent studies. C, tibial metaphyseal osteoclast numbers in the rats from B. D, serum OPG levels at the completion of the experiment represented in B. E–H, histological sections showing osteoclast numbers in the tibial metaphysis of PTX rats from B, which were treated with vehicle (E), PTH plus vehicle (F), PTH plus 5 mg/kg/day Cmpd 5 (G) or PTH plus 50 mg/kg/day Cmpd 5 (H). c = P < 0.05 versus PTX control; p = P ≤ 0.05, versus PTX-PTH control. *, indicates osteoclasts.
bone marrow cells and BALC stromal-osteoblasts) and bone resorption in calvariae treated with PTH to stimulate bone resorption. Like exogenous OPG, Cmpds 1 and 5 inhibited osteoclastogenesis (Fig. 4A). Although both compounds inhibited osteoclast formation in a concentration-dependent manner, Cmpd 5 was approximately 20-fold more potent (IC50, 0.022 versus 0.47 μM). Similarly, we also observed a concentration-dependent inhibitory effect of Cmpds 1 and 5 on PTH-induced bone resorption in calvarial organ culture (Fig. 4B) with Cmpd 5 being approximately 20-fold more potent (estimated IC50, 0.93 versus 25.76 μM).

**Cmpd 5 Inhibits Bone Resorption in Vivo.** To demonstrate in vivo efficacy, we evaluated Cmpd 5 for antiresorptive activity in parathyroidectomized (PTX) rats challenged with PTH infusion. Antiresorptive activity was quantitated by inhibition of PTH-induced hypercalcemia. As we have recently shown, PTH-induced resorption in this model is associated with reciprocal regulation of OPG and RANKL (decreased OPG and increased RANKL) (Ma et al., 2001). PTX rats were treated once a day for 3 days with Cmpd 5 and were then challenged with PTH infusion for 6 h at 2 h after injection on the 3rd day (50 h). Blood ionized calcium levels were determined before and after PTH infusion (50, 53, and 56 h). As expected, PTH challenge caused an increase in blood ionized calcium and increased osteoclast number (Fig. 5, A–C and F; data not shown). In this model, exogenous OPG blocks the effects of PTH infusion (data not shown). Cmpd 5 dose-dependently inhibited PTH-induced hypercalcemia and also inhibited basal blood ionized calcium levels to below normal range at the higher doses (15, 35, and 50 mg). The effects on basal blood ionized calcium were observed in the absence of PTH infusion (50 h). In a separate experiment with three doses of Cmpd 5 (low, medium, and high doses, Fig. 5B), we confirmed that the inhibitory effect on PTH-induced hypercalcemia was associated with decreased osteoclast number (Fig. 5, C, G, and H). At the highest dose (50 mg/kg/day), the histologic sections suggested a decrease in cellularity which might indicate toxicity (Fig. 5, F versus H). PTH decreased serum OPG levels in the PTX rats and a trend toward an increase in serum OPG in Cmpd 5-treated rats was observed (Fig. 5D, compared with PTH control); however, the effect did not reach statistical significance.

Previous studies have demonstrated that exogenously administered OPG inhibits osteoclastogenesis and bone resorption in experimental models of arthritis and tumor metastasis (Kong et al., 1999a; Capparelli et al., 2000). Therefore, we explored the ability of Cmpd 5 to inhibit tumor-induced osteoclastogenesis, osteolysis, and skeletal tumor burden in a rat model of experimental bone metastasis. Injection of rat mammary carcinoma cell line 13762 into the left ventricle of Fischer-344 rats resulted in the development of radiographically evident lytic bone lesions in the tibiae, fibulae, and femurs. Treatment with Cmpd 5, 0.1 or 1 mg/kg/day for 3 to 4 weeks significantly decreased the number and area of radiographically evident lytic bone lesions (Fig. 6, A–C). Histologic evaluation of the proximal tibiae confirmed that the decrease in lytic lesion number and area was associated with decreased osteoclastic bone resorption (data not shown). No additional clinical toxic syndromes were seen in Cmpd 5-treated rats when compared with vehicle controls during the 4-week experimental period.

Last, the efficacy of Cmpd 5 in a lipoidal amine-induced adjuvant arthritic rat model was evaluated (Benslay and Bendele, 1991; Bendele, 2001). Lipoidal amine-induced adjuvant arthritis is characterized by severe inflammation, increased osteoclast number, extensive local bone and cartilage destruction, loss of bone density/volume, and crippling. At the onset of disease (day 9), animals were treated with vehicle or Cmpd 5 (1 or 3 mg/kg) for 5 days. Cmpd 5 partially blocked the severity of inflammation as measured by paw and spleen weight (Fig. 7A). Histologically (when compared...
with control) lipoidal amine-treated animals showed a decreased trabecular area and increased osteoclast number in the calcaneum (Fig. 7, A and B). Cmpd 5 treatment partially blocked the lipoidal amine-induced decrease in trabecular area and increase in osteoclast number (Fig. 7A).

**Discussion**

We have successfully used a high-throughput screen to identify novel nonpeptidyl small molecules capable of stimulating the transcription and protein expression of OPG. Of the three compound classes identified, the benzamide chemical class represented by Cmpd 5 was shown to increase OPG expression and to mimic the antiresorptive activities normally associated with OPG both in vitro and in vivo. Osteoclast differentiation was effectively inhibited both in vitro and in vivo by Cmpd 5 and, in vivo efficacy was demonstrated in a PTH infusion model where osteoclast formation and bone resorption-induced hypercalcemia were decreased by Cmpd 5 treatment. Most importantly, efficacy was observed in two disease models in which osteoclast formation plays a critical role in accelerating bone resorption. Each of these experimental approaches, the growth of tumor deposits in bone and the periarticular bone resorption of adjuvant arthritis, have been important in establishing the therapeutic efficacy of OPG-Fc. In several different animal models, OPG-Fc treatment limits hypercalcemia and osteolysis induced by myeloma, breast or lung cancer, and reduces tumor establishment in bone (Morony et al., 1999; Capparelli et al., 2000; Croucher et al., 2001). A considerable body of evidence has established the central importance of osteoclast formation in the processes of formation and maintenance of bone metastases and the effectiveness of inhibitors of bone resorption in prevention and treatment (Mundy, 2002). The results obtained with the breast cancer-bone metastasis model indicate that stimulating OPG production locally in bone might provide an effective therapeutic approach to decreasing and/or preventing osteolysis in metastatic bone disease.

Adjuvant arthritis induced in the rat by lipoidal amine injection results in severe inflammation and bone destruction. The bone protective effect of Cmpd 5 in these experiments is consistent with its promotion of OPG production, but the mechanism for the partial inhibition of inflammation is intriguing and deserves further investigation. Since OPG neutralizes RANKL and given the important role of the RANKL/RANK pathway in immunomodulation, it is tempting to speculate that this effect may be mediated via increased OPG production. This thesis is supported in part by the finding that RANKL-null T cells have a significantly reduced production of both Th1 and Th2 cytokines (Kong et al., 1999b). By contrast, previous studies have shown in experimental models in the rat of adjuvant-induced arthritis (Kong et al., 1999a) and of collagen-induced arthritis (Romas et al., 2002) that OPG-Fc treatment at onset of disease prevents bone and cartilage destruction but not inflammation. However, the exogenous OPG used in these studies (OPG-Fc) is a shortened long-acting peptide (stabilized by the Fc fragment) and might not have all the biological properties of the endogenous full-length protein.

The results of this study provide proof of the concept that small molecular weight compounds that stimulate the OPG gene can be discovered. Additionally, we provide evidence that such molecules inhibit bone resorption in three in vivo models. Cmpd 5, which was most extensively tested, does possess HDAC inhibitory activity. Inhibition of HDAC activity induces

<table>
<thead>
<tr>
<th>A</th>
<th>Paw Weight (mg)</th>
<th>Spleen Weight (mg)</th>
<th>Trabecular area calcaneum (%)</th>
<th>Osteoclast number calcaneum (#/mm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1766.6 ± 962.9</td>
<td>559.2 ± 4.8</td>
<td>41.8 ± 10.6</td>
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<td>Lipoidal amine</td>
<td>6744.4 ± 514.4*</td>
<td>1324.0 ± 58.3*</td>
<td>11.3 ± 8.4*</td>
<td>27.6 ± 12.7*</td>
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<tr>
<td>Cmpd 5 (1 mg/kg/d)</td>
<td>5654.0 ± 384.1*</td>
<td>811.6 ± 82.5*‡</td>
<td>22.8 ± 8.0</td>
<td>27.1 ± 4.4*</td>
</tr>
<tr>
<td>Cmpd 5 (3 mg/kg/d)</td>
<td>4762.7 ± 281.2*‡</td>
<td>797.0 ± 27.4*‡</td>
<td>28.2 ± 7.4</td>
<td>10.1 ± 3.4‡</td>
</tr>
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![Lipoidal Amine control](image1.png)

![Cmpd 5-3 mg/kg/d](image2.png)

**Fig. 7.** Efficacy of Cmpd 5 in a lipoidal amine-induced adjuvant arthritis rat model. A, effects of Cmpd 5 on paw weight, spleen weight, and trabecular area and osteoclast number. *, P < 0.05 versus normal control; ‡, P < 0.05 versus lipoidal amine-treated control. B, histological section showing bone volume and osteoclast numbers in lipoidal amine-induced adjuvant arthritis with and without treatment with Cmpd 5.
histone hyperacetylation and regulates transcription of some genes through alteration in chromatin structure. HDAC inhibitors are being evaluated for treatment of cancer (Johnstone, 2002). Based on our evaluation of trichostatin A and sodium butyrate, we concluded that the effects of Cmpd 5 on OPG promoter activity and protein secretion are probably not due to inhibition of HDAC activity since trichostatin A and sodium butyrate did not mimic the effects of Cmpd 5. Additonally, trichostatin A and sodium butyrate did not inhibit calvarial bone resorption, which suggests that inhibition of HDAC activity alone is not sufficient to block bone resorption.

It is concluded that modulation of OPG in the bone microenvironment can be as effective as exogenously administered OPG-Fc in inhibiting the growth of tumor deposits in bone and the periosteal resorption of adjuvant arthritis. The described small molecules increase OPG transcription, mRNA, and protein, but it is not yet known whether these effects are explained entirely by the enhanced OPG promoter activity. Together, these data strongly support the concept that small molecules that selectively stimulate endogenous OPG expression could have therapeutic benefits similar to those of native OPG in reducing both the formation and the activity of osteoclasts. This provides a new approach to development of drugs for treatment of diseases of increased bone resorption, such as osteoporosis, Paget’s disease, osteolytic bone metastases, hypercalcemia of malignancy, and rheumatoid arthritis.

References


Gill J, Caruso K, McElhinney K,2002). Based on our evaluation of trichostatin A and sodium butyrate, we concluded that the effects of Cmpd 5 on OPG promoter activity and protein secretion are probably not due to inhibition of HDAC activity since trichostatin A and sodium butyrate did not mimic the effects of Cmpd 5. Additonally, trichostatin A and sodium butyrate did not inhibit calvarial bone resorption, which suggests that inhibition of HDAC activity alone is not sufficient to block bone resorption.

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References


