Peptidomimetic antagonists of $\alpha\text{v}\beta 3$ inhibit bone resorption by inhibiting osteoclast bone resorptive activity, not osteoclast adhesion to bone

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Abstract

Osteoclasts are actively motile on bone surfaces and undergo alternating cycles of migration and resorption. Osteoclast interaction with the extracellular matrix plays a key role in the osteoclast resorptive process and a substantial body of evidence suggests that integrin receptors are important in osteoclast function. These integrin receptors bind to the Arg-Gly-Asp (RGD) sequence found in a variety of extracellular matrix proteins and it is well established that the interaction of osteoclast $\alpha\text{v}\beta 3$ integrin with the RGD motif within bone matrix proteins is important in osteoclast-mediated bone resorption. In this study, we characterized the effects of two synthetic peptidomimetic antagonists of $\alpha\text{v}\beta 3$, SC-56631 and SC-65811, on rabbit osteoclast adhesion to purified matrix proteins and bone, and on bone resorption *in vitro*. SC-56631 and SC-65811 are potent inhibitors of vitronectin binding to purified $\alpha\text{v}\beta 3$. Both SC-56631 and SC-65811 inhibited osteoclast adhesion to osteopontin- and vitronectin-coated surfaces and time-lapse video

microscopy showed that osteoclasts rapidly retract from osteopontin-coated surfaces when exposed to SC-56631 and SC-65811. SC-56631 and SC-65811 blocked osteoclast-mediated bone resorption in a dose-responsive manner. Further analysis showed that SC-65811 and SC-56631 reduced the number of resorption pits produced per osteoclast and the average pit size. SC-65811 was a more potent inhibitor of bone resorption and the combination of reduced pit number and size led to a 90% inhibition of bone resorption. Surprisingly, however, osteoclasts treated with SC-65811, SC-56631 or the disintegrin echistatin, at concentrations that inhibit bone resorption did not inhibit osteoclast adhesion to bone. These results suggest that $\alpha\text{v}\beta 3$ antagonists inhibited bone resorption by decreasing osteoclast bone resorptive activity or efficiency but not by inhibiting osteoclast adhesion to bone *per se*.

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Introduction

Osteoclasts attach to the bone surface and develop polarized and specialized cell membrane areas. The osteoclast ruffled border and circumferential sealing zone juxtaposed with the bone surface define the limits of the resorption pit. The sealing zone forms a tight attachment of the cell membrane to the bone surface isolating the resorption lacunae from the extracellular environment, permitting development and maintenance of an acidic, protease-rich microenvironment favorable for bone degradation (Horton & Rodan 1996).

Osteoclasts actively migrate on bone surfaces and undergo alternating cycles of migration and resorption (Kanehisa & Heersche 1988, Lakkakorpi & Vaananen 1991). Ruffled borders and sealing zones are seen only in

non-motile osteoclasts during the resorption phase and subsequently disappear during the course of osteoclast migration (Kanehisa & Heersche 1988). Osteoclast interaction with extracellular matrix (ECM) plays a key role in the migratory and resorptive phases of the osteoclast cycle and a substantial body of evidence suggests that integrin receptors play a key role in the osteoclast resorption process (Horton & Rodan 1996).

Integrins are transmembrane α/β heterodimers that mediate cellular interactions with other cells and ECM components. The integrin family contains at least 12 α subunits that associate with at least nine β subunits, producing a variety of heterodimer combinations with different cell expression patterns and ligand-binding activity. Many integrins bind to the Arg-Gly-Asp (RGD) consensus sequence found in a variety of ECM proteins

(Ruoslahti 1996). Bone contains a variety of RGD-containing proteins including collagen, thrombospondin, fibronectin, vitronectin, osteopontin and bone sialoprotein (Cheresh & Spiro 1987, Butler 1989, Clezardin *et al.* 1989, Robey *et al.* 1989, Reinholt *et al.* 1990) and it is clear that the interaction of osteoclast integrins with the RGD motif within bone matrix proteins plays an important role in osteoclast-mediated bone resorption (Cheresh & Spiro 1987, Butler 1989, Clezardin *et al.* 1989, Robey *et al.* 1989, Reinholt *et al.* 1990, Flores *et al.* 1992, Helfrich *et al.* 1992, Ross *et al.* 1993, Grano *et al.* 1994, Rodan & Rodan 1997).

Osteoclasts express high levels of $\alpha\text{v}\beta 3$ (the vitronectin receptor), $\alpha 2\beta 1$ (the collagen/laminin receptor) and $\alpha\text{v}\beta 1$ (Horton & Rodan 1996). Horton and colleagues were the first to demonstrate that integrins play an important role in osteoclastic bone resorption (Chambers *et al.* 1986, Davies *et al.* 1989). These investigators showed that the monoclonal antibody 13C2 recognized the osteoclast integrin receptor $\alpha\text{v}\beta 3$ and blocked bone resorption *in vitro*. Various studies have shown that RGD peptides, disintegrins, anti- $\alpha\text{v}\beta 3$ antibodies and RGD-peptidomimetic antagonists of $\alpha\text{v}\beta 3$ inhibit osteoclast adhesion to surfaces coated with RGD-containing proteins, detach adherent osteoclasts from surfaces coated with matrix protein and prevent bone resorption *in vitro* and *in vivo* (Sato *et al.* 1990, 1994, Horton *et al.* 1991, 1993, Helfrich *et al.* 1992, Chuntharapai *et al.* 1993, Fisher *et al.* 1993, Ross *et al.* 1993, King *et al.* 1994, Crippes *et al.* 1996, Engleman *et al.* 1997, Masarachia *et al.* 1998, Yamamoto *et al.* 1998). These results demonstrate that $\alpha\text{v}\beta 3$ plays a critical role in osteoclast-mediated bone resorption, although its precise role in the process is not yet understood.

The purpose of the present studies was to characterize the effects of two synthetic peptidomimetic antagonists of $\alpha\text{v}\beta 3$, SC-56631 and SC-65811, on bone resorption *in vitro*. In agreement with previous studies (Sato *et al.* 1990, 1994, Horton *et al.* 1991, Fisher *et al.* 1993, Ross *et al.* 1993, King *et al.* 1994, Crippes *et al.* 1996, Engleman *et al.* 1997, Masarachia *et al.* 1998, Yamamoto *et al.* 1998) we show that $\alpha\text{v}\beta 3$ antagonists inhibit osteoclast adhesion to purified bone matrix protein and osteoclast-mediated bone resorption. However, we found that these same inhibitors do not significantly inhibit osteoclast adhesion to bone. We conclude that $\alpha\text{v}\beta 3$ antagonists inhibit bone resorption by inhibiting osteoclastic bone resorptive activity but not by inhibiting osteoclast adhesion to bone *per se*, suggesting that the role of $\alpha\text{v}\beta 3$ integrin in osteoclast bone resorption is more complex than previously believed.

Materials and Methods

Reagents

Human plasma was obtained from the American Red Cross (St Louis, MO, USA). Frozen human placentas

were obtained from the Department of Pathology, Jewish Hospital (St Louis, MO, USA). NHS-biotin was from Pierce Chemical Company (Rockford, IL, USA). Tris buffer, O-phenylenediamine (OPD) substrate tablets and RIA grade BSA were obtained from Sigma (St Louis, MO, USA). Peroxidase-labeled and affinity-purified anti-biotin antibody was obtained from Calbiochem (San Diego, CA, USA). Pentex BSA fraction V was used in both cell adhesion and bone resorption assays and pre-screened to demonstrate that osteoclasts did not adhere to plastic surfaces coated with protein (Cat No. 81-053-1, lot No. 40; Miles, Kanakee, IL, USA). α -MEM was obtained from Sigma; penicillin and streptomycin were obtained from Gibco Brl (Grand Island, NY, USA).

The $\alpha\text{v}\beta 3$ antagonists β -[2-[[5-[[aminoiminomethyl] amino]-1-oxopentyl]amino]-1-oxoethyl]amino-3-pyridine propanoic acid, bistrifluoroacetate (SC-56631; see Engleman *et al.* 1997, Carron *et al.* 1998, Wong *et al.* 1998) and β -[[1-oxo-2-[[[3-[[[(phenylmethyl)amino]carbonyl] amino] phenyl]carbonyl]amino]ethyl]amino]-pyridine-3-propanoic acid, trifluoroacetate salt (SC-65811) were synthesized and obtained from the Department of Medicinal Chemistry, G D Searle (St Louis, MO, USA).

Purification of proteins

Human vitronectin fibrinogen and osteopontin were purified and biotinylated as previously described (Engleman *et al.* 1997). Full-length human osteopontin cDNA was expressed in *E. coli* as a $6 \times$ His-fusion protein using the QIA express pQE expression system as described by the manufacturer (Qiagen, Chatsworth CA, USA). Osteopontin was purified by nickel-nitriloacetic acid agarose affinity chromatography according to the manufacturer's recommendations (Qiagen) and then further purified by MonoQ (Pharmacia, Piscataway, NJ, USA) chromatography. Integrin $\alpha\text{v}\beta 3$ and the platelet receptor $\alpha\text{IIb}\beta 3$ were purified from human placenta and outdated platelets, respectively, as previously described (Pytela *et al.* 1987, Engleman *et al.* 1997).

Solid-phase receptor assay

SC-56631 and SC-65811 binding to $\alpha\text{v}\beta 3$ and $\alpha\text{IIb}\beta 3$ were characterized using a solid-phase competitive displacement assay as previously described (Engleman *et al.* 1997). Briefly, 96-well plates were coated with purified human vitronectin receptor ($\alpha\text{v}\beta 3$) or human fibrinogen ($\alpha\text{IIb}\beta 3$) receptor. Plates were washed with Tris-buffered saline containing 1 mM Ca^{2+} , Mg^{2+} , and Mn^{2+} , pH 7.4 (TBS+++) and blocked with TBS+++ containing 1% BSA before use. Serial dilutions of SC-56631 and SC-65811 were diluted in 2 nM biotinylated vitronectin in TBS+++ / 0.1% BSA and then added to plates coated with integrin receptor. The plates were washed with TBS+++ / 0.1% BSA and bound biotinylated vitronectin

detected with affinity-purified horseradish peroxidase (HRP)-labeled goat anti-biotin by incubation with HRP substrate (OPD/ H_2O_2 in 100 mM citrate buffer, pH 5.0) and the absorbance measured at 450–630 nm.

Isolation of rabbit osteoclasts

Animal work was carried out in the G D Searle animal facility in accordance with institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee and conform to the NIH Guidelines for the Ethical Care and Treatment of Animals. Animals were killed by decapitation and bone cells were isolated from the long bones of 2- to 6-day-old rabbits using sterile techniques. Rabbit osteoclasts were obtained as previously described (Kanehisa & Heersche 1988). Long bones were dissected free of soft connective tissues and the cartilaginous epiphyseal portions removed with a scalpel in ice-cold α -MEM containing 1% BSA, 100 U/ml penicillin and 100 U/ml streptomycin and kept on ice. The remaining metaphyseal portions were flushed with media and the bones were curretted and minced in 100 mm sterile bacteriological grade plastic Petri dishes and transferred to 50 ml conical centrifuge tubes. Cells and bone were triturated repeatedly in 25 ml media/rabbit to remove adherent cells, the bone fragments were allowed to settle for 5 min and the osteoclast-containing supernatant was used for experiments.

Osteoclast adhesion assay

Bovine femurs were obtained fresh and then stored frozen (-20°C). Cortical bone slices were cut using a low-speed diamond saw (Isomet Buehler Ltd, Lake Bluff, IL, USA) and 6 mm discs were punched out, sterilized and sonicated in 70% ethanol and then stored frozen until required for use. Bone discs were incubated in α -MEM containing 1% BSA, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 7% CO_2 , for 1 h before use. Osteopontin-coated Costar 96-well plates and wells containing 6 mm circular bone slices were incubated in α -MEM containing 1% BSA and 20 mM HEPES buffer for 1 h at 37°C ; one 6 mm circular bone disc completely occupied the surface area of a Costar 96-well plate. Osteoclasts were diluted in α -MEM containing various concentrations of SC-56631, SC-65811 or echistatin (BACHEM, Torrance CA, USA) and added to osteopontin-coated wells or wells containing bone and incubated for 60 min at 37°C . A tartrate-resistant acid phosphatase (TRAP) microtiter assay was used to quantitate the number of osteoclasts bound to osteopontin-coated plates or to bone slices (Minkin 1982, Collin-Osdoby *et al.* 1995). The plates were washed three times with PBS to remove unbound cells. One hundred microliters soluble TRAP substrate buffer (*n*-nitrophenyl phosphate (NPP)/tartrate lysis buffer: 50 mM sodium acetate pH 5.0, 0.5% Triton X-100, 0.25 mg/ml NPP,

6.7 mM tartrate) were added to the wells and the plates incubated for 1 h at 37°C . Fifty microliters 0.1 N NaOH were added to each well and the absorbance at 405 nm determined. The number of bound osteoclasts was determined by comparison with a standard curve. Various numbers of osteoclasts were plated in osteopontin-coated wells and then fixed and stained for cellular TRAP activity and the cell number counted. Osteoclasts were defined as multinucleated (≥ 3 nuclei/cell), TRAP+ cells. Duplicate wells were incubated with NPP/tartrate lysis buffer. A standard curve was generated by plotting the number of osteoclasts against absorbance at 405 nm.

Video microscopy of live rabbit osteoclasts

Osteoclast suspensions were added to dishes containing sterile glass coverslips coated with vitronectin (10 $\mu\text{g}/\text{ml}$). Coverslip cultures were mounted in an open perfusion microincubator stage (Medical Systems, Greenvale, NY, USA) for phase microscopy. Time-lapse video phase contrast microscopy was performed on a Nikon Diaphot 200 inverted light microscope equipped with CCD camera and signal processor (Dage-MTI, Michigan City, IN, USA) attached to a workstation-based analytical imaging system (Inovision Corp., Raleigh, NC, USA). Typically, sequential images obtained with $10\times$, $20\times$, or $40\times$ objectives were acquired and stored to disk at 10–30 s intervals for periods up to 30 min. Test articles were added after the first four or five frames. Montage images from selected time points were assembled to demonstrate the effect of various treatments.

Quantitative pit resorption assay

Bone slices were transferred to 12-well plates, 0.5 ml osteoclast-containing cell suspension were added and the cells allowed to settle for 2 h at 37°C . Bone pieces were rinsed to remove non-adherent cells and transferred to fresh media and incubated for 2 or 3 days. In some experiments, the $\alpha\text{v}\beta 3$ antagonist was added to the suspension and the cells were plated on bone in the presence of compound; in other cases, compound was added 2 h after cell plating (see figure legends for details). In other instances, $\alpha\text{v}\beta 3$ antagonist was added to the suspension, the cells were plated on bone in the presence of test compound, incubated for 6 h and then transferred to fresh media or fresh media containing compound and incubated for an additional 3 days.

Osteoclasts cultured on bones slices were rinsed with Hanks' balanced salt solution, pH 7.2 (HBSS), fixed in 1% formalin/HBSS, rinsed and stained for TRAP according to the manufacturer's recommendations (Sigma, St Louis MO, USA). The number of TRAP+, multinucleated cells for each treatment was obtained by examination of 10 random fields per bone disc. Two bone pieces were used for each compound and concentration tested. Bone pieces

A. SC-56631 **B. SC-65811**

Figure 1 Structures of synthetic chemical Arg-Gly-Asp (RGD) mimetics: the peptidomimetic antagonists β -[2-[[5-[(aminoiminomethyl)amino]-1-oxopentyl]amino]-1-oxoethyl]amino-3-pyridinepropanoic acid, bistrifluoroacetate (SC-56631) and β -[[1-oxo-2-[[[3-[[[(phenylmethyl)amino]carbonyl]amino]phenyl]carbonyl]amino]ethyl]amino]-pyridine-3-propanoic acid, trifluoroacetate salt (SC-65811).

were then washed in 10 mM NH_4OH to remove cells and the number and area of resorption pits quantitated using dark-field reflective microscopy. Osteoclast number and the number and area of resorption pits were measured and recorded. All measurements were performed on video-captured images linked to a Leica Quantimet image analysis program.

Data evaluation

Data are expressed as means $\pm$ S.E.M. Statistical significance was determined using the InStat software package (GraphPad Software Inc., San Diego, CA, USA).

Results*Identification of RGD peptidomimetic inhibitors of $\alpha\text{v}\beta 3$*

Figure 1 shows the two $\alpha\text{v}\beta 3$ antagonists: β -[2-[[5-[(aminoiminomethyl)amino]-1-oxopentyl]amino]-1-oxoethyl]amino-3-pyridinepropanoic acid, bistrifluoroacetate (SC-56631) and β -[[1-oxo-2-[[[3-[[[(phenylmethyl)amino]

carbonyl]amino]phenyl]carbonyl]amino]ethyl]amino]-pyridine-3-propanoic acid, trifluoroacetate salt (SC-65811).

As seen in Fig. 2, SC-56631 inhibited both vitronectin binding to $\alpha\text{v}\beta 3$ and fibrinogen binding to $\alpha\text{IIb}\beta 3$ in a dose-responsive manner with IC_{50} values of 4.8 nM and 5.9 nM respectively. In contrast, SC-65811 was a more selective inhibitor of $\alpha\text{v}\beta 3$ and inhibited vitronectin binding to $\alpha\text{v}\beta 3$ in a dose-responsive manner with an IC_{50} of 0.8 nM. SC-65811 was much less potent as an inhibitor of $\alpha\text{IIb}\beta 3$ binding to fibrinogen and inhibited $\alpha\text{IIb}\beta 3$ binding to fibrinogen with an IC_{50} of 3100 nM.

Inhibition of osteoclast adhesion to osteopontin

The $\alpha\text{v}\beta 3$ antagonists SC-56631 and SC-65811 inhibited rabbit osteoclast attachment to surfaces coated with osteopontin. Figure 3 shows that SC-65811 inhibited osteoclast adhesion with an IC_{50} of approximately 2 μM and was approximately 10-fold more potent than SC-56631. These results are consistent with the data in Fig. 2 showing that SC-65811 was 6-fold more potent than SC-56631 as an inhibitor of $\alpha\text{v}\beta 3$ binding to vitronectin. Identical results were obtained when serum- or vitronectin-coated surfaces were substituted in place of osteopontin (data not shown).

Figure 4 shows representative results demonstrating the effect of SC-65811 on osteoclasts that were adherent to glass surfaces coated with purified vitronectin. Osteoclasts were plated on vitronectin-coated glass coverslips for 18 h and then the coverslips were mounted in an open perfusion microincubator stage for time-lapse video phase microscopy. Tissue culture media were replaced with fresh media containing SC-65811 (20 μM final concentration) and recording (1 frame/30 s) commenced immediately. Figure 4 shows that osteoclasts rapidly rounded up and retracted on the addition of SC-65811. Initial shape change was detected within 2 min and retraction appeared to be complete by 15 min. In contrast, adherent contaminating fibroblast-like cells remained well spread and did not appear to respond to the addition of SC-65811 over the course of the experiment. Similar results were obtained when SC-56631 was used in place of SC-65811 (data not shown). Taken together, the results shown in Figs 2 and 3 suggest that, like echistatin and other RGD-containing peptides (Flores *et al.* 1992, Horton *et al.* 1993, Ross *et al.* 1993), the $\alpha\text{v}\beta 3$ peptidomimetic antagonists SC-56631 and SC-65811 block osteoclast interaction with the ECM.

In vitro bone resorption

To test the concept that $\alpha\text{v}\beta 3$ antagonists inhibit bone resorption by blocking osteoclast adhesion to bone, osteoclasts were plated on bone slices in media containing either vehicle or SC-65811. Osteoclasts were plated in 20 μM SC-65811 for 6 h and then the bone slices were either

Figure 2 SC-56631 and SC-65811 are potent inhibitors of vitronectin binding to the integrin $\alpha\text{v}\beta 3$. Integrins $\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$ were incubated with 2 nM biotinylated vitronectin in the presence of various concentrations of SC-56631 and SC-65811. Bound vitronectin was detected with peroxidase-labeled goat anti-biotin antibody. Each data point represents the mean of three independent determinations and the error bars indicate the standard deviation of the mean.

transferred to wells containing fresh media and SC-65811 and incubated for an additional 3 days, or washed twice with fresh media to remove compound and non-adherent cells and then transferred to wells containing fresh media lacking SC-65811 and incubated for an additional 3 days. Bone pieces were fixed and TRAP-stained and the resorptive pit area and number were quantitated by computer-assisted image analysis. We anticipated that osteoclasts plated on bone slices in the presence of SC-65811 at concentrations that inhibit osteoclast adhesion to surfaces coated with purified ECM protein would prevent osteoclast adhesion to bone and, as a consequence, bone resorption. Figure 5 shows that continuous treatment of cells with SC-65811 blocked bone resorption by 90% ($P < 0.001$) compared with osteoclasts treated with vehicle. Osteoclasts plated on bone slices in media containing vehicle resorbed $17112 \mu\text{m}^2$ bone/field, whereas osteoclasts plated on bone in the presence of $20 \mu\text{M}$ SC-65811 and maintained in SC-65811 for the duration of the experiment resorbed $1679 \mu\text{m}^2$ bone/field. Surprisingly, osteoclasts that were plated on bone in media containing SC-65811 for 6 h, washed to remove compound and incubated in fresh media for an additional 3 days resorbed $13271 \mu\text{m}^2$ bone/field. These results suggested that the bone resorptive activity was not significantly affected by transient exposure to SC-65811 ($17112 \mu\text{m}^2$ compared with $13271 \mu\text{m}^2$; $P > 0.05$). However, continuous treatment of osteoclasts with SC-65811 reduced the number of resorption pits produced by 67% (from 128 pits to 43 pits; $P < 0.01$) and average resorptive pit area by 70% (from $114 \mu\text{m}^2$ to $34 \mu\text{m}^2$; $P < 0.01$) compared with vehicle-treated cells (data not shown). These results suggest that

SC-65811 inhibition of bone resorption resulted from the production of smaller and fewer resorption pits.

To investigate whether osteoclasts adhere to bone in the presence of $\alpha\text{v}\beta 3$ antagonists, osteoclasts were added to wells containing bone pieces in the presence of SC-56631 or SC-65811, incubated for 2 h, and the bone pieces with adherent cells transferred to fresh wells and media containing $\alpha\text{v}\beta 3$ antagonist and incubated for an additional 3 days. Cells and bone pieces were fixed, TRAP-stained and the number of osteoclasts and the resorption pit area and number quantified by computer-assisted image analysis. Not surprisingly, SC-56631 and SC-65811 inhibited bone resorption in a dose-dependent manner (Fig. 6). Compared with vehicle-treated osteoclasts, SC-65811 inhibited bone resorption by 54% ($P > 0.05$), 79% ($P < 0.001$) and 88% ($P < 0.001$) at $1 \mu\text{M}$, $10 \mu\text{M}$ and $100 \mu\text{M}$ respectively. SC-56631 inhibited bone resorption less potently than SC-65811 (Fig. 6). Compared with vehicle-treated osteoclasts, SC-56631 significantly inhibited resorption by 53% at $100 \mu\text{M}$ ($P < 0.05$). In spite of the fact that osteoclasts were added to bone slices in the presence of $\alpha\text{v}\beta 3$ antagonists, SC-56631 and SC-65811 did not significantly reduce the number of osteoclasts adherent to bone: the number of osteoclasts adherent to bone slices treated with $\alpha\text{v}\beta 3$ antagonist or vehicle was not significantly different at any concentration of SC-56631 or SC-65811 that was tested (Kruskal–Wallis test, $P = 0.166$).

Because these results suggested that SC-56631 and SC-65811 inhibited bone resorption by interfering with aspects of osteoclast resorption other than by simply inhibiting osteoclast adhesion to bone, we considered whether SC-65811 treatment inhibited bone resorption,

Figure 3 SC-56631 and SC-65811 inhibit rabbit osteoclast adhesion to osteopontin. Osteoclasts suspensions were combined with various concentrations of SC-56631 and SC-65811 diluted in α -MEM containing 1% BSA, added to osteopontin-coated wells (5 $\mu\text{g}/\text{ml}$) and incubated at 37 °C for 1 h. The plates were washed to remove non-adherent cells and TRAP substrate (NPP/tartrate lysis buffer: 50 mM Na acetate, pH 5.0, 0.5% Triton X-100, 0.25 mg/ml NPP, 6.7 mM tartrate) was added, the plates incubated for 1 h at 37 °C, and absorbance measured 405 nM. Osteoclasts were defined as multinucleated (≥ 3 nuclei/cell), TRAP+ cells. A standard curve was generated by plotting number of osteoclasts against absorbance at 405 nM. Each data point represents the mean of three determinations and the error bars represent the mean $\pm$ S.E.M.

in the experiment described above, by decreasing osteoclast resorptive activity or efficiency. Data in Table 1 show that plating osteoclasts on bone slices in the presence of SC-65811 produced fewer and smaller resorption pits per osteoclast in a dose-dependent manner. Compared with vehicle-treated osteoclasts, those treated with SC-65811 decreased the number of resorption pits produced by 31% ($P>0.05$), 77% ($P<0.001$) and 69% ($P<0.001$), the number of pits produced per osteoclast by 10%, 65% and 58%, and the resorption pit area produced per osteoclast by 41%, 69% and 83%, at 1 μM , 10 μM and 100 μM respectively. Together, these data indicated that SC-65811 inhibited osteoclast bone resorptive activity, but not osteoclast adhesion to bone, in a dose-dependent manner. Further, multiple regression analysis of the relationship between osteoclast cell number, dose of SC-65811 and pit area failed to demonstrate a statistically significant relationship between pit area and cell number at any dose of SC-65811 tested. Similar results were obtained when SC-56631 was used in place of SC-65811 and in other experiments in which cells were plated on bone slices in the absence of compound and then transferred to fresh media containing either SC-56631 or SC-65811 (data not shown).

Inhibition of osteoclast adhesion to osteopontin or bone discs

To test and compare SC-56631 and SC-65811 directly as inhibitors of osteoclast adhesion to bone and ECM, osteoclasts were diluted in α -MEM containing SC-56631 (100 μM), SC-65811 (100 μM) or echistatin (100 nM) and added to osteopontin-coated 96-well plates or to wells containing 6 mm bone discs. The cells were incubated at 37 °C for 1 h and then the wells were washed to remove non-adherent cells. The number of bound osteoclasts was determined as described in the Materials and Methods. Figure 7 shows that SC-56631, SC-65811 and echistatin inhibited completely the binding of osteoclasts to osteopontin-coated wells, but did not inhibit osteoclast adhesion to bone slices.

Discussion

Osteoclasts migrate on the bone surface and initiate the resorption process in cycles of migration and resorption (Kanehisa & Heersche 1988, Lakkakorpi & Vaananen 1991). Adhesion molecules and integrin receptors are believed to play a role in various steps of the osteoclast resorption cycle. There is now a significant body of evidence that suggests that antagonists of the integrin $\alpha\text{v}\beta 3$ may provide an effective therapeutic approach for the treatment of excessive osteoclast activity and bone loss in osteoporosis, metastatic bone disease or Paget's disease (Horton & Rodan 1996).

Chambers and coworkers (1986) and Davies and colleagues (1989) were the first to implicate integrins in the process of bone resorption. These workers showed that a monoclonal antibody, 13C2, raised against rat osteoclasts recognized $\alpha\text{v}\beta 3$ and inhibited bone resorption *in vitro*. Many of the known integrins, including $\alpha\text{v}\beta 3$, bind to the Arg-Gly-Asp (RGD) consensus sequence found in ECM ligands. Subsequent work by a number of investigators showed that synthetic RGD-based peptides, in addition to the RGD-containing disintegrins inhibit osteoclast binding to immobilized ECM proteins (Flores *et al.* 1992, Helfrich *et al.* 1992, Ross *et al.* 1993, Sato *et al.* 1994) and bone resorption *in vitro* (Sato *et al.* 1990, 1994). Fisher and colleagues (1993) showed that the RGD-containing disintegrin echistatin inhibited osteoclast-mediated bone resorption in the thyroparathyroidectomized rat. More recently, Crippes *et al.* (1996) showed that a monoclonal antibody that recognizes and neutralizes $\alpha\text{v}\beta 3$ blocked the osteoclast-mediated bone resorption in the same model. In addition, Engleman *et al.* (1997) and Yamamoto *et al.* (1998) and others (Masarachia *et al.* 1998) have shown that the RGD peptidomimetic SC-56631 and echistatin inhibited bone loss in ovariectomized rodents.

In an effort to develop a non-toxic small-molecule pharmacologic antagonist of integrin $\alpha\text{v}\beta 3$, peptidomimetic inhibitors of $\alpha\text{v}\beta 3$ were identified and modified to enhance potency and selectivity. Integrin selectivity was

Figure 4 SC-65811-induced retraction of osteoclasts adherent to vitronectin. Osteoclasts were plated on vitronectin-coated glass coverslips for 18 h and the coverslips were examined by time-lapse video microscopy. Tissue culture media were replaced with fresh media containing SC-65811 (20 μ M final concentration) and images were acquired and stored to disk at 30-s intervals. Frames 1–25 show the effects of SC-65811 on a single osteoclast. Frame 1 was captured 30 s after addition of SC-65811; frames 4–25 were acquired at 90-s intervals thereafter. The osteoclast in frame 25 had been incubated in SC-65811 for 12.5 min at the time that this image was acquired. The solid arrows in frame 1 indicate the margin of the osteoclast. The open arrows in frames 1 and 25 point to a fibroblast of which the morphology was only minimally changed over the course of the experiment. Bar represents 20 μ m.

considered important because RGD-binding integrins are widely distributed and participate in various physiologic and pathologic processes. Of particular concern was the platelet fibrinogen receptor, α IIb β 3. Integrins α IIb β 3 and $\alpha v \beta 3$ are related in that they share the α subunit and have homologous β subunits (Pytela *et al.* 1986). More importantly, α IIb β 3 is a key player in platelet aggregation and antagonism of α IIb β 3 would be expected to cause unwanted bleeding problems (Coller *et al.* 1989). Through screening and medicinal chemistry, SC-56631 and SC-65811, two potent antagonists of $\alpha v \beta 3$, were produced. SC-56631 inhibited vitronectin and fibrinogen binding to $\alpha v \beta 3$ and α IIb β 3 respectively with similar potency, but SC-65811 was at least 100 times more potent as an inhibitor of $\alpha v \beta 3$ than of α IIb β 3. As described above, Engleman and colleagues (1997) showed that SC-56631

inhibited bone loss in ovariectomized rodents and therefore it was not surprising to observe that SC-56631 and SC-65811 inhibited osteoclast adhesion and bone resorption *in vitro*.

SC-56631 and SC-65811 inhibited rabbit osteoclast adhesion to immobilized ECM, caused adherent and well-spread osteoclasts to retract and round up from surfaces coated with matrix and inhibited bone resorption. On the basis of these results, the activities of SC-56631 and SC-65811 appeared to be similar to the anti- $\alpha v \beta 3$ and bone resorption inhibiting activities previously reported for RGD-based peptides and echistatin, with the distinction that SC-65811 is clearly both more potent than SC-56631 and selective for $\alpha v \beta 3$ rather than α IIb β 3. In contrast, however, we were surprised to note that SC-56631 and SC-65811 inhibited bone resorption in a dose-responsive

Figure 5 Inhibition of osteoclast bone resorptive activity by SC-65811. Osteoclasts were plated on bone slices in the presence of 20 μM SC-65811 and incubated for 6 h at 37 °C. Bone slices were transferred to wells containing a mixture of fresh media and compound and incubated for an additional 3 days or they were washed twice with fresh media to remove compound and non-adherent cells and incubated in media lacking compound for additional 3 days. At the end of the incubation period, bone pieces were washed with NH_4OH to remove adherent cells and the number of resorptive pits and the resorptive pit area per field quantitated using dark-field microscopy. The inset photomicrograph shows representative reflected-light images of the bone surface after removal of cells. The darkened areas represent osteoclast resorption lacunae. The error bars represent the mean $\pm$ S.E.M. Osteoclasts treated continuously with SC-65811 resorbed significantly less bone than cells treated with vehicle ($P < 0.001$). In contrast, resorptive activity was not significantly affected by transient exposure to SC-65811 (17112 μm^2 compared with 13271 μm^2 ; $P > 0.05$). However, continuous treatment of osteoclasts with SC-65811 reduced the number of resorption pits produced by 67% ($P < 0.01$) and average resorptive pit area by 70% ($P < 0.01$) compared with vehicle-treated cells (data not shown).

manner but did not significantly affect osteoclast adhesion to bone. Further analysis showed that SC-65811 decreased the number and average pit area produced per osteoclast and suggested that $\alpha v \beta 3$ antagonists inhibit bone resorption by decreasing osteoclast bone resorptive activity or efficiency.

Rodan and colleagues have recently reported that echistatin inhibited bone resorption in thyroparathyroid-

ectomized mice (Masarachia *et al.* 1998) and ovariectomized mice and rats (Yamamoto *et al.* 1998). Using histomorphometric techniques, these investigators showed that echistatin treatment did not detectably alter osteoclast morphology but increased the bone surface covered by osteoclasts – results suggesting that echistatin treatment did not reduce osteoclast formation, recruitment, or attachment to bone but did, nevertheless, inhibit bone resorption. Our *in vitro* observations on the effects of SC-56631 and SC-65811 on osteoclast bone resorption agree with these findings of Rodan and colleagues and are consistent with the hypothesis that RGD-containing proteins and peptidomimetics inhibit bone resorption by reducing osteoclast bone resorptive activity.

Previous studies showed that RGD peptides and echistatin inhibited chicken and rat osteoclast adhesion to bone and bone resorption *in vitro* (Sato *et al.* 1990, 1994, Ross *et al.* 1993). In contrast, we have shown that osteoclasts can adhere to bone in the presence of echistatin, SC-56631 and SC-65811. The differences between the reported effects that these antagonists have on osteoclast adhesion to bone could be due to differences in the response of rat, chicken and rabbit osteoclasts to the test compounds utilized in the various studies or to the manner in which osteoclast attachment was determined. In the studies by Sato *et al.* (1990) and Ross *et al.* (1993) [^3H]-labeled bone particles were added to chicken osteoclasts attached to ECM or bone. The hydrolysis of bone and release of ^3H from bone particles, presumably bound to the basolateral osteoclast surface, were determined by scintillation counting. Alternatively, Sato *et al.* (1994) used laser confocal microscopy and epifluorescence to show that treatment of rat osteoclasts with echistatin caused changes in cellular morphology, cell rounding, echistatin receptor redistribution and alterations in morphology suggestive of cell detachment. These techniques differ significantly from the direct determination of osteoclasts adherent to bone substrate that was used in the studies presented herein. It should be noted that, in contrast to the reports of Sato *et al.* (Sato *et al.* 1990, 1994) and Ross *et al.* (1993), Rodan and co-workers (Masarachia *et al.* 1998, Yamamoto *et al.* 1998) recently showed that osteoclasts of mice and rats treated with echistatin *in vivo* appeared normal and showed no signs of altered cell morphology or detachment but, rather, the bone surface area covered by osteoclasts was increased in animals treated with echistatin, implying that echistatin did not impair osteoclast recruitment and attachment to bone. Together, these findings support the notion that bone resorption is more sensitive to echistatin than is cell attachment (Sato *et al.* 1994).

These results underscore the notion that integrin-mediated mechanisms function in the bone resorptive process. The integrin $\alpha v \beta 3$ has been implicated in several aspects of osteoclast interaction with bone, including the formation of the osteoclast clear zone and cell migration. Resorbing osteoclasts develop a specialized membrane

Figure 6 SC-56631 and SC-65811 inhibit bone resorption. Various concentrations of SC-56631 and SC-65811 were combined with osteoclasts and then added to 12-well plates containing bovine bone pieces. The cells were allowed to adhere and then the bone pieces were transferred to fresh media containing SC-56631 or SC-65811 and incubated for an additional 3 days. Osteoclasts cultured on bones slices were fixed and stained for TRAP. The number of TRAP⁺, multinucleated cells for each treatment was obtained by examination of 10 random fields per bone piece; two bone pieces were used for each compound concentration tested. Bone pieces were then washed with NH_4OH to remove cells and the number and the resorptive pit area were quantified using dark-field reflective microscopy. The number of osteoclasts per field and the resorptive pit area per field were determined. The error bars represent the mean $\pm$ S.E.M. Compared with vehicle-treated osteoclasts, SC-65811 inhibited bone resorption by 54% ($P>0.05$), 79% ($P<0.001$) and 88% ($P<0.001$) at 1 μM , 10 μM and 100 μM respectively. SC-56631 significantly inhibited resorption by 53% at 100 μM ($P<0.05$). The number of osteoclasts adherent to bone slices treated with various concentrations of SC-56631 and SC-65811 compared with vehicle was not significantly different (Kruskal–Wallis test, $P=0.166$).

domain termed the clear zone or tight sealing zone, which is believed to mediate cell attachment to the bone matrix isolating the acidic resorption lacunae from the extracellular environment. Localization studies showed that $\alpha v \beta 3$ and osteopontin were enriched in the clear zone of resorbing osteoclasts and the underlying bone matrix (Reinholt *et al.* 1990, Hultenby *et al.* 1993) and, together with the observations that integrin antagonists inhibit

osteoclast attachment to surfaces coated with ECM proteins, suggested that $\alpha v \beta 3$ plays a role in the formation or maintenance of the tight sealing zone. However, more recent localization studies have been unable to confirm the presence of $\alpha v \beta 3$ within the sealing zone and it is now clear that osteopontin is not found exclusively in the bone matrix beneath the sealing zone (Lakkakorpi *et al.* 1991, 1993, Rodan & Rodan 1997). Indeed, more recent studies

Table 1 SC-65811 inhibits osteoclast bone resorption by isolated rabbit osteoclasts

	Cell no. per field [#]	Pit area per field [¶]	% of control	Pit no. per field [*]	Pit no. per cell [‡]	Pit area per cell [†]
Vehicle	3.45	20 345	100	6.75	1.96	5938
SC-65811 [§]						
1 μM	2.65	9365	46	4.70	1.77	3533
10 μM	2.25	4195	20	1.55	0.69	1864
100 μM	2.50	2532	12	2.10	0.84	1013

[#]Total number of osteoclasts counted in 20 fields divided by the number of fields.

[¶]Total area (μm^2) counted in 20 fields divided by the number of fields.

^{*}Total number of resorptive pits counted in 20 fields divided by the number of fields.

[‡]Pit no. divided by the cell no.

[†]Pit area per field divided by the cell no. per field.

[§]SC-65811 1 μM , 10 μM and 100 μM decreased the number of resorption pits produced by 31% ($P>0.05$), 77% ($P<0.001$) and 69% ($P<0.001$) respectively.

Figure 7 SC-56631, SC-65811 and echistatin inhibit osteoclast adhesion to osteopontin but not to bone. Osteoclasts were diluted in α -MEM containing SC-56631 (100 μ M), SC-65811 (100 μ M) or echistatin (100 nM) and added to osteopontin-coated 96-well plates or to wells containing bone slices. The cells were incubated at 37 °C for 1 h and then washed to remove non-adherent cells. The number of bound osteoclasts was determined as described in the Materials and Methods. The error bars represent the mean $\pm$ S.E.M.

demonstrate that $\alpha v\beta 3$ is localized to the osteoclast basolateral surface and ruffled border (Lakkakorpi *et al.* 1991, 1993, Helfrich *et al.* 1996) – a location consistent with a role for $\alpha v\beta 3$ in osteoclast cell migration.

Classically, the role ascribed to integrins has been that of anchoring cells to the ECM. However, recent reports showed that the interaction between the ECM and cell surface integrins leads to intracellular signaling events that affect cell migration (Uhm *et al.* 1999). There is abundant evidence that the integrin $\alpha v\beta 3$ plays a role in the migration of a variety of cells including tumor cells (Leavesley *et al.* 1992, Filardo *et al.* 1995, 1996, Zheng *et al.* 1999), smooth muscle cells (Brown *et al.* 1994, Yue *et al.* 1994, Liaw *et al.* 1995, Jones *et al.* 1996, van Leeuwen *et al.* 1996, Senger & Perruzzi 1996, Stefansson & Lawrence 1996), neutrophils (Lawson & Maxfield 1995) and endothelial cells (Leavesley *et al.* 1993, Senger *et al.* 1996). It seems likely that antagonists of $\alpha v\beta 3$ reduce the ability of the osteoclast to migrate on bone substrates and thereby interfere with the bone resorptive process. It is important to note that treatment of osteoclasts with SC-65811 significantly reduced the number of resorption pits produced per osteoclast and the average pit size, and it is conceivable that the reduction in pit number and size is a consequence of inhibiting osteoclast cell migration.

In summary, we show that $\alpha v\beta 3$ antagonists potently inhibit osteoclast adhesion to purified bone matrix protein and osteoclast-mediated bone resorption but do not inhibit osteoclast adhesion to bone. We conclude that $\alpha v\beta 3$ antagonists inhibit bone resorption by inhibiting osteoclastic bone resorptive activity. Antagonists of $\alpha v\beta 3$ may offer an exciting new therapeutic opportunity for the prevention of bone loss in disease states that include osteoporosis and metastatic bone cancer.

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