

Herbimycin A, a pp60^{c-src} Tyrosine Kinase Inhibitor, Inhibits Osteoclastic Bone Resorption In Vitro and Hypercalcemia In Vivo

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Abstract

Since absence of expression of the c-src gene product in mice indicates that the pp60^{c-src} tyrosine kinase is required and essential for osteoclastic bone resorption, we tested the effects of the antibiotic herbimycin A, which is an inhibitor of pp60^{c-src} on osteoclastic bone resorption in vitro and on hypercalcemia in vivo. We examined the effects of herbimycin A on the formation of bone resorbing osteoclasts in mouse long-term marrow cultures, on isolated rodent osteoclasts and on bone resorption in organ cultures of fetal rat long bones stimulated by parathyroid hormone. We found that herbimycin A in concentrations of 1–100 ng/ml inhibited bone resorption in each of these systems. We determined the effects of herbimycin A (100 ng/ml) on src tyrosine kinase activity in mouse marrow cultures and found that it was decreased. Herbimycin A also decreased elevated blood calcium levels that were induced either by repeated subcutaneous injections of recombinant human interleukin-1 α or by a human tumor. There was no evidence for toxicity in any of these culture systems or in mice treated with herbimycin A. A different tyrosine kinase inhibitor that does not inhibit pp60^{c-src} was used as a control and caused none of these effects. These data suggest that pp60^{c-src} tyrosine kinase inhibitors may be useful pharmacologic inhibitors of osteoclastic bone resorption and hypercalcemia. (*J. Clin. Invest.* 1993. 91:2791–2795.) Key words: pp60^{c-src} • tyrosine kinase inhibitors • osteoclast • bone resorption • hypercalcemia

Introduction

Recent observations suggest that different tyrosine kinases (TKs)¹ may be essential for normal osteoclastic bone resorption. Mice deficient in expression of the c-src protooncogene by introduction of the null mutation into the germ line by homologous recombination develop osteopetrosis (1). These data indicate that the TK encoded by src is required for normal osteoclast function. Recently, an antibiotic herbimycin A that inhibits a number of intracellular TKs, including the src TK, has been identified. This antibiotic has been shown to reverse temperature sensitive respiratory syncytial virus (RSV)-transformed rat cells (ts/NRK) morphology to normal, concomitant with a marked reduction in pp60^{c-src} kinase activity. Herbimycin A irreversibly and directly inhibits pp60^{c-src} TK but has no effects on serine/threonine kinase, protein kinase A, and protein kinase C (2). To determine if herbimycin A could be a useful inhibitor of osteoclastic bone resorption, we tested its effects on bone resorption stimulated by parathyroid hormone (PTH) in three different in vitro systems that examine different aspects of osteoclast function, namely osteoclast formation, as well as osteoclastic activation and resorption. We also found that herbimycin A inhibited hypercalcemia in mice that was induced either by repeated injections of recombinant human IL-1 α (rhIL-1 α) or by a human tumor. We examined another TK inhibitor, RG-13022 (3, 4), as a control. RG-13022 showed none of these effects.

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1. Abbreviations used in this paper: α PY, antiphosphotyrosine antibody; Ca²⁺, whole-blood ionized calcium; PTH, parathyroid hormone; rh-IL-1 α , recombinant human IL-1 α ; TK, tyrosine kinase; TRAP(+)MNC, tartrate-resistant acid phosphatase-positive multinucleated cells; 1,25D₃, 1,25-dihydroxyvitamin D₃.

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Methods

Drugs

RG-13022 was kindly supplied by Dr. A. Zilberstein (Rhône-Poulenc Rorer Biotechnology, Collegeville, PA). Fig. 1 shows the structure of herbimycin A and RG-13022. Stock solutions of the drugs were made in 100% DMSO and diluted with the culture medium before addition to cells. The culture medium containing the equivalent concentrations of DMSO served as vehicle controls.

Fetal rat long bone assay

This assay measures the release of previously incorporated ⁴⁵Ca from fetal rat long bones in organ culture, and has been described previously (5). Four bones were cultured per group, and bone resorbing activity was expressed as treated: control ratios of ⁴⁵Ca released from test and control bones. Statistical significance was assessed using the Student's *t* test for unpaired samples.

Murine marrow cell culture system

We used a modification of the murine marrow culture technique described by Takahashi et al. (6). Marrow cells were collected from the femora and tibiae, washed twice with α MEM (Hazleton Biologics Inc., Lenexa, KS) and resuspended in α MEM supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and without antibiotics at a final density of 4×10^6 cells/ml. 0.5 ml of cell suspension per well was inoculated onto 24-well plates (Corning Inc., Corning, NY) and 10^{-8} M 1,25-dihydroxyvitamin D₃ (1,25D₃) (Biomol Research Laboratories Inc., Plymouth Meeting, PA) in 10 μ l was added to each well. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were fed every 2 d with 0.3 ml of fresh α MEM with 10% FCS and 10^{-8} M 1,25D₃. After 6 d, the cultures were washed in PBS, fixed in 60% acetone in citrate buffer pH 5.4 for 30 s, air dried, and stained for tartrate-resistant acid phosphatase (TRAP) using a

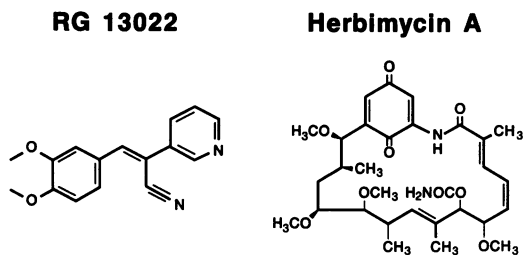


Figure 1. Chemical structure of RG-13022 and herbimycin A.

commercially available kit (Sigma Chemical Co., St. Louis, MO). Stained cultures were examined under light microscopy at a magnification of 40 TRAP-positive (red-staining) multinucleated (five or more nuclei) cells (TRAP(+))MNC were easily distinguishable from other cells present. All TRAP(+))MNCs in each well were counted by manually scanning across the entire well in a systematic fashion.

Assay for bone-resorbing activity of disaggregated rat osteoclasts

Osteoclasts obtained for these experiments were prepared from the femora and tibiae of newborn rats according to techniques previously described (7, 8). The bones of each rat were curetted with a scalpel blade into 1 ml of medium and vigorously agitated with a pipette. The supernatants were added to slices of sperm whale dentine. The cells were then incubated for 15 min at 37°C. Dentine slices were then removed, washed, and placed in separate wells containing 100 μ l of α MEM supplemented with 10% FCS. The wells were incubated in the absence or presence of bovine PTH (1-34; Peninsula Laboratories Inc., Belmont, CA) for 24 h before quantification of cell numbers and assessment of bone resorption.

The disaggregated rat osteoclast pit assay was performed as described by Boyde et al. (9). Slices of sperm whale dentine (0.25 \times 4 \times 6 mm) were prepared using a low speed diamond saw (Buehler Ltd., Lake Bluff, IL), followed by sonication (15 min) in several changes of distilled water. Slices were smoothed between two glass plates and sterilized under ultraviolet light for 4 d. Before all experiments, slices were incubated in α MEM supplemented with 5% FCS and 1% penicillin-streptomycin solution (Gibco Laboratories, Grand Island, NY) for \geq 48 h. Cultures were performed in humidified air (10% CO₂) at 37°C (one slice per well). A single experiment used 16–20 slices, with a minimum of three to four slices per treatment. Experiments were repeated at least three to four times. For examination of resorption lacunae, the slices are sonicated in 0.1 M NaOH and stained with 0.1% (wt/vol) toluidine blue. Lacunae were counted using light microscopy, and the plan area of matrix resorbed was quantitated using a computer-assisted morphometric program on an image and process analysis system (Olympus Corp., Lake Success, NY). Data were analyzed by pattern analysis of variance (8).

Cell lysis

Mouse bone marrow cells were cultured in the presence of 10⁻⁸ M 1,25D₃ for 6 d in six-well plates as described above. The cells were then treated with or without herbimycin A for 24 h, followed by 50 ng/ml PTH, which is known to cause increased resorption pit formation by TRAP-positive MNC (6), for 30 min. At the end of PTH incubation, cells were washed with ice-cold PBS twice, lysed at 4°C with 300 μ l solubilization buffer (10 mM Tris, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μ M sodium orthovanadate, 2 mM iodoacetic acid, 2 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 5 μ M ZnCl₂, and 1% Triton X-100, pH 7.1) (10), centrifuged at 13,000 *g* for 25 min at 4°C with an Eppendorf centrifuge, and the supernatants were immediately frozen by liquid nitrogen. Protein concentration in the lysates was measured (protein DC assay; BioRad, Richmond, CA) according to the manufacturer's protocol or

by AuroDye (Amersham Corp., Arlington Heights, IL) staining of protein blots as described by Li et al. (11).

Immunoprecipitation

Cell lysates (130 μ l) containing equivalent amounts of protein were mixed with 25 μ l of antiphosphotyrosine (α PY)-agarose beads (Oncogene Science, Inc., Uniondale, NY) for 4 h at 4°C. The α PY beads were centrifuged, washed six times with the solubilization buffer and proteins bound to α PY were eluted by the treatment with the solubilization buffer containing 1 mM phenylphosphate for 10 min at 4°C. The eluted material was mixed with an equal volume of 2 \times sample buffer (1% SDS, 0.1% β -mercaptoethanol, 0.15 M Tris, 10% glycerol, 0.02% bromophenol blue, 1 mM EDTA, pH 6.8), boiled for 5 min, and spun down for 10 min. The samples were then subjected to SDS (12.5%) PAGE (Phast System; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to manufacturer's instructions.

Immunoblotting

The proteins separated on SDS-PAGE were transblotted to nitrocellulose membrane (ProBlot membrane; Applied Biosystems Inc., Foster City, CA) in 3-[cyclohexylamide]-1-propanesulfonic acid buffer at 20 V, 25 mA at 15°C for 20 min. The membranes were immunostained with the 327 monoclonal antibody to pp60^{v-src}.

Assay for pp60^{c-src} protein kinases

The assays were performed as described (12). In brief, 130 μ l cell lysate prepared as above were incubated with 15 μ l 327 monoclonal antibody (Oncogene Science) (13) for 50 min at 4°C and successively with 40 μ l agarose-bound protein G (Immuno Pure Plus Immobilized Protein G; Pierce, Rockford, IL) for 40 min at 4°C. The immunoprecipitates were washed in the solubilization buffer three times and then incubated in 30 μ l of reaction mixture of 10 mM Tris (pH 7.4), 5 mM MnCl₂, 1 μ M ATP, 10 μ Ci [γ -³²P]ATP (New England Nuclear, Boston, MA, 5,000 Ci/mmol) and 1 μ g enolase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 5 min at 30°C. The reaction product was mixed with equal volume of 2 \times sample buffer, boiled for 5 min, and subjected to SDS-PAGE as described above. The gel was dried and exposed to film (XAR-5; Kodak, Rochester, NY) with intensifying screens (Fischer Biotech, Pittsburgh, PA) for 24 h at -70°C for autoradiography.

Administration of herbimycin A and RG-13022 to animals

rhIL-1 α -injected mice. ICR Swiss white mice (Harlan Sprague-Dawley, Indianapolis, IN) were injected subcutaneously over the right side of the calvariae with 10 μ g herbimycin A or RG-13022 in 10 μ l DMSO or DMSO alone four times a day using 27-gauge needles. 30 min later of each injection of herbimycin A or RG-13022, some of the mice received subcutaneous injections of 0.1 μ g recombinant human IL-1 α , kindly supplied by Dr. Lomedico (Hoffman-La Roche, Nutley, NJ) in 10 μ l PBS four times a day over the right side of the calvariae as described previously (14).

Tumor-bearing mice. Nude mice (BALB/c nu/nu; Sprague-Dawley) bearing a well-characterized human squamous cancer MH-85 (15) that manifested hypercalcemia were injected intraperitoneally with 100 μ g herbimycin A in 0.1 ml DMSO twice a day.

Determination of blood-ionized Ca (Ca²⁺) and body weight. Ca²⁺ was determined using a calcium pH analyzer (model 634; Corning, Medfield, MA) as described (15). Body weight of animals was measured (Lum-O-Gram; Ohaus Scale Corporation, Florham Park, NJ). These measurements were carried out at 9:00 a.m. every other day before the first injection of agents under anesthesia.

Statistical analysis. All data shown are mean \pm SE and were analyzed by ANOVA followed by a paired *t* test.

Results

First, we examined the effects of herbimycin A and RG-13022 on the formation of TRAP(+))MNC stimulated with 1,25D₃

(10^{-8} M) in mouse long-term marrow cultures. Herbimycin A reduced the number of TRAP(+)MNC in $1,25D_3$ -treated cultures in a dose-dependent manner (Fig. 2). This effect is unlikely to be caused by the cytotoxicity of herbimycin A. Number of stromal cells harvested by trypsinization in control and herbimycin A-treated (100 ng/ml) marrow cultures at day 6 was not different (control/treated = $3.2 \pm 0.3:2.9 \pm 0.3 \times 10^6$ /well, $n = 4$) and 3H -amino acid incorporation into TCA-insoluble protein in herbimycin A-treated (100 ng/ml) cultures was not changed (control/treated = $4.2 \pm 0.5:3.9 \pm 0.2 \times 10^3$ dpm/well, $n = 4$). In the same cultures, PTH increased apparent activity of pp60^{c-src} TK, which was measured using enolase as a substrate in bone marrow cell lysates immunoprecipitated with the 327 monoclonal antibody to pp60^{c-src}. Herbimycin A markedly decreased the activity of pp60^{c-src} TK, which was increased by PTH (Fig. 3). There were no significant differences in size, shape and nuclear number of TRAP(+)MNC formed between cultures treated with herbimycin A and control cultures (data not shown). In contrast, 20 μ M (5.36 μ g/ml) RG-13022, which markedly suppressed epidermal growth factor-stimulated TK and growth in several cancer cell lines (16), did not decrease TRAP(+)MNC formation and PTH-stimulated pp60^{c-src} TK activity in mouse marrow cultures (Fig. 2 B and Fig. 3). Both herbimycin A and RG-13022 did not change

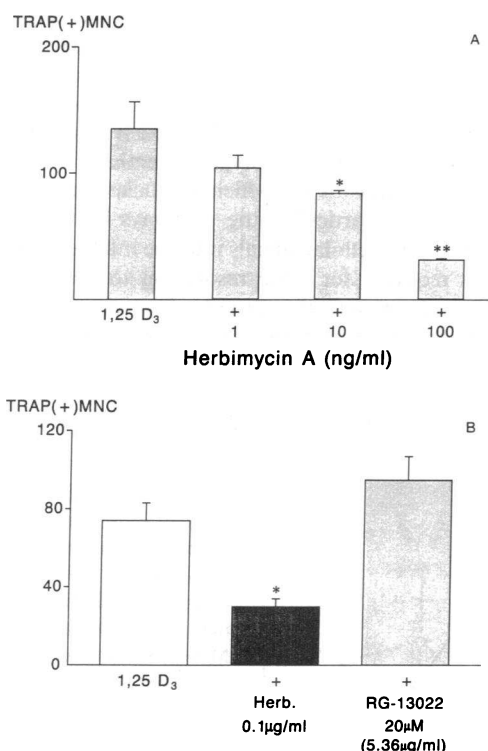


Figure 2. (A) Effect of herbimycin A on TRAP(+)MNC formation in mouse bone marrow cultures. Bone marrow cells (2×10^6 /well, 24-well) were cultured as described in the text in the presence of 10^{-8} M $1,25D_3$ with or without increasing concentrations of herbimycin A for 6 d. Data shown are mean \pm SE. *Significantly different from cultures treated with $1,25D_3$ alone ($P < 0.05$). **Significantly different from cultures treated with $1,25D_3$ alone ($P < 0.01$). (B) Effect of herbimycin A and RG-13022 on TRAP(+)MNC formation in mouse bone marrow cultures. Data shown are mean \pm SE. *Significantly different from cultures treated with $1,25D_3$ alone ($P < 0.01$).

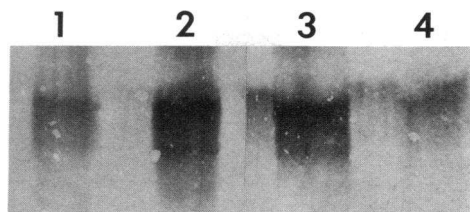


Figure 3. Effect of herbimycin A and RG-13022 on pp60^{c-src} TK activity. Bone marrow cells were cultured in the presence of $1,25D_3$ for 6 d as described in the text. From day 5 of the culture, cells were incubated with or without 0.1 μ g/ml herbimycin A or 20 μ M (5.36 μ g/ml) RG-13022 for 24 h until the end of the culture. At the end of the culture, cells were incubated with or without 50 ng/ml PTH for 30 min, lysed, and immunoprecipitated with 327 monoclonal antibody. pp60^{c-src} TK activity was then determined using enolase as a substrate as described in the text. Lane 1, untreated (DMSO); lane 2, PTH (50 ng/ml); lane 3, RG-13022 (20 μ M) + PTH; and lane 4, herbimycin A (0.1 μ g/ml) + PTH. Bands shown are phosphorylated enolase.

pp60^{c-src} TK protein levels determined by Western analysis in mouse marrow cultures (data not shown).

Next, we examined the effects of herbimycin A on the capacity of disaggregated rat osteoclasts to form resorption pits on dentine. Herbimycin A decreased the area of pits formed by disaggregated osteoclasts treated with PTH in a dose-dependent manner from 1 to 100 ng/ml (Fig. 4). Herbimycin A showed no effects on the number of pits formed (Fig. 4). Herbimycin A also inhibited PTH-stimulated bone resorption of fetal rat long bones in organ cultures (Fig. 5). RG-13022 did not show such an effect in the same assay (Fig. 5, data not shown). Bone resorption in untreated cultures was not affected by herbimycin A, and none of the cells (including osteoclasts and stromal cells) in marrow cultures or disaggregated osteoclast assays treated with herbimycin A showed morphologic or numerical changes.

Repeated subcutaneous injection of rhIL-1 α (0.1 μ g/mouse, four times a day) induced hypercalcemia in ICR Swiss

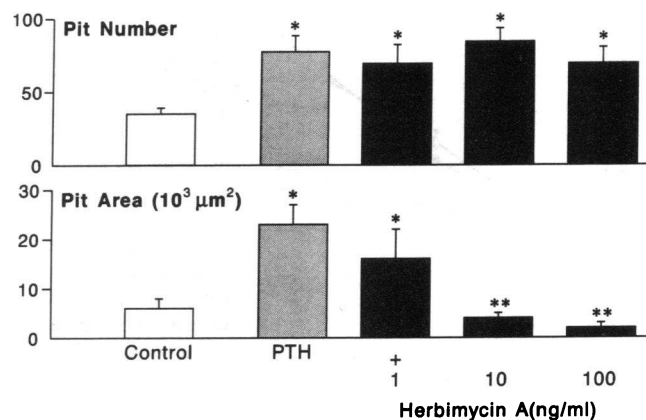


Figure 4. Effect of herbimycin A on resorption pit formation by isolated rat osteoclasts. Isolated osteoclasts were cultured on the dentine slices in the absence or presence of 50 ng/ml PTH with or without herbimycin A for 24 h. Pit number (top) and pit area (bottom) were then determined as described in Methods. Data shown are mean \pm SE. *Significantly greater than control ($P < 0.05$). **Significantly less than cultures treated with PTH alone ($P < 0.01$).

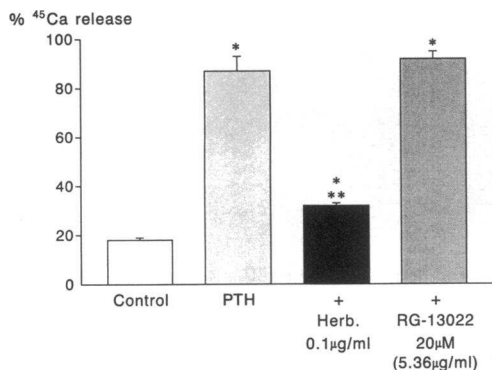


Figure 5. Effect of herbimycin A and RG-13022 on bone resorption in organ cultures of fetal rat long bones. Fetal rat long bones were cultured in the absence or presence of 50 ng/ml PTH with or without 0.1 µg/ml herbimycin A or 20 µM (5.36 µg/ml) RG-13022 for 120 h. Values shown are mean ± SE. *Significantly higher than control ($P < 0.01$). **Significantly less than cultures treated with PTH alone ($P < 0.01$).

mouse (Fig. 6). Previous studies have shown this is caused by increased osteoclastic bone resorption (14). Simultaneous treatment with subcutaneous injections of herbimycin A (10 µg/mouse per injection, four injections a day) markedly prevented the development of hypercalcemia induced by rhIL-1α (Fig. 6).

In preliminary experiments, we found that repeated subcutaneous injections of 400, 200, and 100 µg herbimycin A/mouse per day into untreated ICR Swiss mice killed the animals after 1–3 d. However, administration of herbimycin A at 40 µg/mouse per day as shown in Fig. 6 did not show any significant effects on viability, food consumption, body weight, and behavior of animals (data not shown).

RG-13022 (10 µg/mouse per day) administered in the same manner as that of herbimycin A did not decrease Ca²⁺ in rhIL-1α-treated mice (Fig. 6).

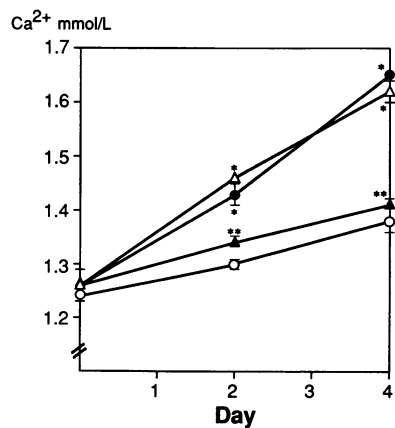


Figure 6. Effect of herbimycin A and RG-13022 on rhIL-1α-induced hypercalcemia. ICR Swiss mice (M, 5–6 wk-old; $n = 20$) were divided into four groups (five mice per group). Open circle, DMSO; closed circle, rhIL-1α alone (0.1 µg/mouse per injection, four injections a day, subcutaneously on calvariae); closed triangle, rhIL-1α + herbimycin A (10 µg/mouse/injection,

four injections a day subcutaneously on calvariae); open triangle, rhIL-1α + RG-13022 (10 µg/mouse per injection, four injections a day subcutaneously on calvariae). Ca²⁺ was measured at 9:00 a.m. every other day before injection of agents. Normal Ca²⁺ range: 0.95–1.28 mmol/liter. Values shown are mean ± SE. *Significantly more hypercalcemic than DMSO-treated mice ($P < 0.01$). **Significantly lower than animals treated with rhIL-1α alone ($P < 0.01$). ○ — ○, DMSO; ● — ●, IL-1; ▲ — ▲, herbimycin A + IL-1; △ — △, RG-13022 + IL-1.

When herbimycin A (200 µg/mouse per day) was administered intraperitoneally to hypercalcemic nude mice bearing the human tumor MH-85, which previously was shown to induce hypercalcemia partly by TNF-mediated mechanisms (17), Ca²⁺ was lowered by day 4 of injection and significantly decreased by day 8 of the administration of the drug (Fig. 7). In contrast, hypercalcemic MH-85 tumor-bearing nude mice that were not treated with herbimycin A showed a progressive increase in Ca²⁺. Herbimycin A treatment for 8 d did not show any effect on MH-85 tumor growth (data not shown).

Discussion

The data reported in this paper show that herbimycin A, which is known to inhibit pp60^{c-src} TK activity (2), decreased pp60^{c-src} TK activities in mouse bone marrow cells and inhibited the formation of cells with osteoclast characteristics and their bone resorbing capacity in the same culture system. Furthermore, herbimycin A inhibited the capacity of mature osteoclasts to form pits on dentine slices and suppressed bone resorption in organ cultures of fetal rat long bones. More importantly, we found that herbimycin A decreased and prevented rhIL-1α- or tumor-induced hypercalcemia, both of which are associated with increased osteoclastic bone resorption in vivo (14, 15). Inhibition of bone resorption by herbimycin A is likely to be, at least in part, associated with suppression of pp60^{c-src} TK activity, since RG-13022, which failed to decrease pp60^{c-src} TK activity, did not inhibit bone resorption. These results are consistent with other observations that suggest that pp60^{c-src} TK is essential and required for normal osteoclast formation, as well as osteoclastic bone resorption, and point to the potential of pharmacologic inhibition of pp60^{c-src} TK in bone resorbing cells as a therapeutic approach to inhibition of increased bone resorption and hypercalcemia.

The molecular mechanisms by which osteoclasts resorb bone are still unclear. In particular, the pathways by which osteoclasts transduce extracellular signals into the intracellular machinery that is required for bone resorption to occur is poorly understood. However, the recent observation that pp60^{c-src} TK-deficient mice manifest osteopetrosis in which osteoclast function is impaired has raised the possibility that pp60^{c-src} TK might mediate signal transduction in osteoclasts (1). Consistent with this notion, our data have shown that

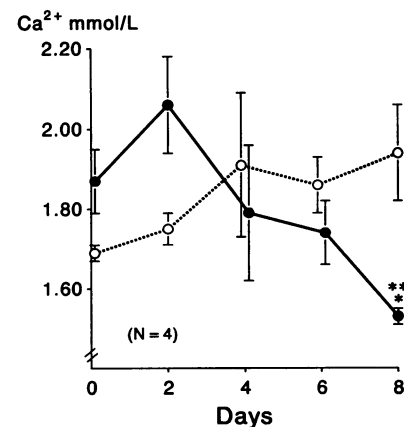


Figure 7. Effect of herbimycin A on tumor-induced hypercalcemia. Nude mice ($n = 8$) were inoculated with a human squamous cancer MH-85 subcutaneously. 6 wk later, when animals manifested hypercalcemia, herbimycin A (closed circles) 100 × µg/mouse per injection, two injections per day or DMSO (open circles) was given to these mice intraperitoneally every day for 8

d. Data shown are mean ± SE. *Significantly lower than DMSO-treated group ($P < 0.05$). **Significantly lower than animals before herbimycin A treatment ($P < 0.05$). — ○ —, DMSO; — ● —, herbimycin A (200 µg/mouse per day).

PTH, a potent stimulator of osteoclastic bone resorption, increased pp60^{c-src} TK levels in marrow cell cultures in which there were considerable numbers of osteoclasts. In addition, we have recently found that calcitonin inhibits pp60^{c-src} TK activity in marrow cell cultures and decreases resorption pit formation by TRAP(+)MNC (manuscript submitted for publication). Furthermore, we have also found recently that osteoclasts in pp60^{c-src} TK-deficient mice are not able to form ruffled borders and resorption pits in response to PTH and IL-1 (18). Taken together, these findings indicate that pp60^{c-src} TK mediates osteoclastic bone resorption and makes it feasible to use pp60^{c-src} TK inhibitors as potent inhibitors of bone resorption.

None of the currently available inhibitors of bone resorption are entirely satisfactory. Estrogens have only been used effectively in postmenopausal females and the mode of action of estrogen remains unclear. Bisphosphonates are effective, but are absorbed poorly when used orally. Again, their mode of action to inhibit bone resorption remains uncertain. In the case of herbimycin A, its inhibitory effects on bone resorption are most likely due to its capacity to inhibit pp60^{c-src} TKs, and this or related TK inhibitors that can be used orally may be useful therapies in common diseases of increased osteoclast activity such as postmenopausal osteoporosis, hypercalcemia of malignancy, and Paget's disease.

Although herbimycin A has been shown to inhibit pp60^{c-src} TK and have no effects on protein kinase A and protein kinase C (2), its absolute specificity for pp60^{c-src} TK is unknown. Since pp60^{c-src} TK is increased in active osteoclasts during bone resorption, it seems likely that the effects of herbimycin A to inhibit bone resorption are related to its capacity to inhibit pp60^{c-src} TK.

Toxicity was not likely to account for the effects we observed. Since herbimycin A is an irreversible inhibitor of pp60^{c-src}, we were not able to exclude toxicity by showing that transient exposure to herbimycin A would not impair bone resorption stimulated by PTH. However, herbimycin A did not affect the numbers of stromal cells and pits formed by osteoclasts, decrease protein synthesis by marrow cells, cause detachment of osteoclasts or stromal cells from either plastic culture dishes or dentine, or reduce control resorption in the organ culture assays. Herbimycin A-treated animals did not manifest any marked changes in food consumption, body weight, and motional activity.

In conclusion, our data suggest that herbimycin A may have therapeutic potential as a bone resorption inhibitor in disease states where bone resorption is enhanced.

Acknowledgments

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