Identification of a Novel Phosphonocarboxylate Inhibitor of Rab Geranylgeranyl Transferase That Specifically Prevents Rab Prenylation in Osteoclasts and Macrophages

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Fraser P. Coxon§§, Miep H. Helfrich§, Banafshe Larijani¶, Mariusz Muzyjak**, James E. Dunford§, Deborah Marshall‡‡, Alastair D. McKinnon§§, Stephen A. Nesbitt**, Michael A. Horton**, Miguel C. Seabra†, Frank H. Eabeto∥∥, and Michael J. Rogers‡†

From the §Department of Medicine and Therapeutics, ‡‡Medical Microbiology, and §§Pathology, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom, the †Cell and Molecular Biology Section, Division of Biomedical Sciences, Imperial College School of Medicine, London SW7 2AZ, United Kingdom, the **Bone & Mineral Centre, University College, London WC1E 6JJ, United Kingdom, and ¶∥∥Procter & Gamble Pharmaceuticals, Cincinnati, Ohio 45204

Nitrogen-containing bisphosphonate drugs inhibit bone resorption by inhibiting FPP synthase and thereby preventing the synthesis of isoprenoid lipids required for protein prenylation in bone-resorbing osteoclasts. NE10790 is a phosphonocarboxylate analogue of the potent bisphosphonate risedronate and is a weak anti-resorptive agent. Although NE10790 was a poor inhibitor of FPP synthase, it did inhibit prenylation in J774 macrophages and osteoclasts, but only of proteins of molecular mass ~22–26 kDa, the prenylation of which was not affected by peptidomimetic inhibitors of either farnesyl transferase (FTI-277) or geranylgeranyl transferase I (GGTI-298). These 22–26-kDa proteins were shown to be geranylgeranylated by labelling J774 cells with [3H]geranylgeraniol. Furthermore, NE10790 inhibited incorporation of [14C]mevalonic acid into Rab6, but not into H-Ras or Rap1, proteins that are modified by FTase and GGTase I, respectively. These data demonstrate that NE10790 selectively prevents Rab prenylation in intact cells. In accord, NE10790 inhibited the activity of recombinant Rab GGTase in vitro, but did not affect the activity of recombinant FTase or GGTase I. NE10790 therefore appears to be the first specific inhibitor of Rab GGTase to be identified. In contrast to risedronate, NE10790 inhibited bone resorption in vitro without markedly affecting osteoclast number or the F-actin “ring” structure in polarized osteoclasts. However, NE10790 did alter osteoclast morphology, causing the formation of large intracellular vacuoles and protrusion of the basolateral membrane into large, “domed” structures that lacked microvilli. The anti-resorptive activity of NE10790 is thus likely due to disruption of Rab-dependent intracellular membrane trafficking in osteoclasts.

Protein prenylation is a post-translational modification involving the transfer of an isoprenoid lipid moiety from farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) to a conserved C-terminal cysteine residue contained within characteristic prenylation motifs of target proteins, mostly small GTP-binding proteins (small GTPases). Prenylation (either farnesylation or geranylgeranylation) is essential for the function of the modified proteins, because it is required both for membrane association and for specific protein-protein interactions (1). FPP and GGPP, which are sequentially produced by condensation reactions involving FPP synthase and GGPP synthase, are products of the mevalonate pathway, which is also responsible for the biosynthesis of cholesterol. The process of prenylation is carried out by one of three protein:prenyl transferase enzymes, the specificity being determined by the prenylation motif in the protein substrate. Proteins with a cysteine residue four positions from the C terminus (CAAX motif) are modified by either protein:farnesyl transferase (FTase), which farnesylates proteins such as Ras and lamina, or protein:geranylgeranyl transferase I (GGTIase I), which geranylgeranylates small GTPase proteins of the Rho family (e.g. Rho, Rac, and Cdc42); molecular mass, ~21 kDa) and others such as Rap. A distinct protein:geranylgeranyl transferase (Rab GGTase, also known as GGTase II) geranylgeranylates small GTPases of the Rab family (molecular mass, 22–26 kDa) on two C-terminal cysteine residues contained in motifs such as CXXC, CXXX, or XXCC (2). This modification also requires the participation of an additional protein, Rab escort protein (REP), which binds unprenylated Rab and presents it to Rab GGTase (3).

Several effective and specific inhibitors of both FTase and GGTase I have been developed, such as the peptidomimetic inhibitors FTI-277 and GGTI-298 (4, 5). However, a specific inhibitor of Rab GGTase has not yet been identified. Although metabolites of the monoterpene limonene are able to inhibit one or more protein:prenyl transferase, none of these specifically inhibits Rab GGTase. For example, perillyl alcohol inhibits Rab GGTase, but also GGTase I in cell-free lysates and

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† Present address: Cell Biophysics Laboratory ICRF, 44 Lincoln’s Inn Fields, London, UK.
‡ To whom correspondence should be addressed: Dept. of Medicine & Therapeutics, University of Aberdeen Medical School, Polwarth Bldg., Foresterhill, Aberdeen AB25 2ZD, UK. E-mail: f.p.coxon@abdn.ac.uk.

1 The abbreviations used are: FPP, farnesyl diphosphate; BP, bisphophonate; FTase, protein:farnesyl transferase; FTI, protein: farnesyl transferase inhibitor; GGPP, geranylgeranyl diphosphate; GGOH, all-trans geranylgeranol; GGTase, protein:geranylgeranyl transferase; REP, Rab escort protein; BIS, (2-[3-pyridinyl]-1-hydroxyethylidene-1,1-bisphophonate); SEM, scanning electron microscopy; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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intact 3T3 cells (6), whereas other monoterpenes, such as perillyl acid, inhibit FTase and GGTase I only (7).

Recently, some bisphosphate drugs have been shown to act by preventing protein prenylation (8). Bisphosphonates (BPs) are synthetic inhibitors of bone resorption, a property that has led to their use in the treatment of bone diseases characterized by excessive resorption, such as post-menopausal osteoporosis and tumor-associated bone disease (9). BPs that contain a nitrogen in the structure of one of their two side chains (10), such as risedronate (RIS), inhibit the function of bone-resorbing osteoclasts by preventing protein prenylation in these cells because of inhibition of FPP synthase (11-13). This results in the depletion of FPP and GGPP required for prenylation of small GTPase proteins that are essential for osteoclast function. Loss of geranylgeranylated proteins appears to be the cause of the anti-resorptive effects of these BPs on osteoclasts, because loss of osteoclast function can be overcome by the addition of geranylgeraniol, which bypasses inhibition of FPP synthase and replenishes the cells with a substrate for protein geranylgeranylation (14, 15). In addition, we have suggested that bisphosphonates disrupt osteoclast function as a result of loss of geranylgeranylation of small GTPases by GGTase I, such as Rho, Rac, and Cdc42, because an inhibitor of GGTase I (GGTI-298) closely mimics the effects of BPs (16). Because of the lack of a specific inhibitor of Rab GGTase, the effect of loss of prenylation of Rab small GTPases in osteoclasts could not be assessed. There is evidence that some bisphosphonates and their analogues may be able to inhibit other enzymes of the mevalonate pathway. Some nitrogen-containing BPs can also inhibit squalene synthase (17, 18), which is involved in the synthesis of cholesterol and also uses FPP as a substrate. Furthermore, a recent study has demonstrated that certain bisphosphonate analogues of FPP are able to inhibit FTase (19).

NE10790 (see Fig. 1A) is an analogue of the nitrogen-containing BP RIS, in which one of the phosphate groups is replaced with a carboxylate group (20). NE10790 retains the ability to inhibit bone resorption in vivo, although its anti-resorptive potency in rodents is markedly reduced compared with RIS (20). At least part of this loss of potency is due to the fact that NE10790 has reduced affinity for bone, because the loss of one of the phosphate groups allows binding of only one calcium ion (21). However, it remains unclear whether this compound is also less effective at affecting osteoclast function at the cellular level or indeed whether it inhibits bone resorption by the same molecular mechanism as nitrogen-containing BPs (that is, by inhibition of FPP synthase). NE10485 (see Fig. 1A) is an analogue of NE10790 in which the nitrogen of the heterocyclic group is methylated and the hydroxyl group attached to the central carbon is replaced with hydrogen. The anti-resorptive potency of this compound has not been characterized.

In this study we demonstrate that NE10790 is a poor inhibitor of FPP synthase and does not inhibit protein prenylation indiscriminately. Rather, this compound selectively prevents the geranylgeranylation of Rab small GTPases in several cell types in vitro, including osteoclasts, as a result of specific inhibition of Rab GGTase.

**EXPERIMENTAL PROCEDURES**

**Reagents**—NE10790, NE10485, and RIS (see Fig. 1A) were provided by Procter and Gamble Pharmaceuticals (Cincinnati, OH). The drugs were dissolved in PBS, and the pH was adjusted to 7.4 with 1 N NaOH and then filtered-sterilized by using a 0.2-μm filter. Mevastatin was purchased from Sigma and converted from the lactone as described by Luckman et al. (8). [3H]GGPP was from American Radiochemicals Ltd. (St Louis, MO). [14C]Mevalonic acid lactone, Enhance reagent, [1-3H]trans-PPP, and [1-3H]trans-GGPP were purchased from PerkinElmer Life Sciences. Solvent was removed from [3H]GGPP and [14C]mevalonic acid lactone by evaporating in nitrogen. Recombinant human K-Ras, Rho, Rab1a, Rab escort protein (REP1) and recombinant human FTase, GGTase I, and Rab GGTase were purified as described previously (22). All other reagents were from Sigma unless stated otherwise.

**MTA Method of Viable Cell Number by MTT Assay**—The number of viable J774 macrophages was determined by MTT assay as previously described (23). J774 cells were seeded at a density of 10^6 cells/well into 96-well plates and then treated with RIS, NE10790, or NE10485 for the following day in replicates of six wells. 48 h later, the reduction of MTT reagent was measured.

**Inhibition and Purification of Rabbit Osteoclasts**—Mature osteoclasts were isolated from 2-day-old New Zealand White rabbits as previously described (16). Briefly, the long bones from each rabbit were removed and minced in α-minimum essential medium (Life Technologies, Inc.). After allowing the bone pieces to settle, the supernatant was transferred to a fresh tube, and fetal calf serum was added to a final concentration of 10%. The cells were seeded into 6-well or 96-well plates (Costar, Cambridge, MA). The following day, contaminating adherent cells in 6-well plates were removed by treatment with 0.001% Pronase, 0.002% EDTA in PBS. The remaining adherent cells (>95% tartrate-resistant acid phosphatase-positive osteoclasts) were rinsed twice in PBS and then cultured in fresh α-minimum essential medium containing 10% fetal calf serum plus treatments under investigation.

**Bisphosphonate and [14C]Mevalonate Into Prenylated Proteins in Intact Cells**—Detection of prenylated proteins in J774 macrophages and purified rabbit osteoclasts was carried out as described previously (16, 24). Briefly, the cells were depleted of mevalonate by incubation with 5 μM mevastatin for 4 h and then transferred into fresh medium containing 5 μM mevastatin and either 7.5 μCi/ml [14C]mevalonic acid lactone or 30 μCi/ml [1H]GGPP, plus RIS, NE10790, NE10485, FTI-277, or GGTI-298. After 18 h the cells were lysed in RIPA buffer (150 mM NaCl, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate in PBS, plus 1% (v/v) Sigma protease inhibitor mixture), and then 50 μg of cell lysate from each treatment were electrophoresed on 12% polyacrylamide-SDS gels under reducing conditions. After electrophoresis, the gels were fixed in 10% (v/v) acetic acid, 40% (v/v) methanol, 50% (v/v) distilled water, and then 1C-labeled gels were dried, and labeled proteins were visualized on a Bio-Rad Personal FX Imager after exposure to a Kodak phosphorimaging screen. H-Labeled gels were incubated in Enhance for 30 min prior to drying. H-Labeled proteins were then visualized by exposing the gel to preflashed Hyperfilm-MP (Amersham Pharmacia Biotech) for 6 days at 70°C.

**Inmunoprecipitation of [14C]Mevalonic Acid-labeled Small GTPases**—J774 cells were seeded at 2 × 10^6 cells/well in a 6-well plate and then metabolically labeled the following day with [14C]mevalonic acid as described above. Whole cell lysates were prepared in 1 ml of RIPA buffer and assayed for protein content, and then equal amounts of protein (~500 μg) were incubated with 2 μg of rabbit polyclonal Rab6 antibody or 1 μg/ml goat polyclonal Rap1A antibody (Santa Cruz Bio- technology, Santa Cruz, CA) at 4°C for 1 h. 20 μl of protein A-Sepharose were then added, and incubation was continued overnight. For immunoprecipitation of Ras, lysates were incubated at 4°C overnight with 20 μl of a-Ras antibody conjugated to agarose beads (Oncogene Science, Manhasset, NY). The beads were pelleted by centrifugation at 2,500 rpm for 5 min in a microcentrifuge and washed four times in RIPA buffer. 20 μl of 2% Laemmli sample buffer were added to each Sepha- rose pellet and boiled for 5 min, and the entire sample was electrophoresed on 12% polyacrylamide-SDS gels. Radiolabeled bands were then visualized as described above.

**Western Blot Analysis of Rab 6 and Unprenylated Rap1A**—Purified rabbit osteoclasts and J774 macrophages were treated for 24 h and then lysed in RIPA buffer. After determining the protein content, 20 μg (J774 lysate) or 60 μg (osteoclast lysate) from each sample were electrophoresed under reducing conditions on 12% polyacrylamide-SDS gels. The proteins were transferred on to polyvinyl difluoride membrane by semidry transfer and blocked overnight with 5% (w/v) skimmed milk in TBS with Tween 20. Membranes were then hybridized with 0.5 μg/ml rabbit polyclonal Rab6 antibody or 1 μg/ml goat polyclonal Rap1A antibody (Santa Cruz Biotechnology), at 4°C for 1 h. 20 μg/ml of protein A-Sepharose were then added, and incubation was continued overnight. For immunoprecipitation of Ras, lysates were incubated at 4°C overnight with 20 μl of a-Ras antibody conjugated to agarose beads (Oncogene Science, Manhasset, NY). The beads were pelleted by centrifugation at 2,500 rpm for 5 min in a microcentrifuge and washed four times in RIPA buffer. 20 μl of 2% Laemmli sample buffer were added to each Sepha- rose pellet and boiled for 5 min, and the entire sample was electrophoresed on 12% polyacrylamide-SDS gels. Radiolabeled bands were then visualized as described above.

**FPP synthase assay—**FPP synthase was assayed as described previously (11). Briefly, 40 μl of assay buffer (50 mM Tris, pH 7.7, 10 mM NaF, 2 mM MgCl2, 1 mM bovine serum albumin, 0.5 mM dithiothreitol) containing 2 nmol [1-3H]isopentenyl diphosphate (4 μCi/nmol) and 2 nmol GPP were prewarmed to 37°C. The assay was initiated by the
addition of 1 μl of recombinant human FPP synthase (with an activity of 8 pmol FPP/min) diluted to 10 μl with assay buffer. The assay was allowed to proceed for 30 min and was terminated by the addition of 200 μl of saturated NaCl. The samples were then extracted with 1 ml of water-saturated butan-1-ol, and the amount of radioactivity in the upper phase was measured by mixing 0.5 ml of the butanol phase with 4 ml of general purpose scintillant. This was then counted using a Packard Tricarb 1900CA scintillation counter. To determine the effects of NE10790, RIS, and NE10485 on FPP synthase activity, the compounds were diluted to 5× final concentration in assay buffer and were preincubated with the enzyme preparation for 10 min prior to initiation of the reaction.

**Protein:Farnesyl Transferase Assay**—The activity of recombinant human farnesyl transferase was determined by assessing the amount of [3H]farnesyl transferred from [3H]FPP to recombinant K-Ras (22). The final concentrations of reagents in a standard reaction mix were 50 mM Tris-Cl, pH 7.2, 150 mM KCl, 5 mM MgCl₂, 0.3 mM Nonidet P-40, 1 mM dithiothreitol, 5 mM GGPP, 0.5 μM [3H]GGPP (15–30 Ci/mmol), 58 mM GGTase I, and 10 μM RhoA. Final concentrations in the Rab GGTase reaction mix were 25 mM Tris, pH 7.2, 5 mM MgCl₂, 1 mM dithiothreitol, 3.6 μM GGPP, 0.4 μM [3H]GGPP, 0.04 μM Rab GTase, 10 μM Rab1a, and 2.5 μM REP1. The assay was carried out as outlined above for the FTase assay, including a negative control (lacking REP1) for the Rab GGTase assay. The assays were in duplicate and were repeated three times independently to verify reproducibility.

**Protein:Geranylgeranyl Transferase Assay**—The activity of recombinant human GGTase I and Rab GGTase were determined by assessing the amount of [3H]geranylgeranylated transferred from [3H]GGPP to Rho A and Rab1a, respectively (22). The final concentrations in the GGTase I reaction mix were 50 mM Tris-Cl, pH 7.2, 150 mM KCl, 5 mM MgCl₂, 0.3 mM Nonidet P-40, 1 mM dithiothreitol, 5 mM GGPP, 0.5 μM [3H]GGPP (15–30 Ci/mmol), 58 mM GGTase I, and 10 μM RhoA. Final concentrations in the Rab GGTase reaction mix were 25 mM Tris, pH 7.2, 5 mM MgCl₂, 1 mM dithiothreitol, 3.6 μM GGPP, 0.4 μM [3H]GGPP, 0.04 μM Rab GTase, 10 μM Rab1a, and 2.5 μM REP1. The assay was carried out as outlined above for the FTase assay, including a negative control (lacking REP1) for the Rab GGTase assay. The assays were in duplicate and were repeated three times independently to verify reproducibility.

**Analysis of Osteoclast Polarization and Resorption**—Osteoclast number, F-actin “rings,” and resorptive activity of mature rabbit osteoclasts in vitro were assessed as described previously (16). Briefly, rabbit osteoclasts were allowed to adhere for 2 h on 5-mm-diameter elephant tusk dentine discs in 96-well plates and then cultured with fresh α-minimum essential medium in the presence or absence of RIS, NE10790, or NE10485. The cultures were fixed for 10 min in a 1:1 (v:v) mixture of minimum essential medium and fixation buffer (3.5% (w/v) paraformaldehyde, 2% (w/v) sucrose in PBS). The samples were then critical point dried from CO₂, glued onto aluminum stubs with colloidal silver adhesive, and sputter coated with 20 nm platinum and examined in a Jeol JSM-35CF scanning electron microscope operating at 10 kV.

To quantify the results, osteoclasts throughout the whole dentine disc were identified and scored according to their morphology. “Normal osteoclasts were defined as those that were well spread, with numerous microvilli on the basolateral surface (the membrane not apposed to the bone surface). “Retracted” osteoclasts were defined as those in which the peripheral membrane adjacent to the dentinal surface was completely retracted or showed evidence of retraction fibers. “Domed” osteoclasts were defined as those in which one or more regions of the basolateral membrane were both raised and devoid of microvilli.

**Assessment of Osteoclast Morphology by Immunostaining and Confocal Microscopy**—For morphological studies, rabbit osteoclasts were cultured as above for 48 h on dentin slices without reagents or with 10–100 μM RIS or 500–1000 μM NE10790. The cultures were fixed for 10 min in a 1:1 (v:v) mixture of minimum essential medium and fixation buffer (3.5% (w/v) paraformaldehyde, 2% (w/v) sucrose in PBS). The cells were then permeabilized in Triton buffer (20 mM Heps, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) Triton X-100, 0.5% (v/v) sodium azide, in PBS at pH 7.0) at 4 °C and then immunostained for paxillin using a mouse monoclonal antibody (10 μg/ml) and secondary fluorescein isothiocyanate-conjugated anti-mouse Ig polyclonal antibodies (1:40 dilution) (Dako, Denmark). Resorbing osteoclasts were identified by their characteristic F-actin ring structure (described above) after staining with TRITC-phalloidin conjugate (Molecular Probes) at 5
RESULTS

**NE10790 Reduces Viability in J774 Cells**—RIS dramatically and dose-dependently reduced the number of viable J774 cells with an IC\textsubscript{50} of approximately 30 \mu M (Fig. 1B). NE10790 also reduced viable J774 cell number but was ~40 times less potent than RIS (IC\textsubscript{50} ~1.2 \mu M). By contrast, NE10485 had no effect on cell viability at concentrations up to 3 \mu M.

**NE10790 Is a Weak Inhibitor of FPP Synthase**—RIS potently inhibited recombinant human FPP synthase in vitro with an IC\textsubscript{50} value of 10 \mu M \pm 10 \mu M (n = 4; Fig. 1C). By contrast, neither NE10790 nor NE10485 affected FPP synthase at concentrations up to 100 \mu M, although at higher concentrations up to 1 mM, both NE10790 and NE10485 partially inhibited (30–50%) FPP synthase activity in vitro.

**NE10790 Prevents Prenylation of 22–26-kDa Proteins That Are Not Modified by FTase or GGTase I**—The effect of NE10790, RIS, and NE10485 on protein prenylation was investigated by examining the incorporation of \[^{14}C\]mevalonic acid into prenylated proteins in J774 cells in vitro. Whereas 100 \mu M RIS inhibited the incorporation of \[^{14}C\]mevalonic acid into all prenylated small GTPases (21–26 kDa) and higher molecular mass proteins (~60 kDa, most likely farnesylated lamin B and prelamin A), 1.5 mM NE10790 inhibited incorporation of \[^{14}C\]mevalonic acid into bands of prenylated small GTPases of higher molecular mass only (22–26 kDa proteins; most likely Rab GTPases based on molecular mass) (Fig. 2A). The effect of NE10790 was dose-dependent, with complete inhibition of incorporation of \[^{14}C\]mevalonic acid into 22–26-kDa proteins at a concentration of 1 mM (Fig. 2B). The IC\textsubscript{50} for inhibition of Rab prenylation in J774 cells was calculated from densitometric analysis of the 22–26-kDa bands and was determined to be 560 \pm 120 \mu M (n = 3). The intense radiolabel at the dye front, which probably represents isoprenoids such as GGPP (8), was unaffected by treatment with NE10790 but was completely absent in lysates of RIS-treated cells (Fig. 2A). 1.5 mM NE10485 had no effect on either protein prenylation or the abundance of radiolabeled isoprenoids at the dye front (Fig. 2A).

The effect of NE10790 on incorporation of \[^{14}C\]mevalonic acid into prenylated proteins in J774 cells was compared with the effects of peptidomimetic inhibitors of FTase and GGTase I. An FTase inhibitor, FTI-277 (10 \mu M), prevented incorporation of \[^{14}C\]mevalonic acid into bands of around 60 kDa (farnesylated lamins), whereas 15 \mu M GGTTI-298, an inhibitor of GGTase I, reduced incorporation of \[^{14}C\]mevalonic acid into a broad band with a molecular mass of 21 kDa, most likely consisting of geranylgeranylated Rho family proteins (Fig. 2C). Treatment with a combination of FTI-277 and GGTTI-298 (i.e. inhibition of FTase and GGTase I) prevented the incorporation of \[^{14}C\]mevalonic acid into all protein bands except for the...
of lowest molecular mass, whereas the two bands of higher molecular mass were more intense than in the control (Fig. 3A). Conversely, treatment with 1.5 mM NE10790 completely inhibited the incorporation of [3H]GGGHOH into the two bands of higher molecular mass (22–26 kDa) (that is, the only bands that were not affected by GGTI-298), whereas the bands of lower molecular mass appeared slightly more intense than in the control. By contrast, neither 1.5 mM NE10485 nor 100 μM RIS (a concentration that completely inhibits the incorporation of [14C]mevalonate into prenylated proteins; Fig. 2A) had any effect on the incorporation of [3H]GGGHOH into geranylgeranylated proteins in J774 cells (Fig. 3A). These observations demonstrate that, in intact cells, NE10790 prevents geranylgeranylation of proteins that are substrates for Rab GGTase I but does not affect prenylation of proteins that are substrates for Rab GGTase I.

To further confirm that NE10790 does not inhibit the prenylation of substrates for GGTase I, cell lysates of J774 cells were analyzed by Western blotting using an antibody that specifically recognizes the unprenylated form of Rap1A (26), a substrate for GGTase I. Unprenylated Rap1A was absent in untreated J774 cells but accumulated markedly in cells after treatment with either 100 μM RIS or 15 μM GGTI-298 for 24 h (Fig. 3C). However, neither NE10790 nor NE10485 caused accumulation of unprenylated Rap1A. Similarly, treatment of purified rabbit osteoclasts for 24 h with 100 μM RIS or 10 μM GGTI-298 caused accumulation of unprenylated Rap1A, whereas 1 mM NE10790 had no effect (Fig. 3D).

NE10790 Prevents Prenylation of Rab6 but Not Rap1 or Ha-Ras—The effect of NE10790 on prenylation of substrates
for FTase, GGTase I, and Rab GGTase in intact J774 cells was investigated by specifically immunoprecipitating Ha-Ras, Rap1, and Rab6 from lysates of cells that had been metabolically labeled with [14C]mevalonic acid. Neither 1.5 mM NE10790 nor 15 μM GGTI-298 markedly affected the incorporation of [14C]mevalonic acid into Ha-Ras (a substrate for FTase) (Fig. 4A). Geranylgeranylation of Rap1 (a substrate for GGTase I) was not affected by treatment of cells with NE10790 but was reduced by treatment with GGTI-298 (an inhibitor of GGTase I). By contrast, geranylgeranylation of Rab6 (a substrate for Rab GGTase) was completely inhibited by NE10790 but was not affected by GGTI-298 (an inhibitor of GGTase I). By contrast, geranylgeranylation of Rab6 (a substrate for Rab GGTase) was completely inhibited by NE10790 but was not affected by GGTI-298 (an inhibitor of GGTase I). NE10790 specifically prevented the prenylation of Rab6 but not Rap1 or Ha-Ras. To exclude the possibility that NE10790 could decrease the synthesis of Rab proteins rather than directly affect their prenylation, cell lysates were analyzed by Western blotting using the anti-Rab6 antibody. The amount of Rab6 protein present in J774 cells or osteoclasts was unaffected by treatment with NE10790 or GGTI-298 (Fig. 4B).

**NE10790 Inhibits Rab GGTase in Vitro**—To confirm that NE10790 prevents Rab prenylation by inhibiting Rab GGTase, we used an in vitro assay to measure the transfer of [3H]GGPP to Rab1a by recombinant human Rab GGTase. NE10790 dose-dependently inhibited the activity of Rab GGTase at concentrations >100 μM, with an IC50 of ~600 μM (Fig. 5A). By contrast, neither RIS nor NE10485 inhibited geranylgeranylation of Rab1a by Rab GGTase at concentrations up to 3 mM (Fig. 5A). To confirm that NE10790 is a selective inhibitor of Rab GGTase, we investigated the ability of NE10790 to affect prenylation of K-Ras and Rho A by FTase and GGTase I, respectively. Concentrations of NE10790 up to 1.2 mM, which inhibited Rab GGTase activity, had no effect on either FTase or GGTase I activity in vitro (Fig. 5B).

**NE10790 Inhibits Bone Resorption without Affecting Actin Organization**—The effect of NE10790, RIS, and NE10485 on actin organization in osteoclasts seeded onto dentine discs was determined by counting the number of osteoclasts with actin rings, a characteristic feature of resorbing osteoclasts. Typically, ~50 osteoclasts on each dentine disc exhibited actin rings; this represents ~30–50% of the total number of osteoclasts with actin rings, a characteristic feature of resorbing osteoclasts. Typically, ~50 osteoclasts on each dentine disc exhibited actin rings; this represents 30–50% of the total number of osteoclasts with actin rings, 100 μM completely disrupting these structures (Fig. 6). RIS also dose-dependently reduced resorption pit area, an effect that closely matched the reduction in actin ring number. Osteoclasts
number was less markedly reduced, by only 20% at a concentration of 100 μM RIS. By contrast, NE10790 inhibited resorption at concentrations (0.5–1 mM) that did not affect the number of actin rings or the number of osteoclasts (Fig. 6), although 1 mM NE10790 had subtle effects on the structure of the actin rings, some of which appeared less dense with distinct F-actin containing visible podosomes (see Fig. 8, A versus C and H). At a higher concentration of 1.5 mM, NE10790 reduced both the number of actin rings and the number of adherent osteoclasts, suggesting a toxic effect at this concentration. NE10485 had little effect on actin ring number, osteoclast number, or resorption pit area at concentrations up to 1.5 mM (Fig. 6).

**NE10790 Causes Morphological Changes in the Basolateral Membrane of Osteoclasts**—The effects of NE10790 and RIS on osteoclast morphology were examined by SEM of osteoclasts cultured on dentine discs (Fig. 7). The majority of untreated cells or cells treated with NE10485 (>90%) were attached and were well spread on the dentine surface, and many of these were in the process of forming a resorption pit (typical cell shown in Fig. 7A, left panel). A small proportion (5–7%) of osteoclasts in untreated cultures and cultures treated with 1 mM NE10790 or NE10485 showed signs of membrane retraction (Fig. 7B). However, following treatment with 1 mM NE10790, ~12% of osteoclasts developed raised, dome-shaped basolateral surfaces devoid of ruffles (typical cell shown in Fig. 7A, right panel), features that were rarely seen in untreated osteoclasts or osteoclasts treated with 20 μM RIS or 1 mM NE10485. These domed osteoclasts remained spread and did not show signs of membrane retraction, unlike cultures treated with RIS in which ~40% showed signs of membrane retraction (Fig. 7B, typical retracting cell shown in Fig. 7A, middle panel). Furthermore, domed osteoclasts were usually associated with a smaller resorption pit than normal osteoclasts.

**NE10790 Causes the Formation of Large Intracellular Vacuoles in Osteoclasts**—Standard confocal zx images confirmed that osteoclasts treated with 1 mM NE10790 for 48 h had extensive basolateral membrane protrusions, which occurred in up to 55% of cells, whereas such structures were only infrequently seen in untreated osteoclasts or osteoclasts treated with 100 μM RIS (Fig. 8, D compared with B and F). The same features could be identified en face in xy images where the basolateral membrane domes are seen as empty or paxillin-containing structures enclosed by F-actin (typical examples are shown in Fig. 8, compare C and G with A and E). Osteoclasts containing these structures maintained an intact F-actin ring at the apical membrane (Fig. 8, C and H), although these were often less dense than in control cells (e.g., Fig. 8, H compared with A). By contrast, 100 μM RIS caused a breakdown of the actin ring, with the appearance of dispersed dot-like podosomes throughout the cell (Fig. 8E). The disparity between the number of osteoclasts displaying domed morphology detected by confocal analysis and SEM (55% versus 12% respectively) is probably due to differences in the way this morphology was detected. Osteoclasts scored as domed by SEM analysis had lost surface microvilli in addition to a raised basolateral surface (Fig. 7), whereas confocal analysis detected only osteoclasts with large vacuoles and a raised basolateral surface. The quantitation of domed osteoclasts by SEM is therefore likely to be an underestimate of osteoclasts with large intracellular vacuoles, because osteoclasts treated with NE10790 developed large vacuoles while retaining microvilli (see below).

In TEM sections of rabbit osteoclasts cultured on dentine discs, both untreated (Fig. 9A) and NE10790-treated (Fig. 9B) osteoclasts were closely apposed to the mineral surface, indicating that adhesion to the substrate was not affected by NE10790 (consistent with the lack of effect of NE10790 on actin ring integrity). Untreated osteoclasts contained small vacuoles, which were concentrated near the ruffled border (the site of resorption; Fig. 9A, arrowhead). By contrast, some osteoclasts that had been treated with 1 mM NE10790 contained one or more large, membrane-bound vacuoles, which were not observed in untreated osteoclasts (Fig. 9B). In the osteoclast shown in Fig. 9B, microvilli were still present on the basolateral surface, suggesting that the development of large vacuoles occurred prior to the appearance of the smooth, domed basolateral membranes observed by SEM (Fig. 7). This osteoclast also lacks a ruffled border membrane (arrow) and does not resorb the mineral substrate.

**DISCUSSION**

We and others recently demonstrated that nitrogen-containing BP drugs (such as RIS), widely used for the treatment of metabolic bone diseases, are inhibitors of FPP synthase and thus prevent the formation of FPP and GGPP that are required for protein prenylation (11–14). RIS therefore indirectly prevents the prenylation of both farnesylated and geranylgeranylated proteins (8). In this study, we show that NE10790 (a phosphonocarboxylate analogue of RIS) inhibits the prenylation of a subset of proteins with molecular masses of 22–26 kDa without affecting the prenylation of proteins with molecular masses of 21 kDa. These 22–26-kDa prenylated proteins were the only ones unaffected by treatment of cells with FTI-277 or GTTI-298, specific peptidomimetic inhibitors of FTase and GGTTase I, respectively (4, 5). These data strongly suggest that NE10790 specifically inhibits the prenylation of Rab proteins, because the molecular mass of Rab GTPases corresponds to the molecular mass of the proteins affected by NE10790, and Rab proteins are the only prenylated small GTPases that are not modified by either FTase or GGTTase I. This conclusion was supported by metabolically labeling cells with [3H]GGOH to specifically identify proteins that are geranylgeranylated by GGTTase I and Rab GTGTase. In J774 macrophages and rabbit osteoclasts, NE10790 inhibited the incorporation of [3H]GGOH into the only proteins (22–26 kDa) that were not affected by GTTI-298, confirming that NE10790 inhibits prenylation of proteins modified by Rab GTGTase (i.e., Rab GTPases) but does not affect proteins prenylated by GGTTase I. In support of this, NE10790 had no effect on the prenylation of Rap1A (a GGTTase I substrate) in J774 cells or osteoclasts, whereas RIS and GTTI-298 caused accumulation of unprenylated Rap1A.

The specific effect of NE10790 on Rab GTGTase was further demonstrated by examining the prenylation of proteins modified by FTase, GGTTase I, and Rab GTGTase (Ha-Ras, Rap1, and Rab6, respectively). As expected, GTTI-298 inhibited the prenylation of Rap1 but did not affect either the prenylation of Rab6 or the prenylation of Ha-Ras. By contrast, NE10790 completely inhibited the prenylation of Rab6 without markedly affecting the prenylation of Ha-Ras or Rap1 in J774 cells. Therefore, NE10790 effectively and specifically inhibits Rab GTGTase and prevents Rab prenylation in intact cells.

The ability of NE10790 to inhibit Rab GTGTase without affecting either FTase or GGTTase I in vitro was confirmed using assays with recombinant enzymes. We observed in vitro a relatively weak inhibition (at least, under the in vitro assay conditions we used, IC50 was ~600 μM of Rab GTGTase, a value surprisingly similar to the IC50 in cultured cells (560 μM)). Although NE10790 was able to inhibit recombinant FPP synthase in vitro at these concentrations, it did not inhibit FPP synthase activity in intact cells, because inhibition of this enzyme would also inhibit the formation of isoprenoid lipids and prevent prenylation of all small GTPases (as with RIS, a potent inhibitor of FPP synthase (11)). Clearly, NE10790 did not affect the prenylation of 21-kDa small GTPases (Rap1A, Ha-Ras, or...
FIG. 7. NE10790 causes morphological changes at the basolateral membrane of osteoclasts. Rabbit osteoclasts were seeded onto dentine discs and then treated in the presence or absence of 20 μM RIS or 1 mM NE10790 for 48 h, and then the morphology of the cells was analyzed by scanning electron microscopy. A, representative images of individual osteoclasts. Bar, 10 μm. Control shows a typical actively resorbing osteoclast, RIS shows a typical retracting osteoclast, and NE10790 shows a domed osteoclast (note lack of microvilli on the basolateral surface). Asterisks denote areas of resorption, which can be distinguished by their irregular surface compared with the smooth unresorbed dentine; arrows indicate the basolateral surface of the osteoclasts. B, osteoclasts on each disc were scored according to their morphology: normal, retracted, or domed, as shown in A. The results are the means ± S.E. of three independent experiments.

Fig. 8. NE10790 causes the formation of multiple large vacuoles within osteoclasts. Rabbit osteoclasts were cultured on dentine slices for 48 h in the presence of no additional compounds (A and B), 1 mM NE10790 (C, D, G, and H), or 100 μM RIS (E and F) and stained for F-actin with phalloidin-TRITC (red) and paxillin (green); the dentine surface was identified by reflection imaging (not shown), the surface being marked in the zx sections (B, D, and F) by arrows. A, C, and E are extended focus images; B, D, F, G, and H are 1-μm sections. Untreated cells have a typical dense F-actin ring structure (arrowheads in A and B) associated with a resorbing cell (see dentine excavation below the level of the dentine surface in B). Treatment with NE10790 caused characteristic alterations in osteoclasts (C, D, G, and H). A particular feature was the presence of dome-like protrusions in the basolateral membrane (marked with an asterisk in the zx section, D), which could also be identified in xy views as sharply defined empty and paxillin-filled areas encompassed by F-actin (asterisks in C and G). Whereas NE10790-treated osteoclasts maintained an intact actin ring (arrowheads in C and H), RIS caused a breakdown of the actin ring with the appearance of numerous dot-like podosomes (star in E shows podosomes with co-localized actin and paxillin as yellow) and dissociation of paxillin from actin (seen as “clumps” of actin, in red, in E and F).

Fig. 9. Transmission electron microscopy analysis of the effect of NE10790 on osteoclast ultrastructure. Rabbit osteoclasts were seeded onto dentine discs and then cultured in the presence or absence of 1 mM NE10790 for 48 h. Ultrathin sections were then cut, and osteoclasts were analyzed by transmission electron microscopy. Representative images of osteoclasts are shown. A, control; B, 1 mM NE10790. The arrowhead in A shows the ruffled border beneath which resorption has occurred. The arrowhead in B shows the absence of the ruffled border in the apical membrane of this osteoclast. Bar, 10 μm.

Rap1) or the formation of isoprenoid lipids, which migrated with the dye front when lysates from [14C]mevalonic acid-labeled J774 cells and osteoclasts were analyzed by SDS-poly-
sary for the reaction (such as GGPP and REP) in the \textit{in vitro} assay could be much greater than their concentration within cells. Alternatively, the \textit{in vitro} assay could be lacking some other factor that contributes to inhibition of Rab GGTase by NE10790. Future studies are intended to address this issue.

Inhibition of Rab prenylation in J774 cells was associated with a decrease in viable cell number, most likely as a result of decreased cell proliferation rather than increased cell death, because NE10790 had little effect on J774 apoptosis at concentrations that inhibited Rab prenylation (data not shown). RIS was considerably more potent than NE10790 at reducing J774 cell viability, whereas NE10485 (which did not inhibit protein prenylation) had no effect on J774 cells. NE10790 was also ~100 times less potent than RIS at inhibiting bone resorption by rabbit osteoclasts \textit{in vitro}, in agreement with another \textit{in vitro} study using organ cultures of bone (21). However, unlike RIS, NE10790 had little effect on the actin cytoskeleton of osteoclasts. Concentrations of RIS that inhibited resorption of dentine disrupted F-actin rings in osteoclasts and caused these cells to retract. By contrast, concentrations of NE10790 that inhibited resorption (0.5–1 mM) had little effect on F-actin organization at the mineral surface, with no significant reduction in the number of F-actin rings. This is consistent with the evidence that regulation of the osteoclast cytoskeleton, required for bone resorption, involves small GTPases prenylated by GGTase I, such as Rh and Rac (16, 27–29). NE10790 specifically inhibits Rab prenylation by Rab GGTase, demonstrating for the first time that Rab proteins are also required for osteoclastic bone resorption. Although high concentrations (1.5 mM) of NE10790 did reduce the actin ring number, this appeared to be a toxic effect, because the number of adherent osteoclasts was also reduced at this concentration. At present it is unclear whether this toxicity was due to loss of Rab prenylation or to disruption of other aspects of osteoclast function.

Analysis of cell morphology by SEM demonstrated that NE10790 caused profound morphological changes in osteoclasts. In a proportion of osteoclasts, the basolateral surface became raised into single or multiple dome shapes and was completely devoid of the microvilli that were seen on the surface of all untreated osteoclasts. Confocal and TEM analysis suggested that these dome structures were the result of the formation of multiple, large vacuoles within the cell. Because Rab proteins play a fundamental role in vesicular trafficking, it is tempting to speculate that the formation of these vacuoles occurred as a direct consequence of the loss of prenylation of Rab proteins, resulting in aberrant vesicular fusion intracellularly. In osteoclasts, normal membrane trafficking is likely to be tightly controlled and to be essential for formation of the ruffled border (30) and for trafficking of intracellular vesicles containing proteins involved in the resorptive process, such as the vacuolar H+–ATPase, which is responsible for acidification beneath the osteoclast, and cathepsin K, a proteolytic enzyme important for the degradation of collagen. A role for Rab proteins in the formation of the ruffled border is supported by the demonstration that Rab3, which is expressed in both osteoclasts and their precursors, is associated with the vacuolar H+–ATPase and c-Src (31), and that Rab7, which is known to be involved in lysosome biogenesis (32), has been localized to the ruffled border within osteoclasts (33).

To our knowledge, NE10790 is the first specific inhibitor of Rab prenylation to be identified. Other inhibitors of Rab GGTase, such as the monoterpene perillyl alcohol, are less specific and also affect GGTase I and FTase (6, 7). NE10790 will therefore be a useful tool for further characterization of the role that Rab proteins play in cellular processes necessary for resorption by osteoclasts. Furthermore, the ability of NE10790 to specifically inhibit Rab prenylation in J774 macrophages, osteoclasts, and MC3T3 osteoblast-like cells suggests that this compound will be useful for examining the role of Rab proteins in a variety of cell types \textit{in vitro}. Until now, the only insights into the consequences of loss of Rab prenylation have come from studies on the effect of genetic mutations in the Rab prenylation machinery. For example, a mutation in REP1 leads to a retinal degenerative disease called choroideremia (34). The loss of REP1 is partially compensated by the homologous REP2 protein but results in a selective defect in prenylation of a subset of Rab proteins (35). In addition, mutations in the yeast REP (mrs6-2) results in reduced prenylation of Rab proteins, causing failure of the polarized transport of vesicles toward the bud (36). Finally, mice with a mutation known as \textit{gunmetal} have a 4-fold decrease in Rab GGTase activity in platelets as a result of a single base substitution that disrupts the splicing of the \textit{Rabgga} mRNA (37). These mice exhibit prolonged bleeding, thrombocytopenia, and reduced platelet contents, suggesting that Rab GGTase could be a novel target for the treatment of clotting disorders such as myocardial infarction and stroke.

The mechanism by which NE10790 inhibits Rab GGTase remains to be determined. Because the closely related analogue NE10485 (which differs only in that the nitrogen in the side chain is methylated and the hydroxyl group attached to the geminal carbon atom is replaced by a hydrogen) did not inhibit Rab GGTase and had no effect on protein prenylation, the use of other phosphonocarboxylate analogues similar to NE10790 may help to shed light on the molecular structures necessary for interaction with Rab GGTase. Furthermore, although the low potency of NE10790 could limit its potential therapeutic use, future studies addressing the inhibitory mechanism of NE10790 will ultimately help in the rational design of new, more potent inhibitors of this Rab GGTase that have potential for the treatment of thrombotic disorders in addition to diseases of excessive bone resorption such as post-menopausal osteoporosis.

In summary, we demonstrate in this study that NE10790, a phosphonocarboxylate analogue of the potent anti-resorptive drug RIS, inhibits Rab GGTase \textit{in vitro} and specifically prevents the prenylation of Rab GTPases. NE10790 is therefore a useful new tool that provides a novel approach for investigating the function of Rab proteins in osteoclasts and other cell types.

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