Lowering Bone Mineral Affinity of Bisphosphonates as a Therapeutic Strategy to Optimize Skeletal Tumor Growth Inhibition in vivo

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Abstract
Bisphosphonates bind avidly to bone mineral and are potent inhibitors of osteoclast-mediated bone destruction. They also exhibit antitumor activity in vitro. Here, we used a mouse model of human breast cancer bone metastases to examine the effects of risedronate and NE-10790, a phosphonocarboxylate analogue of the bisphosphonate risedronate, on osteolysis and tumor growth. Osteolysis was measured by radiography and histomorphometry. Tumor burden was measured by fluorescence imaging and histomorphometry. NE-10790 had a 70-fold lower bone mineral affinity compared with risedronate. It was 7-fold and 8,800-fold less potent than risedronate at reducing, respectively, breast cancer cell viability in vitro and bone loss in ovariectomized animals. We next showed that risedronate given at a low dosage in animals bearing human B02-GFP breast tumors reduced osteolysis by inhibiting bone resorption, whereas therapy with higher doses also inhibited skeletal tumor burden. Conversely, therapy with NE-10790 substantially reduced skeletal tumor growth at a dosage that did not inhibit osteolysis, a higher dosage being able to also reduce bone destruction. The in vivo antitumor activity of NE-10790 was restricted to bone because it did not inhibit the growth of subcutaneous B02-GFP tumor xenografts nor the formation of B16-F10 melanoma lung metastases. Moreover, NE-10790, in combination with risedronate, reduced both osteolysis and skeletal tumor burden, whereas NE-10790 or risedronate alone only decreased either tumor burden or osteolysis, respectively. In conclusion, our study shows that decreasing the bone mineral affinity of bisphosphonates is an effective therapeutic strategy to inhibit skeletal tumor growth in vivo. [Cancer Res 2008;68(21):8945–53]

Introduction
Cancer cells that metastasize to the skeleton are, on their own, rarely able to destroy bone (1). Instead, they stimulate the function of bone-degrading cells, the osteoclasts, leading to the formation of osteolytic lesions (1). In this respect, bisphosphonate drugs are potent inhibitors of osteoclast-mediated bone resorption, and have shown clinical utility in the palliative treatment of patients with osteolytic metastases (2).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Chemically, bisphosphonates are all characterized by two phosphonate groups linked to a central carbon atom, forming a P-C-P structure (3). Two chains (called R1 and R2) are covalently bound to the carbon atom of the common P-C-P structure. This common backbone and the R1 side chain (preferably a hydroxyl group) allow bisphosphonates to bind avidly to hydroxyapatite crystals in the skeleton (3). The R2 side chain determines the potency of bisphosphonates to inhibit osteoclast-mediated bone resorption (3). Bisphosphonates that lack a nitrogen functional group in the R2 side chain (e.g., etidronate or clodronate) cause the intracellular accumulation of nonhydrolyzable, cytotoxic ATP analogues that subsequently induce osteoclast apoptosis (3). Bisphosphonates with an R2 side chain containing a nitrogen atom either in an alkyl chain (e.g., pamidronate, alendronate, ibandronate) or within a heterocyclic ring (e.g., risedronate or zoledronate) target osteoclast farnesyl diphosphate (FPP) synthase, a key enzyme in the mevalonate pathway, and inhibit its activity to varying degrees, depending on the overall molecular structure of these nitrogen-containing bisphosphonates (4). The inhibition of FPP synthase activity by nitrogen-containing bisphosphonates leads to impaired prenylation and prevents correct function of small GTPases that are essential for osteoclast activity (3).

There is now extensive in vivo preclinical evidence that bisphosphonates can reduce skeletal tumor burden and inhibit the formation of bone metastases in animal models (5). Several mechanisms have been proposed to explain these observations. For example, bisphosphonates may render the bone a less favorable microenvironment for tumor cell colonization by reducing osteoclast-mediated bone resorption, which, in turn, deprives tumor cells of bone-derived growth factors that are required for tumor cell proliferation (5). In addition, bisphosphonates seem to have direct antitumor effects (5). They have been shown to inhibit tumor cell adhesion, invasion, and proliferation, and they induce apoptosis of a variety of human tumor cell lines in vitro (5). Inhibition of the prenylation of small GTPases in tumor cells is thought to be responsible for many of the observed in vitro antitumor effects of nitrogen-containing bisphosphonates (5). However, whereas bisphosphonates clearly exhibit a direct antitumor potential, these drugs that are used for the treatment of patients with skeletal metastases to date have shown no convincing antitumor effects (2). Thus, it is important to define new strategies to optimize the direct antitumor properties of bisphosphonates in vivo.

We have previously shown that soluble bisphosphonates are significantly more potent than mineral-bound bisphosphonates at inhibiting tumor cell adhesion to bone in vitro (6). Moreover, it has been recently reported that osteoclasts internalize bisphosphonates from bone, whereas nonresorbing breast cancer cells only take up small amounts of these bisphosphonates that become...
available due to their natural desorption from the bone surface (7). Overall, these findings (6, 7) suggest that the higher the affinity of a bisphosphonate is for bone mineral, the more limited their direct antitumor potential is in vivo. Therefore, we reasoned that a bisphosphonate possessing weak bone mineral affinity could be released in higher concentration near the bone mineral surface and might act directly on tumor cells that reside in the bone marrow.

To address this question, we used a phosphonocarboxylate analogue of risedronate, NE-10790, in which one of the phosphonate groups of the P-C-P moiety is replaced with a carboxyl group. NE-10790 has a low bone mineral affinity and a poor antiresorptive activity in vitro and in the Schenk growing rat model (8, 9). In addition, this compound inhibits breast and prostate cancer cell invasion and induces apoptosis of myeloma cells in vitro (10, 11). At the molecular level, NE-10790 does not inhibit FPP synthase activity but blocks Rab geranylgeranyl transferase in several cell types, including osteoclasts, macrophages, and myeloma cells (11, 12). Here, using a mouse model of human breast cancer bone metastasis, we compared the effects of risedronate and NE-10790 on osteolysis and skeletal tumor growth.

Materials and Methods

Cell lines. MDA-MB-231, MDA-MB-435s, MCF-7, HS 578T, T-47D, and ZR-75-1 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The MDA-MB-231/B02 human breast cancer cell line is a subpopulation of the MDA-MB-231 cancer line that was selected for the high efficiency with which it metastasizes to bone after i.v. inoculation (13). Tumor cells were routinely cultured in RPMI 1640 (Invitrogen) or α-MEM (Invitrogen) at 10% (v/v) heat-inactivated FCS (Invitrogen) and 1% (v/v) penicillin/streptomycin (Invitrogen) in a humidified atmosphere of 5% CO2 in air.

Drugs and reagents. Risedronate [2-(3-pyridinyl)-1-hydroxyethylidene-bisphosphonic acid] and NE-10790 [2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonocarboxylic acid] were obtained from Procter and Gamble Pharmaceuticals. These drugs were resuspended in PBS or cell culture medium and stored at 4°C. All transgeranylgeranyl (GGOH) was purchased from Sigma-Aldrich, diluted at 10−1 mol/L in absolute ethanol, and stored at −20°C.

Measurement of the bone mineral affinity of risedronate and NE-10790. Binding affinity constants (Kd) for the adsorption of risedronate and NE-10790 were calculated from kinetic studies on hydroxyapatite crystal surfaces using Langmuir isotherm plots, as previously described (14, 15).

Cell viability assay. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (13, 16). Briefly, breast tumor cells were cultured in 96-well plates at a density of 180,000 cells per well in complete α-MEM supplemented with murine macrophage-colony stimulating factor (M-CSF; 25 ng/mL; Preprotech), receptor activator of nuclear factor κB ligand (RANKL; 0.5% v/v) and the conditioned media from B02-GFP cells previously treated with the vehicle, risedronate, or NE-10790. After 6 d of culture, differentiated osteoclasts were enumerated under a light microscope on the basis of multinuclearity (cells with more than three nuclei) and tartrate-resistant acid phosphatase (TRAP) activity using a microscope (Sigma). Results were expressed as the number of osteoclasts per square centimeter.

Animal studies. All procedures involving animals, including housing and care, were conducted in accordance with the institutional animal care and use committee policies and the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. For bone loss experiments, rats were sham-operated or ovariectomized at 3 mo of age, allowed to lose bone for 7 d, and then treated for 35 d. Treatment consisted of s.c. injection of PBS (vehicle), risedronate (1.4 mg/kg body weight), or NE-10790 (3,700, 12,350, or 37,000 mg/kg body weight) once weekly. Bone mineral density was measured on the proximal tibiae using single-photon absorptiometry. Results were expressed in milligrams per square centimeter.

For bone metastasis experiments, we specifically used B02 cells that had been stably transfected with the gene encoding green fluorescent protein to detect tumor cells in live animals using noninvasive fluorescence imaging. The characteristics of the B02-GFP cell line were described elsewhere (17). B02-GFP cells (5 × 106 in 100 μL of PBS) were injected into the tail vein of nude mice anesthetized with 130 mg/kg ketamine and 8.8 mg/kg xylazine. Based on an average body weight of 20 g for 4-wk-old mice, or NE-10790 (3,700, 12,350, or 37,000 mg/kg body weight) was given daily to animals by s.c. injection beginning on the day of tumor cell inoculation (day 0) and continued until the end of the protocol (day 35). All doses of each drug were given by s.c. injection in 100 μL PBS (vehicle). Control mice received a daily treatment with vehicle only. On day 35 after tumor cell inoculation, radiographs of anesthetized mice were taken using a Faxitron X-Ray Corporation model 9000 film (Kodak) in an MX-20 cabinet X-ray system. Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. The area of the osteolytic lesions was measured using a VisiLab 2000 computerized image analysis system (Explora Nova), and the extent of bone destruction per animal was calculated for 2 h with a cocktail of 79 biotinylated antibodies. Membranes were then washed and incubated for an additional 2 h with a peroxidase-labeled streptavidin solution. Detection of immunoreactive spots was carried out using an enhanced chemiluminescence detection system (GE Healthcare).

Measurement of cytokine production by ELISA. Breast cancer cells (B02-GFP, MDA-MB-231, MDA-MB-435s, MCF-7, HS 578T, T-47D, and ZR-75-1) were seeded in 24-well and 96-well plates at a cell density used for the cell viability assay. Growing cells were cultured for 2 d in complete α-MEM in the presence or absence of drugs. After washing to remove drugs, tumor cells were further cultured for another 2 d, at which time the 96-well- and 24-well plates were used for cell counting and measurement of cytokine production, respectively. For the measurement of cytokines, cell culture supernatants containing protease inhibitors (aprotinin and leupeptin; 1 μg/mL) were centrifuged (1,000 × g, 5 min, 4°C) and then stored at −20°C until use. The measurement of human interleukin-6 (IL-6), IL-8, and MCP-1 was performed by ELISA (Module Set Bender MedSystems, AChy SA). Results were expressed as nanograms per milliliter per 106 cells. Cell culture supernatants used for measurement of cytokine production were also tested in the osteoclastogenesis assay.

In vitro osteoclastogenesis assay. Experiments were carried out using 8-wk-old female OF1 mice (Charles River Laboratories), as previously described (16). Briefly, breast tumor cells were cultured in 96-well plates and then seeded in 12-well plates at a density of 180,000 cells per well in complete α-MEM supplemented with murine macrophage-colony stimulating factor (M-CSF; 25 ng/mL; Preprotech), receptor activator of nuclear factor κB ligand (RANKL; 0.5% v/v) and the conditioned media from B02-GFP cells previously treated with the vehicle, risedronate, or NE-10790. After 6 d of culture, differentiated osteoclasts were enumerated under a light microscope on the basis of multinuclearity (cells with more than three nuclei) and tartrate-resistant acid phosphatase (TRAP) activity using a microscope (Sigma). Results were expressed as the number of osteoclasts per square centimeter.

For bone loss experiments, rats were sham-operated or ovariectomized at 3 mo of age, allowed to lose bone for 3 d, and then treated for 25 d. During treatment, the vehicle (PBS), risedronate (1.4 mg/kg body weight), or NE-10790 (20–37,000 mg/kg body weight) was given daily to animals by s.c. injection in 100 μL PBS (vehicle). Control mice received a daily treatment with vehicle only. On day 35 after tumor cell inoculation, radiographs of anesthetized mice were taken using a Faxitron X-Ray Corporation model 9000 film (Kodak) in an MX-20 cabinet X-ray system. Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. The area of the osteolytic lesions was measured using a VisiLab 2000 computerized image analysis system (Explora Nova), and the extent of bone destruction per animal was measured for 2 h with a cocktail of 79 biotinylated antibodies. Membranes were then washed and incubated for an additional 2 h with a peroxidase-labeled streptavidin solution. Detection of immunoreactive spots was carried out using an enhanced chemiluminescence detection system (GE Healthcare).
Concentrations (IC50) for risedronate and percentage of the control. Half-maximal inhibitory (control), and results were expressed as a mean of two separate experiments; bars. *P < 0.05.

Cell viability was measured using an MTT assay. The viability of untreated cells was set to 100% (control), and results were expressed as a percentage of the control. Half-maximal inhibitory concentrations (IC50) for risedronate and NE-10790 were 0.37 and 2.74 mmol/L, respectively. C, effect of 10 μmol/L geranylgeraniol (GGOH) on viability of B02-GFP cells upon treatment with risedronate (IC50 = 0.37 mmol/L), NE-10790 (IC50 = 2.74 mmol/L), or the vehicle (Ctrl). Columns, mean of two separate experiments; bars, SD. *
P < 0.05.

Figure 1. A, antiresorptive potencies of risedronate and NE-10790 in an animal model of bone loss caused by ovariectomy. The bone mineral density of tibia from female rats 35 d after ovariectomy was statistically significantly decreased when compared with that observed with sham-operated animals. The treatment of ovariectomized animals with a daily dose of 1.4 μg/kg risedronate prevented bone loss, whereas much higher doses of NE-10790 were required to reach this end point. Columns, mean of six animals per group; bars, SD. *, P < 0.05. B, effects of risedronate and NE-10790 on viability of B02-GFP breast cancer cells in vitro. B02-GFP cells were cultured in complete medium and then treated with increasing concentrations of each drug for 6 d. Cell viability was measured using an MTT assay. The viability of untreated cells was set to 100% (control), and results were expressed as a percentage of the control. Half-maximal inhibitory concentrations (IC50) for risedronate and NE-10790 were 0.37 and 2.74 mmol/L, respectively. C, effect of 10 μmol/L geranylgeraniol (GGOH) on viability of B02-GFP cells upon treatment with risedronate (IC50 = 0.37 mmol/L), NE-10790 (IC50 = 2.74 mmol/L), or the vehicle (Ctrl). Columns, mean of two separate experiments; bars, SD. *, P < 0.05.

Bone histology and histomorphometry. Bone histology and histomorphometric analysis of bone tissue sections were performed as previously described (13, 16, 17). Briefly, metastatic animals treated with the vehicle, risedronate, or NE-10790 were killed on day 35 after tumor cell inoculation, and both hind limbs from each animal were dissected, fixed in 80% (v/v) alcohol, dehydrated, and embedded in methylmethacrylate. A microtome (Polycut E, Reichert-Jung) was used to cut 7-μm-thick sections of undecalcified long bones, and the sections were stained with Goldner’s trichrome (13, 16, 17). Histologic and histomorphometric analyses were performed on Goldner’s trichrome-stained longitudinal medial sections of tibial metaphysis with the use of Vioslab 2000 computerized image analysis system, as described previously (13, 16, 17). The in situ detection of osteoclasts was performed on TRAP-stained longitudinal medial sections of tibial metaphysis with the use of a commercial kit (Mercck). Osteoclast number (OcN/BS) and resorption surface (OcS/BS) were calculated as, respectively, the ratio of TRAP-positive cells (OcN) and TRAP-positive trabecular bone surface (OcS) to the total trabecular bone surface using Vioslab 2000 computerized image analysis system (16).

Statistical analysis. All data were analyzed with the use of StatView v5.0 software (version 5.0; SAS Institute, Inc.). Pairwise comparisons were carried out by performing nonparametric Mann-Whitney U test. P values of <0.05 were considered statistically significant. All statistical tests were two-sided.

Results

Structure-activity relationships of risedronate and NE-10790. Phosphonate groups of the P-C-P structure are responsible for the strong affinity of bisphosphonates for bone mineral (3). NE-10790 is a phosphonocarboxylate analogue of risedronate in which one of the phosphonate groups is replaced with a carboxyl group (Fig. 1A). The affinity constant (K_L) for the adsorption of NE-10790 on hydroxyapatite crystals was measured using an experimental procedure similar to that previously described for risedronate (13, 14). Compared with risedronate (K_L = 2.2 μmol/L), we observed that modifications to the P-C-P moiety resulted in a 73-fold decrease in bone mineral affinity for NE-10790 (K_L = 0.03 μmol/L; ref. 14).

We then studied antiresorptive potencies of risedronate and NE-10790 in an animal model of bone loss caused by ovariectomy. The treatment of ovariectomized animals with increasing doses of NE-10790 (3,700–37,000 μg/kg) showed that the lowest effective dose (LED) required to prevent bone loss was 12,350 μg/kg (Fig. 1A). By contrast, risedronate had a LED of 1.4 μg/kg (Fig. 1A). NE-10790 expressed in square millimeters, as described previously (13, 16, 17). Animals analyzed by radiography were also examined by noninvasive, whole-body fluorescence imaging using a fluorescence scanning system (FluorImager, Molecular Dynamics). Tumor burden in animals was expressed in square millimeters, as described previously (13, 16, 17). Animals analyzed by radiography were also examined by noninvasive, whole-body fluorescence imaging using a fluorescence scanning system (FluorImager, Molecular Dynamics). Tumor burden in animals was expressed in square millimeters, as described previously (13, 16, 17). Animals analyzed by radiography were also examined by noninvasive, whole-body fluorescence imaging using a fluorescence scanning system (FluorImager, Molecular Dynamics). Antitumor Activity of Phosphonocarboxylate NE-10790.
was, therefore, 8,800-fold less potent than risedronate as an inhibitor of osteoclast-mediated bone resorption in vivo.

We next examined the potencies of risedronate and NE-10790 at inhibiting the viability of B02-GFP breast cancer cells in vitro. Risedronate reduced the number of viable B02-GFP cells in a time-dependent (data not shown) and dose-dependent manner (Fig. 1B) with a half-maximal inhibitory concentration (IC_{50}) of 0.37 mmol/L (Supplementary Table S1). NE-10790 (IC_{50} = 2.74 mmol/L) was 7-fold less potent than risedronate at reducing tumor cell viability (Fig. 1B and Supplementary Table S1). These effects of risedronate and NE-10790 on tumor cell viability were not restricted to B02-GFP cells because similar results were obtained with MDA-MB-231, MDA-MB-435s, MCF-7, Hs578T, T-47D, and ZR-75-1 breast cancer cell lines (Supplementary Table S1). Risedronate is known for its ability to inhibit protein prenylation by acting as a potent inhibitor of FPP synthase (3). NE-10790 does not inhibit FPP synthase but, instead, inhibits Rab geranylgeranyl transferase, an enzyme downstream of FPP synthase in the mevalonate pathway required for protein prenylation (11). Cell viability experiments were therefore conducted in the presence of exogenous geranylgeraniol (GGOH), an intermediate of the mevalonate pathway downstream of FPP synthase but upstream of Rab geranylgeranyl transferase. As shown in Fig. 1C, the inhibitory effect of risedronate on B02-GFP cell viability was overcome by replenishing tumor cells with 10 μmol/L GGOH (Fig. 1C). As expected, GGOH did not prevent the inhibitory effect of NE-10790 (Fig. 1C).

Effects of risedronate and NE-10790 on the formation of breast cancer bone metastases. We used a mouse model of bone metastasis in which we have previously shown that the bisphosphonate zoledronate, given s.c. at a daily dose of 150 μg/kg, markedly inhibits osteolysis and skeletal tumor burden in animals bearing B02-GFP breast cancer cells (15). The same dosing regimen was therefore chosen to compare the effects of risedronate and NE-10790 on the formation of B02-GFP breast cancer bone metastases.

Radiographical analysis on day 35 after tumor cell injection revealed that metastatic animals treated with risedronate had
osteolytic lesions that were 95% smaller than those of tumor-bearing mice treated with the vehicle (Fig. 2A; Table 1). By contrast, NE-10790 did not inhibit bone destruction (Fig. 2A; Table 1). Noninvasive fluorescence imaging on day 35 after tumor cell injection showed that these metastatic animals treated with risedronate or NE-10790 also had a statistically significantly lower tumor burden than animals treated with the vehicle (Fig. 2B). In this respect, risedronate and NE-10790 decreased tumor burden by 40% and 70%, respectively, compared with vehicle (Table 1).

Histomorphometric analysis of hind limbs with metastases showed that mice treated with risedronate had a bone volume (BV)–tissue volume (TV) ratio that was significantly higher than vehicle-treated mice and mice that had not been injected with tumor cells, indicating a complete prevention of bone loss by the bisphosphonate (Fig. 2C; Table 1). TRAP staining of bone tissue sections of metastatic legs from mice treated with risedronate showed a statistically significant inhibition of the osteoclast number (Oc.N) and active-osteoclast resorption surface (Oc.S) per trabecular bone surface (BS) when compared with vehicle (Fig. 3). By contrast, the BV/TV, Oc.N/BS, and Oc.S/BS ratios for mice treated with NE-10790 did not statistically significantly differ from those observed with vehicle-treated mice (Table 1; Figs. 2C and 3). Yet, mice treated with NE-10790 had a tumor burden (TB)–soft tissue volume (STV) ratio decreased by 75% compared with vehicle (Table 1). Risedronate also reduced the TB/STV ratio by 80%, compared with vehicle (Table 1).

Aside from our observation that these compounds decreased skeletal B02-GFP tumor burden at a dosage of 150 μg/kg/d (Fig. 2B and C; Table 1), we found that the same dosing regimen of NE-10790 or risedronate did not inhibit the growth of s.c. B02-GFP tumor xenografts in animals (Supplementary Fig. S1A). Moreover, risedronate and NE-10790 (used at a dosage of 350 μg/kg/d) did not inhibit the formation of B16-F10 pulmonary metastases in animals (Supplementary Fig. S1B).

A head-to-head comparison of the effects of risedronate and NE-10790 on bone metastasis formation was next conducted using increasing doses of each of these compounds. Radiographical analysis and fluorescence imaging of animals on day 35 after B02-GFP tumor cell injection revealed that risedronate and NE-10790 inhibited both osteolysis and tumor burden in a dose-dependent manner (Fig. 3). The LED of risedronate that inhibited osteolysis (15 μg/kg/d) did not inhibit tumor burden (Fig. 3A and B), indicating that risedronate first inhibited osteoclast-mediated bone resorption and then skeletal tumor growth. Conversely, the LED of NE-10790 that inhibited tumor burden (150 μg/kg/d) did not inhibit osteolysis (Figs. 2A and 3A and B). A higher dose of NE-10790 (37,000 μg/kg/d) did, however, inhibit osteolysis, as judged by radiography and TRAP staining of metastatic bone tissue sections (Fig. 3).

Effects of risedronate and NE-10790 on the production of osteoclast-stimulatory cytokines by breast cancer cells. In vitro B02-GFP cells produced several cytokines, chemokines, and growth factors as detected by RayBio human cytokine antibody arrays (Gro, GM-CSF, IL-6, IL-8, MCP-1, IGFBP-1, VEGF, TIMP-1, and TIMP-2; Fig. 4A). The effects of risedronate and NE-10790 on production of these factors were investigated. B02-GFP cells were cultured for 2 days in the presence of risedronate or NE-10790 using a concentration that induced a 50% reduction in cell viability. After washing to remove drugs, tumor cells were further cultured for another 2 days, at which time conditioned media were collected and incubated with a cytokine antibody array membrane. Compared with vehicle, several cytokines were differentially expressed in the conditioned medium from cells treated with risedronate or NE-10790, including osteoclast-stimulatory cytokines IL-6, IL-8, and MCP-1 (SupplementaryFig. S2). These results were confirmed by ELISA. NE-10790 and, to a lower extent, risedronate stimulated the production of IL-8, IL-6, and MCP-1 by B02-GFP cells when compared with that observed with the conditioned medium from vehicle-treated cells (Fig. 4B).

Ex vivo experiments, using human cytokine antibody arrays and ELISA, were next conducted to measure human cytokine levels in the bone marrow of metastatic hind limbs from animals treated with a daily dose of 150 μg/kg risedronate, NE-10790, or the vehicle. Several human cytokines/growth factors were produced in the bone marrow of metastatic legs from vehicle-treated animals (Fig. 4C), among which human IL-8 was the most abundant. No human IL-8 was detected in the bone marrow cell supernatant of bones from age-matched animals that had not been inoculated with B02-GFP

### Table 1. Effects of risedronate and NE-10790 on the extent of osteolytic lesions and skeletal tumor burden in animals bearing B02-GFP breast cancer cells

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. mice</th>
<th>Radiography (mm²/mouse)</th>
<th>Fluorescence (mm²/mouse)</th>
<th>Histomorphometry ¹</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. legs</td>
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<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
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<td>13.7 ± 2.2</td>
<td>9</td>
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<td>8.3 ± 2.3 ³</td>
<td>7</td>
</tr>
<tr>
<td>NE-10790</td>
<td>20</td>
<td>4.3 ± 0.8</td>
<td>4.1 ± 1.3 ³</td>
<td>7</td>
</tr>
</tbody>
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Abbreviations: BV/TV, bone volume–tissue volume ratio; TB/STV, tumor burden–soft tissue volume ratio.

*Drug administration was initiated from the time of tumor cell inoculation (day 0) to the end of the protocol (day 35). All measurements were made 35 d after tumor cell injection. Results are the mean ± SE of three separate experiments. Risedronate and NE-10790 were given at a daily dose of 150 μg/kg s.c. from days 0 to 35. PBS was used as vehicle. Naive are age-matched animals that had not been injected with tumor cells.

¹ Histomorphometry was performed on legs with bone metastasis.

² P < 0.05, compared with the vehicle-treated group. Statistical pairwise comparisons were made using Mann-Whitney U test.

³ P < 0.005, compared with the vehicle-treated group. Statistical pairwise comparisons were made using Mann-Whitney U test.
cells (naive animals; Fig. 4D), further indicating that the detection of human IL-8 was solely inherent to the presence of human tumor cells in the bone marrow. Furthermore, human IL-8 amounts in bone marrow cell supernatants of metastatic bones from risedronate-treated and NE-10790–treated animals were increased 2-fold to 3-fold compared with vehicle-treated animals (Fig. 4D).

After the observation that risedronate and NE-10790 stimulated the production of osteoclast-stimulatory cytokines by tumor cells, conditioned media used to measure human cytokine levels were tested for their ability to stimulate osteoclastogenesis in vitro. These conditioned media did not, however, induce the development of mature osteoclasts in vitro (Fig. 5). Additional experiments were therefore conducted in the presence of RANKL and M-CSF, which are two hematopoietic factors both necessary and sufficient to induce osteoclastogenesis (18). Compared with the conditioned medium from vehicle-treated tumor cells, the conditioned medium collected from NE-10790–treated B02-GFP cells statistically significantly enhanced the differentiation of osteoclasts induced by RANKL + M-CSF (Fig. 5). By contrast, the conditioned medium from risedronate-treated cells did not promote RANKL + M-CSF-induced osteoclastogenesis in vitro (Fig. 5).

**Effect of a combined treatment with risedronate and NE-10790 on breast cancer bone metastases.** Results obtained with risedronate and NE-10790 on breast cancer bone metastasis formation brought us to the question of whether risedronate might maximize the antitumor activity of NE-10790 when used in combination. As shown in Supplementary Fig. S3, the combination of risedronate (used at its lowest antiresorptive effective dose) with NE-10790 (used at its lowest antitumor effective dose) statistically significantly decreased both osteolysis and skeletal tumor burden when compared with the vehicle-treated animals, whereas risedronate or NE-10790 alone at these doses only decreased either osteolysis or tumor burden, respectively. The use of NE-10790 in combination with risedronate did not further decrease the extent of osteolytic lesions when compared with risedronate alone (1.4 ± 0.4 versus 1.9 ± 1.3 mm², respectively; Supplementary Fig. S3A). Similarly, the antitumor efficacy observed in combining NE-10790 with risedronate did not differ statistically significantly from that obtained with NE-10790 alone (3.7 ± 1.4 versus 4.1 ± 1.3 mm², respectively; Supplementary Fig. S3B).

**Discussion**

Our results first show that the bisphosphonate risedronate given at a low dosage in an animal model of breast cancer bone metastasis inhibited osteolysis by inhibiting bone resorption, whereas therapy with higher dosages also inhibited skeletal tumor burden. These results are in agreement with previous preclinical findings (19), showing that zoledronate therapy with a long dosing
interval inhibits osteolysis, whereas a therapy with more frequent dosing intervals reduces both osteolysis and skeletal tumor burden. It is possible that the antitumor activity of bisphosphonates (as exemplified here by risedronate) in preclinical models of bone metastasis is explained by the inhibition of osteoclast-mediated bone resorption which, in turn, deprives tumor cells of bone-derived growth factors that are required for tumor growth. However, if a bisphosphonate treatment decreased skeletal tumor burden solely by reducing bone loss, we would have expected the lower dosage of risedronate to have inhibited skeletal tumor growth more than what we observed. Similarly, zoledronate therapy with a long dosing interval would have also inhibited skeletal tumor burden (19). Thus, there must be additional reasons explaining the limited antitumor activity of these drugs in vivo. We have previously shown that mineral-bound bisphosphonates are significantly less potent than soluble bisphosphonates at inhibiting breast and prostate cancer cell adhesion to bone in vitro (6). We therefore surmised that, irrespective of the antiresorptive properties of bisphosphonates, the rapid uptake of these drugs in bone might limit their ability to act directly on tumor cells that reside in the bone marrow. Conversely, bisphosphonates with a low bone mineral affinity should be more readily released from the bone surface, enabling a more prolonged exposure of the bone marrow to these drugs and a direct effect on tumor cells. To address this important question, we examined the effects of NE-10790 on osteolysis and skeletal tumor growth. Our results show that, compared with risedronate, NE-10790 poorly inhibited osteoclast-mediated bone resorption in animal models of bone loss caused by ovariectomy or cancer. These results were in complete agreement with a previous report showing that NE-10790 is 8,000-fold less potent than risedronate at inhibiting bone resorption in the Schenk rat growing model (8). Conversely, NE-10790 is only 100-fold less potent than risedronate at inhibiting osteoclastic resorption in vitro (9, 12). The difference in antiresorptive potency between in vitro and in vivo studies is likely explained by the low bone mineral affinity of NE-10790 which, in turn, limits the skeletal uptake of the drug in vivo. Our results also show that a continuous treatment with NE-10790, at a dosage that did not inhibit osteolysis, produced meaningful antitumor effects in a mouse model of breast cancer bone metastasis. Moreover, the in vivo antitumor activity of NE-10790 was restricted to bone because the dosing regimen of NE-10790 that reduced
The number of osteoclasts in the NE-10790-treated group is statistically significantly increased in blue. Results are the mean ± SD of three separate experiments. The number of osteoclasts in the NE-10790-treated group is statistically significantly higher than the vehicle-treated or risedronate-treated group (P < 0.01, Mann-Whitney U test).

The skeletal B02-GFP tumor burden did not inhibit the s.c. growth of B02-GFP tumor xenografts nor the formation of B16-F10 melanoma lung metastases. Thus, our results strongly suggest that, after a transient accumulation in bone, NE-10790 was released from bone mineral, enabling a direct effect of the drug on breast cancer cells that reside in the bone marrow.

How did NE-10790 inhibit skeletal tumor burden in our mouse model of bone metastasis? NE-10790 specifically inhibits Rab geranylgeranyl transferase (Rab GGTase or GG Tase-2) in several cell types, including osteoclasts, macrophages, and myeloma cells (11, 12). In addition, NE-10790 induces apoptosis of myeloma cells (11) and inhibits breast cancer cell invasion in vitro (10). We found that NE-10790 also inhibited the viability of breast cancer cell lines and that GGOH (an intermediate of the mevalonate pathway upstream of Rab GGTase) did not prevent the inhibitory effect of NE-10790 on viability of breast cancer cells. Moreover, NE-10790 inhibited the prenylation of Rab6 (geranylgeranylated by Rab GGTase) but not that of Rap1a (geranylgeranylated by GG Tase-1) in B02-GFP breast cancer cells. These results are in line with the observation that NE-10790 disrupts the prenylation and membrane localization of several Rab proteins (Rab5, Rab6, and Rab27a) in J774 macrophages (20). Evidence is emerging that Rab GGTase plays an important role in cancer cell proliferation and aggressiveness in vivo (21, 22). Our results provide some support for a role of prenylated Rab GGTase in breast cancer bone metastasis. They also suggest that NE-10790 (and potentially to some extent, risedronate and other bisphosphonates, depending on their bone mineral affinity), by preventing Rab prenylation in breast cancer cells, reduced both the invasion and viability of tumor cells, leading to inhibition of skeletal tumor burden.

Whereas NE-10790 was decreasing skeletal tumor growth, it concomitantly stimulated the production of osteoclast-stimulatory cytokine IL-8 by tumor cells resident in the bone marrow. Tumor-derived IL-8 mediates osteolysis in experimental breast cancer bone metastasis (23). These findings (23) may explain why tumor-bearing animals treated with NE-10790 had osteolytic lesions despite a substantial reduction of skeletal tumor burden. Molecular mechanisms through which NE-10790 stimulates IL-8 production by breast cancer cells are unknown. Cyclooxygenase-2 (COX-2) overexpression in MDA-MB-231 breast cancer cells causes an increase production of IL-8 (24). GGTI-286, a selective inhibitor of geranylgeranyltransferases, increases COX-2 expression in smooth muscle cells (25). It is therefore possible that NE-10790 stimulates COX-2 expression in breast cancer cells. Whatever the molecular mechanisms are, a higher dosage of NE-10790 inhibited osteoclast-mediated bone resorption, thereby countering the osteoclast-stimulatory effect of tumor-derived IL-8. The risk associated with IL-8 production by NE-10790-treated tumor cells was also minimized when using NE-10790 in combination with risedronate. Moreover, the combination of NE-10790 with risedronate inhibited both osteolysis and skeletal tumor burden, whereas NE-10790 or risedronate used as a single-agent therapy only decreased either tumor burden or osteolysis, respectively. Our results are reminiscent of those obtained in combining bisphosphonates with chemotherapeutic agents for the treatment of animals with bone metastases, in which drug combinations were shown to provide a greater benefit compared with either drug alone (4, 26). These experimental findings (refs. 4, 26 and this study) are therefore in line with current clinical studies investigating the utility of bisphosphonates as adjuvant therapy for the prevention of bone metastases in several large-scale clinical trials in multiple cancer types (27). For example, it was recently reported that the addition of zoledronate to adjuvant endocrine therapy for premenopausal women with endocrine-responsive breast cancer significantly prolonged the disease-free and relapse-free survival by 35% over a 5-year period (28). Our results suggest that the use of risedronate and NE-10790 in combination with standard cytotoxic and/or endocrine treatments may represent another promising approach to obtain clinically meaningful antitumor effects.

In conclusion, our study shows for the first time that decreasing the bone mineral affinity of bisphosphonates is an effective therapeutic strategy to inhibit skeletal tumor growth in a mouse model of breast cancer bone metastasis. We believe that such compounds with a low bone mineral affinity will open new exciting ways for optimizing antitumor activity of bisphosphonates in oncology.

Disclosure of Potential Conflicts of Interest

P. Clézardin: commercial research grant, P&G Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

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5 S. Gordon and M. Rogers, unpublished results.
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In memoriam: P. Clezardin dedicates this work to the memory of Pierre Delmas who has always been a mentor and friend to him. All other authors also graciously acknowledge the many contributions of Prof. Delmas to the bone field.

References

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