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Variable Effects of Tyrosine Kinase Inhibitors on Avian Osteoclastic Activity and Reduction of Bone Loss in Ovariectomized Rats

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Abstract We compared the effects of the tyrosine kinase inhibitor genistein, a naturally occurring isoflavone, to those of tyrphostin A25, tyrphostin A47, and herbimycin on avian osteoclasts in vitro. Inactive analogs daidzein and tyrphostin A1 were used to control for nonspecific effects. None of the tyrosine kinase inhibitors inhibited bone attachment. However, bone resorption was inhibited by genistein and herbimycin with ID_{50}s of 3 μM and 0.1 μM, respectively; tyrphostins and daidzein were inactive at concentrations below 30 μM, where nonspecific effects were noted. Genistein and herbimycin thus inhibit osteoclastic activity via a mechanism independent of cellular attachment, and at doses approximating those inhibiting tyrosine kinase autophosphorylation in vitro; the tyrphostins were inactive at meaningful doses. Because tyrosine kinase inhibitors vary widely in activity spectrum, effects of genistein on cellular metabolic processes were compared to herbimycin. Unlike previously reported osteoclast metabolic inhibitors which achieve a measure of selectivity by concentrating on bone, neither genistein nor herbimycin bound significantly to bone. Osteoclastic protein synthesis, measured as incorporation of ^{3}H-leucine, was significantly inhibited at 10 μM genistein, a concentration greater than that inhibiting bone degradation, while herbimycin reduced protein synthesis at 10 nM. These data suggested that genistein may reduce osteoclastic activity at pharmacologically attainable levels, and that toxic potential was lower than that of herbimycin. To test this hypothesis in a mammalian system, bone mass was measured in 200 g ovariectomized rats treated with 44 μmol/day genistein, relative to untreated controls. During 30 d of treatment, weights of treated and control group animals were indistinguishable, indicating no toxicity, but femoral weight in the treated group was 12% greater than controls ($P < 0.05$). Our data indicate that the isoflavone inhibitor genistein suppresses osteoclastic activity in vitro and in vivo at concentrations consistent with its ID_{50}s on tyrosine kinases, with a low potential for toxicity.

Key words: bone resorption, tyrphostins, genistein, herbimycin, osteoporosis

Genistein is a naturally occurring trihydroxy isoflavone tyrosine kinase inhibitor. It inhibits tyrosine kinase activity on exogenous substrates of EGF receptor, v-*src*, and pp110$^{gag\text{-}fes}$, all with ID_{50}s of 24–30 μM, and inhibits EGF-receptor autophosphorylation at ~3 μM [Akiyama et al., 1987].

Osteoclasts have an unusual dependence on *src* as shown by knockout experiments [Soriano et al., 1991]. While the function of *src* in osteoclasts is not well characterized, it has been determined that pp60$^{c\text{-}src}$ is required for bone resorption [Boyce et al., 1992]. In keeping with these observations, osteoclastic bone resorption was found to be inhibited by herbimycin A, a tyrosine kinase inhibitor with IC_{50} of ~0.1 μM for *src*, with IC_{50} of ~0.1 μg/ml (0.2 μM) [Yoneda et al., 1993]. However, herbimycin at this dose also dramatically reduced the number of tartrate-resistant acid phosphatase–positive cells, a standard assay for osteoclasts, in 6 d mouse marrow cell cultures [Yoneda et al., 1993]. This suggests that this extremely potent inhibitor, a benzoquinonoid antibiotic that reacts with reactive sulfhydryl groups including those of many tyrosine kinases [Fukazawa et al., 1990], is cytotoxic at concentrations inhibiting osteoclasts.

Additional tyrosine kinase inhibitors include tyrphostins [Levitzki, 1990], cyanoalkyl tyro-

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sine analogs, which are also chemically reactive. However, genistein, 4′,5,7-trihydroxyisoflavone, is structurally less likely to irreversibly react with cellular proteins than benzoquinone or cyanide inhibitors. Thus, while genistein requires higher concentrations to reduce tyrosine kinase activity, it was hypothesized to be less nonspecifically cytotoxic.

We compared the effects of genistein, tyrphostins A25 and A47, and herbimycin, along with inactive genistein and tyrphostin analogs as controls, on avian osteoclasts in vitro. Genistein and herbimycin inhibited osteoclastic bone resorption at concentrations consistent with tyrosine kinase inhibition, but the tyrphostins were not useful inhibitors at doses 10 times their IC_{50}s for tyrosine kinase receptor autophosphorylation. Further tests included recovery of cellular activity and protein synthesis after treatment with tyrosine kinase inhibitors and washout, using avian osteoclasts in vitro, and investigation of the effects of genistein on femoral and body mass in vivo, using ovariectomized rats. We report that genistein reversibly inhibits osteoclastic activity in vitro with minimal effects on cellular viability as compared to herbimycin, and increases bone mass of ovariectomized rats relative to controls without measurable toxic effects.

METHODS

Isoflavonoids were obtained from Protein Technologies International (St. Louis, MO) as partially purified concentrates and isolated as described [Peterson and Barnes, 1991]. Concentrations of isoflavonoids were determined by reversed-phase HPLC analysis [Barnes et al., 1994]. Other tyrosine kinase inhibitors and control substances were from Calbiochem (San Diego, CA). Inhibitors were prepared at > 1,000 times the working concentration in dimethyl sulfoxide, and equal concentrations of dimethyl sulfoxide were added to controls.

Cell Cultures

Osteoclasts were isolated from calcium-starved laying hens, *Gallus domesticus*. A diet without added calcium (Purina 5070-9) causes large numbers of osteoclasts to be produced in the medullary bone to provide calcium for egg production. These osteoclasts, ~ 50% of the cell mass in the medullary bone, were washed through a 110 μm nylon filter, and the preparation was enriched in osteoclasts by sedimenting the cell population through 70% serum as described [Blair et al., 1986], which raises the osteoclast content to ~ 80%. Osteoclast purity was then raised to essential homogeneity by allowing the cells to attach to 500 μg of 20–40 μm bone fragments in 10 cm tissue culture plates. Other cells attach nonspecifically according to where they settle, 99% being cell culture plastic; after 36 hr the bone fragments with attached osteoclasts were collected by washing and separated from unattached cells by sedimentation. Cells were plated in 2 cm^2 wells in Dulbeco's modified Eagle's minimal essential medium with 5% fetal calf serum, 5% chicken serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cultures were maintained at 37°C in humidified air with 5% CO_2.

Osteoclasts in the bone-attached fraction were > 98% in the preparations used, as determined by 121F monoclonal antibody (gift of Dr. Philip Osdoby, Washington University, St. Louis) binding [Oursler et al., 1985]. Briefly, cells fixed 2 hr in 1% phosphate-buffered formalin at 4°C were incubated 30 min at 20°C with 121F antibody diluted 1:100 with phosphate-buffered saline, 0.05% polyoxyethylene sorbitan monooleate, and 1% bovine serum albumin, washed, and incubated with fluoresceinated goat anti-mouse (Sigma) at 1:500 dilution to label 121F bound, washed, and examined by epifluorescence using 450–490 nm excitation and a 520 nm barrier filter.

Bone Attachment of Cells

One hundred micrograms of 20–40 μm bone was added to 10^4 osteoclasts in 2 cm^2 tissue culture wells as for bone resorption, or to no-cell control wells. After 4 hr incubation, during which an insignificant portion (< 2%) of the total labeled material was degraded, the wells were gently washed twice with phosphate-buffered saline at 20°C. Retained bone was dissolved in 100 μl of 6 N HCl overnight, neutralized with NaOH, and measured by scintillation counting relative to no-cell controls. Control (untreated) cells bound 40–60% of added bone fragments; results are indicated as binding relative to matched positive and negative controls run concurrently.

Adsorption of Inhibitors to Bone Substrate

Effect of adsorbed inhibitors was determined as described [Carano et al., 1990] by preincubating bone substrate (100 μg) in the inhibitor at a

known effective concentration (as described in Results), washing the substrate in PBS, and adding the preincubated washed substrate to untreated osteoclasts, with resorption of the preincubated substrate compared to resorption of substrate treated identically except for omission of the inhibitor. To determine directly whether a significant fraction was adsorbed, concentrations of selected inhibitors in supernatant were measured by HPLC essentially as described [Barnes et al., 1994] after preincubation of 10–100 μM inhibitor for 2 d in 1 ml of PBS containing 50 mg of 20–40 μm bone fragments, separation of supernatant from substrate by centrifugation (10,000 × *g*, 1 min), and decanting.

Bone Resorption

One hundred micrograms of 20–40 μm fragments of devitalized rat bone, metabolically labeled with L-[2,3,4,5-^{3}H]-proline as described [Teitelbaum et al., 1979], was added to bone affinity-purified osteoclast cultures with 10^4 cells in 1 ml of medium in 2 cm^2 wells of 24-well tissue culture plates. Bone resorption, relative to culture time, is thus indicated directly by ^{3}H recovered in supernatants of 10^4 osteoclasts. This assay uses the cross-linked, insoluble component of bone to determine resorption; results reflect complete hydrolysis of mineral and nonmineral components, are essentially unaffected by physicochemical exchange, and are highly reproducible; degradation fragment analysis and comparison with results of mineral labeling showing good correlation with other bone degradation assays is reported [Blair et al., 1986]. Activity of cell-free controls was subtracted, and substrate-specific activity was then used to convert counts to micrograms of bone resorbed. Substrate specific activity was determined by total hydrolysis (6 N HCl, 24 hr, 60°C) and scintillation counting.

Protein Synthesis

Protein synthesis by cultured cells was assessed using ^{3}H-leucine incorporation [Blair et al., 1989]. Specific conditions: Cells were incubated in 1 μCi/ml of L-[4,5-^{3}H]-leucine (Amersham), 143 Ci/mM, in complete medium (i.e., also containing 0.5 mM unlabeled leucine) for 6 hr, and washed twice with PBS. Isotope incorporated into macromolecules was fixed 30 min in 10% trichloroacetic acid and 30 min in 5% trichloroacetic acid; lipids were removed with ethanol/ether (3:1, v/v) to reduce background; protein was solubilized by digestion in 0.1 M NaOH and radioactivity determined by scintillation counting.

Ovariectomized Rat Bone Mass

Ovariectomized rats were obtained from Charles River Laboratories (Wilmington, MA) 1 d after operation, weighed, and pair-fed test diet AIN 76A with control diet or diet containing test isoflavonoids at 44 μMol per day per 250 g (average weight in test period) rats to give peak serum levels ~10 μM [Xu et al., 1994]. This diet [American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies, 1977] is a semi-synthetic casein and cornstarch-based mixture. It is soy-free to avoid natural sources of isoflavonoids, and contains standard rat dietary calcium (0.5% elemental calcium as $CaHPO_4$) with 1.25% phosphate. This diet is favorable for the study of rat osteoporosis in that, while the calcium intake is optimal, it avoids the 1:1 to 1:1.5 calcium to phosphate ratios that are protective against osteoporosis in the rat [Draper et al., 1972]. Animals were weighed weekly for 4 weeks and then killed. Femora were dissected, dried 4 weeks in a desiccator, weighed to obtain dry tissue weight, and ashed at 700°C to obtain mineral weight.

Statistics

Data show mean ± standard deviation of quadruplicate determinations unless noted; where a point has no error bar, standard deviation is less than symbol size. Where a difference is concluded, comparisons reject the null hypothesis, at 0.05, by Student's *t* test.

RESULTS

Osteoclastic activity depends on bone attachment, since the degradative process takes place in an isolated extracellular acid compartment. Thus, we first determined the effects of the tyrosine kinase inhibitors genistein, tyrphostin A25, tyrphostin A47, and herbimycin on 4 hr bone binding by avian osteoclasts in vitro. Inactive analogs of genistein and the tyrphostins, daidzein and tyrphostin A1, were used to control for nonspecific effects. None of the tyrosine kinase inhibitors had significant effects on bone attachment at 4 hr when administered at doses approximating the IC_{50}s of typical tyrosine kinase targets (Fig. 1A). However, dose–response

study showed reduced bone binding at 30 μM for the isoflavonoids and tyrosine analogs, an example of which (genistein) is shown (Fig. 1B).

Bone resorption was inhibited by genistein and herbimycin with ID_{50}s of 3.0 and 0.1 μM, respectively (Fig. 2A,B). Daidzein was inactive at concentrations below 30 μM, where inhibition was seen, delimiting nonspecific isoflavonoid effects. Results for herbimycin appeared to show plateauing above 0.1 μM. However, when measurements were made after 2 d of exposure (Fig. 2B, lower trace), complete inhibition was seen above 0.1 μM. The differences between the 0–120 hr and 48–120 hr curves were essentially constant, showing that the effect is mainly confined to the later time period. This finding probably reflects delayed irreversible cell damage (see washout and cell protein synthesis, below). Dose–response profiles of the other compounds were similar whether experiments were run after substance preincubation or if substances were added at the same time as labeled bone. Tyrphostins were inactive at concentrations below 30 μM, where minor (~20%) inhibition was noted, nonspecific as indicated by decreased resorption with all of the compounds including the tyrosine kinase–inactive tyrphostin A1 (Fig. 2C). Possible reasons for inactivity of the tyrphostins include metabolism by the target cell (Discussion).

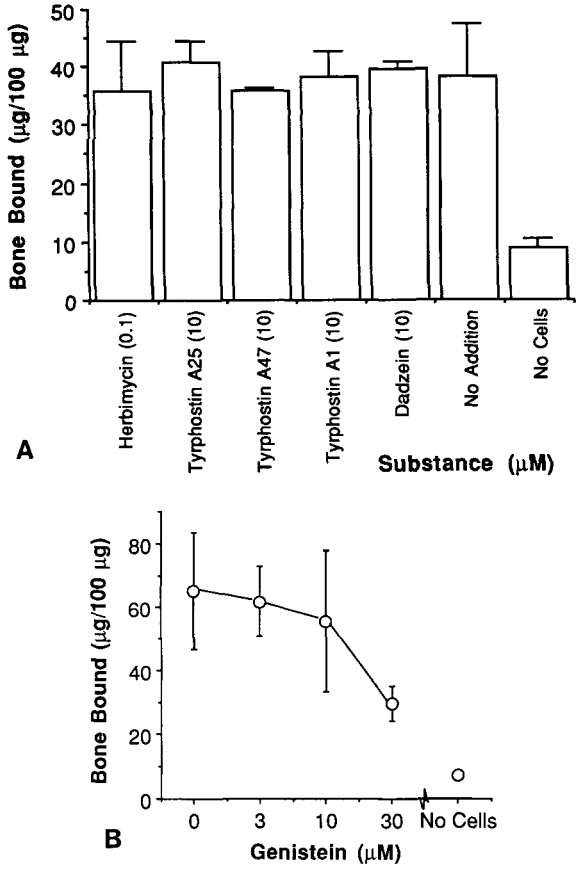

Fig. 1. Effects of tyrosine kinase inhibitors and control compounds on bone attachment by cultured avian osteoclasts. The amount of 100 μg added ^{3}H-proline-labeled bone that was bound during 4 hr incubation by 10^4 avian osteoclasts in 2 cm^2 tissue cultures was determined by washing unbound material, hydrolyzing the retained bone, and scintillation counting. Mean ± standard deviation, n = 4. **A:** Herbimycin and tyrphostins A25 and A47 at several times the IC_{50} for tyrosine kinases, and the control substances daidzein and tyrphostin A1 at matched concentrations, have no effect relative to 4 hr bone attachment of control cells (second column from right). The far right column shows bone nonspecifically retained in the assay, ~20% of cell bound levels. **B:** Dose–response of bone bound at 4 hr as a function of genistein concentration. Similar results were obtained with the tyrphostins including the control compound tyrphostin A1, and daidzein, suggesting that 30 μM represents a toxic level.

The cellular and biochemical mechanisms of genistein and herbimycin inhibition were investigated further. Other osteoclast inhibitors achieve selectivity in part because most of the compounds are adsorbed to bone (which is largely hydroxyapatite), thereby removing the majority of compounds such as bisphosphonates and group III metals [Carano et al., 1990; Blair et al., 1992] from solution. Thus, as an initial step we determined whether genistein, herbimycin, or daidzein would accumulate on bone. Because the only available direct assay, reversed-phase HPLC, has accuracy in the ±10% range, a more stringent test was achieved by using a ratio of bone surface area to inhibitor 5–50 times that used in resorption assays. Ten to one hundred micromolar concentrations were incubated 2 d with 50 mg of 20–40 μm sieve size bone fragments (~150 cm^2 exposed hydroxyapatite surface) in a 1 ml solution, and concentration was measured relative to controls (Fig. 3). Concentrations were unaffected within the accuracy of this measurement. As an additional control, 100 μg aliquots of bone were incubated with 10 μM genistein or 0.1 μM herbimycin, the bone substrates were washed, and osteoclastic bone degradation was measured relative to untreated bone. Differences between groups were insignificant. Thus, bone surface adsorption is probably unimportant for these compounds.

Since significant surface adsorption is effectively excluded, a useful measure of irreversible cellular effects is to preincubate osteoclasts on bone with the tyrosine kinase inhibitors, change

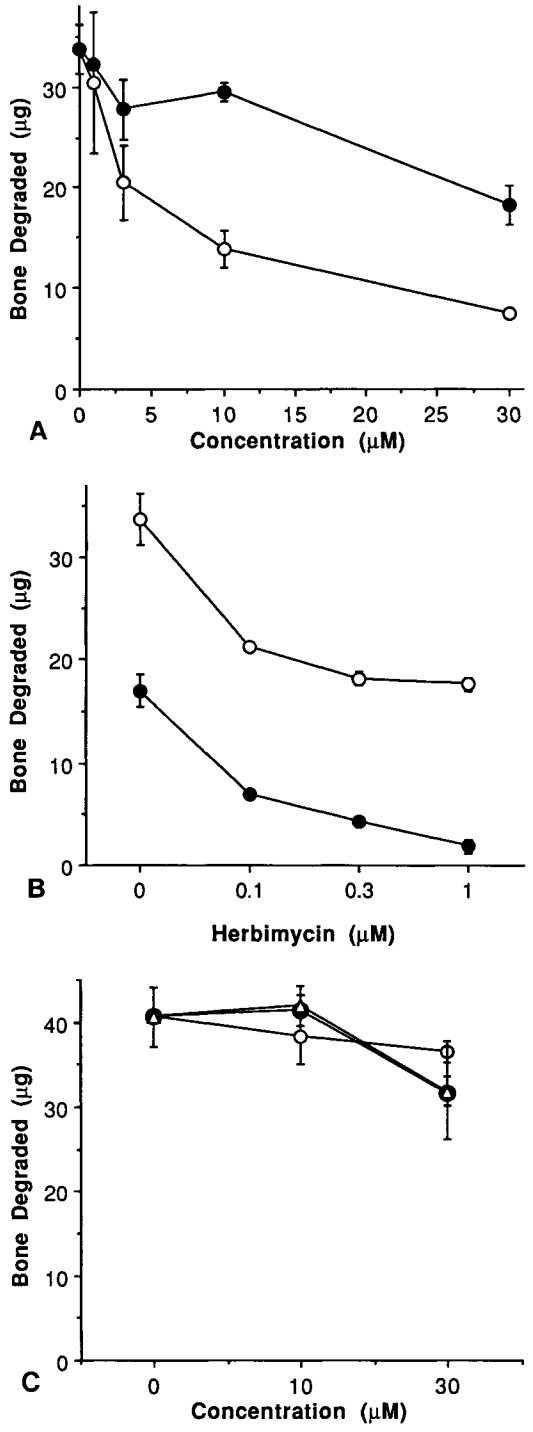

Fig. 2. Effects of tyrosine kinase inhibitors and control compounds on bone resorption by avian osteoclasts. The amount of 100 μg ^{3}H-proline labeled bone substrate that was degraded during incubation was determined by scintillation counting of supernatants. No-cell controls are subtracted; bone degraded is expressed as micrograms dry weight using specific activity determined by total hydrolysis of the substrate (Methods). Mean ± standard deviation, n = 4. **A:** Bone resorption from 0 to 120 hr as a function of genistein (○) and daidzein (●) concentrations. The tyrosine kinase–inactive isoflavone daidzein inhibits at 30 μM. **B:** Bone resorption as a function of concentration for herbimycin at 0–96 hr incubation (○) and from 48 to 96 hr (●). The plateau in resorption at 0.3–1 μM reflects delayed inhibition, as is seen by the complete inhibition in the latter half of this period. Note that to improve clarity in this case, concentrations are in logarithmic scale, except for zero concentration. **C:** Bone resorption from 1 to 96 hr in the presence of tyrphostins A1 (○), A25 (△), and A47 (●). Effects seen are small and not statistically meaningful, although long exposure at 30 μM shows effects in other experiments.

the supernatant, washing out the compounds, and study recovery of bone resorption. The results of this washout experiment (Fig. 4) show activity on washout similar to the inhibition curves for the compounds, but exaggerating the inhibition at high doses, herbimycin above 0.1 μM and genistein above 10 μM.

As a more specific measure of cellular toxicity, 6 hr incorporation of ^{3}H-leucine into protein was measured relative to untreated controls after 60 hr preincubation to indicate long-term effects of the tyrosine kinase inhibitors on this basic cellular synthetic apparatus (Fig. 5). This showed that protein synthesis by cells was moderately but significantly inhibited by genistein at 10 μM, and 40% inhibited at 30 μM, the highest concentration tested. In contrast, herbi-

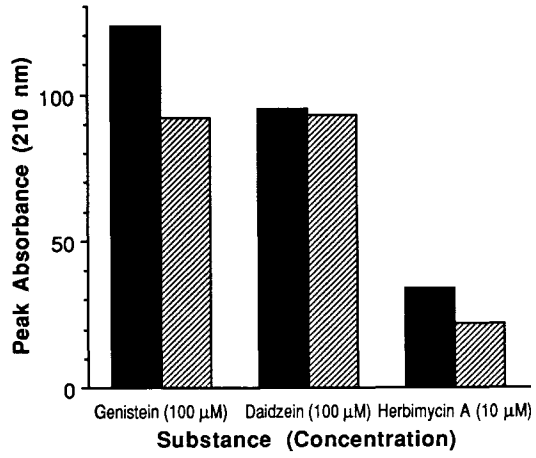

Fig. 3. Genistein, daidzein, and herbimycin do not concentrate on bone. Substances were incubated 2 d at indicated concentrations with 50 mg of 20–40 μm bone, 5–50-fold the bone surface to inhibitor ratio at which 50% inhibition is seen with genistein or herbimycin (Fig. 1), and solution concentration was determined by HPLC relative to no-bone controls (right bar of each pair). No reduction in solution concentration is seen within the accuracy of this test. Bone incubated with these compounds, washed, and then added to resorption assays was also not resorbed at reduced rates (not illustrated, see text).

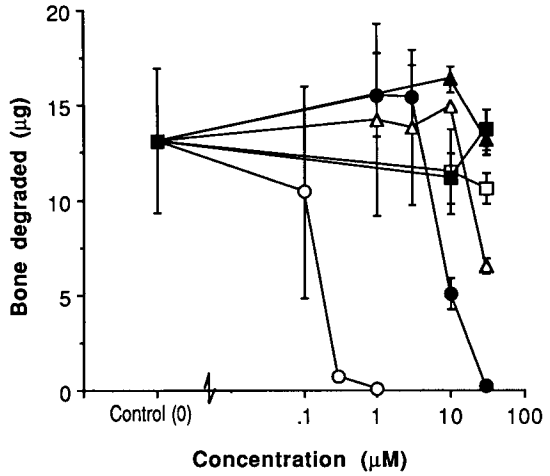

Fig. 4. Irreversible effects of tyrosine kinase inhibitors on osteoclasts. Osteoclasts (10^4/2 cm^2) were incubated on 100 μg of 20–40 μm bone for 48 hr at the indicated concentrations of herbimycin (○), genistein (●), daidzein (△), tyrphostin A1 (▲), tyrphostins A25 (□), and tyrphostin A47 (■). Cultures were washed, media replaced, and resorption measured over an additional 48 hr. Above 0.1 μM herbimycin and 10 μM genistein, there is no measurable activity.

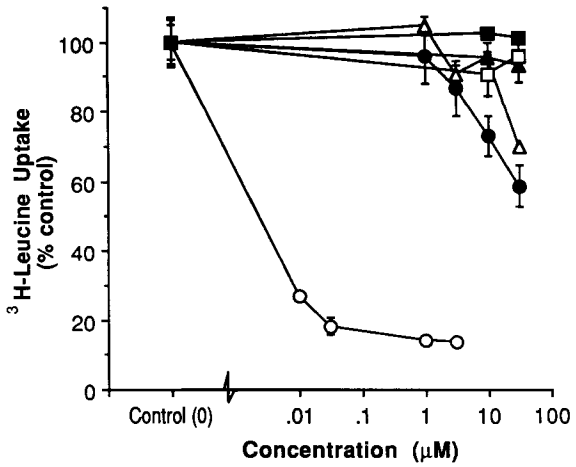

Fig. 5. Effects of tyrosine kinases and control compounds on protein synthesis. Osteoclasts were incubated in the indicated concentrations of added compounds or vehicle alone (control, zero concentration) for 60 hr, and 6 hr ^{3}H-leucine incorporation into trichloroacetic acid–insoluble material was determined. Data indicate percent of control uptake to allow presentation of several experiments on one graph; control uptake varied from 8 to 20 × 10^3 cpm. Herbimycin (○); genistein (●); daidzein (△); tyrphostin A1 (▲); tyrphostin A25 (□); tyrphostin A47 (■). Note that herbimycin inhibits protein synthesis at concentrations below those affecting short-term bone degradation, but genistein does not. In this case, n = 8, mean ± standard error of the mean.

mycin had profound effects, with average protein synthesis reduced 60% after 2.5 d preincubation even at doses of 0.01 μM, the lowest concentration tested, a log order below the concentration reducing short-term bone degradation. In addition, examination of tissue culture plates treated with herbimycin at 30 nM or greater showed cell fragmentation and detachment by 4 d, indicating widespread cell death (not illustrated). These findings together suggested that this highly sulfhydryl-reactive tyrosine kinase inhibitor causes irreversible cell damage at low doses, and is less likely to be useful in studying cells other than for short-term experiments. As with resorption experiments, effects of the tyrphostins were minor at all doses tested. However, the 30 μM doses of all the isoflavone and tyrphostin compounds showed some cell loss relative to controls after 120 hr incubation.

The osteoclast activity data suggested that genistein may be useful as an osteoclastic inhibitor at pharmacologically attainable levels. To test this hypothesis in a mammalian system, 200 g ovariectomized rats were fed a diet containing 44 μmol/day genistein or its 7-0-β-glucoside conjugate genistin, a natural metabolite; controls received an identical diet except that isoflavones were excluded. As a measure of toxicity, weight gain was recorded and compared to controls. During 30 d of treatment, weight of treated and control group animals was indistinguishable (Fig. 6A). Further, behavior and appearance of animals was indistinguishable between groups. Thus, there was no identifiable toxic effect. Dry femoral weight in the genistein-treated group was 12% greater than controls ($P < 0.05$); genistin had a smaller effect that was not statistically different (Fig. 6B). Femoral ash weights gave results similar to those of dry weight (Fig. 6C), although the mean mineral weight of the genistein-treated group was slightly less elevated over the control (9%) than was the dry weight (total bone mass), and standard error in the control group was slightly larger, reducing the statistical difference. These data show, with ~95% confidence, that genistein is effective in reducing bone loss relative to controls in ovariectomized rats.

DISCUSSION

Substances influencing bone resorption are of wide interest because of their potential value as antiosteoporotic agents. The discovery that osteoclasts have an unusual dependence on *src* [Soriano et al., 1991] led to interest in the use of tyrosine kinase inhibitors as osteoclast-modify-

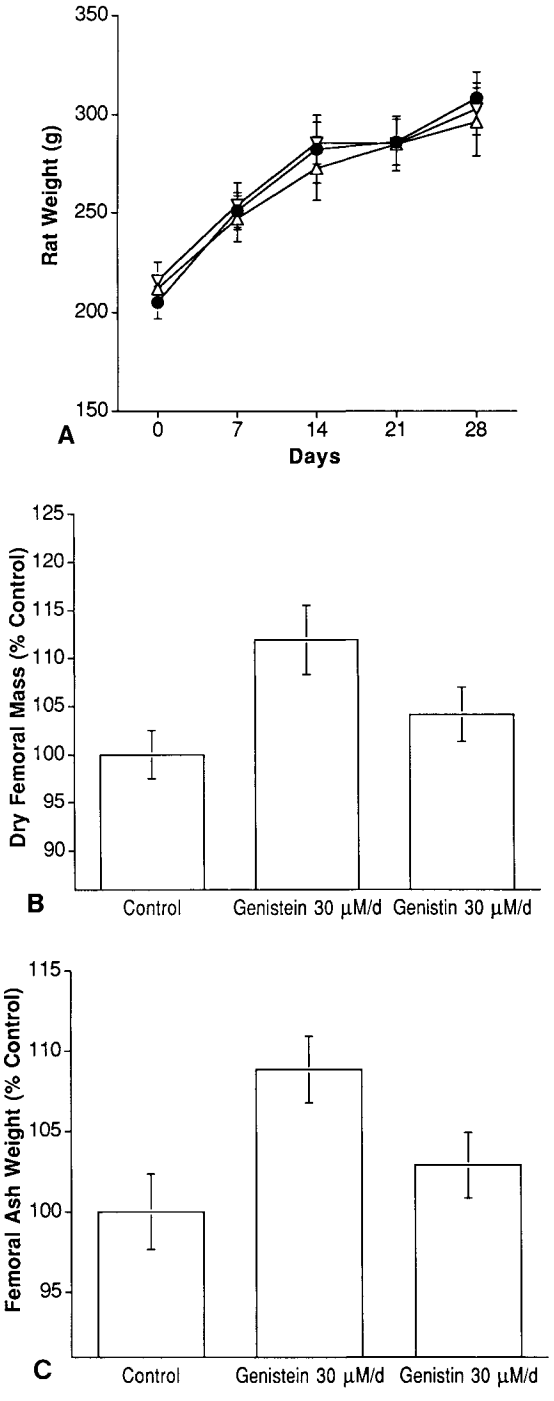

Fig. 6. Effect of genistein and its seven-glucoside conjugate genistin on growth and bone mass of ovariectomized rats. Rats were fed a diet containing 44 μmol/day of the indicated compounds using a semisynthetic diet with no isoflavones other than the added substances. Animals were killed on day 30 and femora dissected for analysis (Methods). **A:** Weights of control and treated groups were invariant over the treatment period, an indicator of low toxicity. Control (●); genistein (△); genistin (▽). n = 4, mean ± standard deviation. **B:** Femoral dry weights as percent control. Dry weights of femora in the genistein-treated group are statistically different from controls ($P < 0.05$), but the genistin group is not. n = 8, mean ± standard error of the mean. **C:** Ash weight of genistein-treated femora is marginally different from controls ($P = 0.07$); genistin gives intermediate values that are not statistically different from controls.

ing compounds for research and therapeutic use. Boyce et al. [1992] showed that $pp60^{c\text{-}src}$ is required for bone resorption, and bone resorption is reduced by the tyrosine kinase inhibitor herbimycin A, which has similar IC_{50}s for *src* and osteoclast activity [Yoneda et al., 1993].

However, tyrosine kinase inhibitors are for the most part extremely toxic. Herbimycin is a benzoquinonoid antibiotic that reacts with sulfydryl groups of tyrosine kinases [Fukazawa et al., 1990], and it is cytotoxic at concentrations inhibiting osteoclasts (cf. Fig. 5). Other tyrosine kinase inhibitors include the tyrphostins, cyanoalkyl tyrosine analogs, which are also chemically reactive and which are proposed as antitumor agents [Levitzki, 1990]. On the other hand, genistein, 4′,5,7-trihydroxyisoflavone, is a widely occurring compound that is relatively chemically nonreactive but which is nevertheless a well characterized tyrosine kinase inhibitor. At concentrations ~10–50-fold greater than those of herbimycin, genistein displays similar tyrosine kinase inhibition, including EGF receptor, v-*src*, and $pp110^{gag\text{-}fes}$ [Akiyama et al., 1987].

We hypothesized that genistein would inhibit osteoclasts at concentrations consistent with its inhibition of *src*, but that it would be less cytotoxic than other inhibitors. We compared the effects of genistein, tyrphostins A25 and A47, and herbimycin on avian osteoclasts in vitro, with inactive isoflavone and tyrphostin controls. None of these compounds affected short-term cell–bone binding (Fig. 1), although genistein and herbimycin inhibited bone degradation (Fig. 2) with half-maximal inhibition at ~3 μM and ~0.1 μM, respectively. The effects of herbimycin on bone degradation observed here are consistent with studies measuring rodent osteoclast pits [Yoneda et al., 1993]. This is the expected result, since osteoclastic cellular processes are conserved in higher vertebrates, but nevertheless a useful positive control; dose–response was essentially identical to that reported for rodent cells. The herbimycin study of Yoneda et al. [1993] used a mixed cell culture system, so it was possible that effects of herbimycin might be mediated by other cells; our study using purified osteoclasts essentially rules out this possibility.

On the other hand, the tyrphostins were surprisingly inactive. Neither tyrphostin A25 nor A47 inhibited osteoclasts at 10 times the IC_{50}s for tyrosine kinase receptor autophosphorylation. Inactivity of the tyrphostins may be due to cellular impermeability to these cyanoalkyl amino acid analogs, which would be expected to be poorly transported. Tyrphostin metabolism by osteoclasts, either elimination of active forms or lack of metabolic activation, is also possible. Further, differences in the spectrum of tyrosine kinase inhibition with those expressed by the osteoclast could produce negative results. The former two possibilities would seem to be more likely since two tyrphostin compounds were tested that have relatively wide tyrosine kinase inhibitor profiles.

We investigated whether the active compounds, genistein or herbimycin, adsorb to bone (80% hydroxyapatite) as do gallium III and several bisphosphonates, a factor important to the specificity of their skeletal effects [Carano et al., 1990; Blair et al., 1992]. Effects of these substances were not reproduced using bone preconditioned with the inhibitors, and solution concentration was not measurably altered after incubation with large surface areas of fragmented bone (Fig. 3). Thus, action of these compounds does not involve significant bone binding, and cellular activity after washout of supernatant should not be affected by significant amounts of substances retained bound to bone. After washout of substances added (Fig. 4), cultures recovered >80% of control activity when treated at doses of genistein up to 10 μM. On the other hand, cells inhibited by herbimycin did not recover their activity above a dose of 0.1 μM.

We studied protein synthesis to determine whether this was differentially affected by genistein and herbimycin, which proved to be the case (Fig. 5). Indeed, long-term exposure to herbimycin doses as low as 0.01 μM, one-tenth the IC_{50}, dramatically reduced cellular protein ^{3}H-leucine uptake. On the other hand, effects of genistein were moderate, with 40% inhibition at 30 μM, 10 times the effective dose for short-term inhibition of osteoclastic activity. Thus, irreversible metabolic inhibition by genistein occurs at 2–10 times the concentration inhibiting osteoclastic activity, whereas long-term exposure to herbimycin is toxic at doses below its IC_{50} for *src* or, in keeping with this, concentrations inhibiting osteoclastic activity over times less than 48 hr.

These results also suggested that genistein might, in addition to its utility as a tool in dissecting osteoclastic biochemical pathways in vitro, be useful in vivo as an antiosteoporotic compound. We therefore measured the effects of genistein and a major form of genistein found in foods, the glycoside conjugate genistin, on ovariectomized rats. We found that genistein had no measurable toxic effect at dietary doses amounting to 0.1% of dietary mass, 44 μmol per day per 250 g rat (Fig. 6A), but increased bone mass relative to controls of ovariectomized rats at the 95% confidence level (Fig. 6B,C). The activity of genistin was much lower, and not statistically different from controls.

There are precedents for isoflavone effects on bone remodeling. 7-Isopropoxyisoflavone (ipriflavone) at 0.6–1.2 g/day gave beneficial effects in human studies of Paget's disease [Agnusdei et al., 1992], primary hyperthyroidism [Mazzuoli et al., 1992], and increased bone mass in postmenopausal women [Nakamura et al., 1992]. The ipriflavone doses reported in these studies emphasize the typically low toxicity of this class of compounds. The mechanism of these effects is poorly understood and somewhat controversial. The major effects of ipriflavone have been attributed to increased osteoblast activity [Benvenuti et al., 1991; Notoya et al., 1994]. Osteoblastic effects of ipriflavone are probably not relevant to our osteoclast findings; this cellular element is unrelated to osteoclasts, and was excluded from our experimental system. However, effects of genestein on osteoblasts and cell types other than bone cells are a potential factor in the results of the ovariectomized rat bone-mass study. Lack of bone binding by tyrosine kinase inhibitors (Fig. 4) indicates that bone specificity of tyrosine kinase inhibitors is unlikely. These possibilities, including assessment of changes in other organs over longer times, will require study beyond the scope of the present work. On the other hand, Tsuda et al. [1986] found that ipriflavone and several of its metabolites at 10 μg/ml inhibited ^{45}Ca release from fetal rat bone, and Albanese et al. [1994] found that ipriflavone reduced spread area and pit formation, and increased intracellular calcium, in rat osteoclasts. These findings are consistent with our results.

We conclude that genistein and herbimycin inhibit osteoclastic activity directly, by a mechanism independent of cellular attachment, at doses approximating those inhibiting tyrosine

kinase autophosphorylation in vitro. The effect on skeletal mass of genistein (Fig. 6) is attributed to the unusual expression of *src* in osteoclasts and their sensitivity to *src* knockout [Soriano et al., 1991]. Our results with genistein do not rule out other osteoclast inhibition mechanisms unrelated to tyrosine kinases. However, since the dose–response matches ID_{50}s of tyrosine kinase inhibition, this is likely a principal mechanism. Since the effects of genistein are observed at doses preserving cell viability, this compound will be particularly useful in the definition of secondary cellular consequences of tyrosine kinase pathway inhibition.

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