Design and Characterization of Orally Active Arg-Gly-Asp Peptidomimetic Vitronectin Receptor Antagonist SB 265123 for Prevention of Bone Loss in Osteoporosis

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ABSTRACT

The Arg-Gly-Asp (RGD)-binding integrin αvβ3 is highly expressed on osteoclasts and has been proposed to mediate cell-matrix adhesion required for osteoclast-mediated bone resorption. Antagonism of this receptor should prevent stable osteoclast adhesion and thereby inhibit bone resorption. We have generated an orally bioavailable, nonpeptide RGD mimetic αvβ3 antagonist, SB 265123, which prevents bone loss in vivo when dosed by oral administration. SB 265123 binds αvβ3 and the closely related integrin αvβ5 with high affinity (K_i = 3.5 and 1.3 nM, respectively), but binds only weakly to the related RGD-binding integrins αIβ3 (K_i > 1 μM) and αvβ1 (K_i > 1 μM). The compound inhibits αvβ3-mediated cell adhesion with an IC_{50} = 60 nM and more importantly, inhibits human osteoclast-mediated bone resorption in vitro with an IC_{50} = 48 nM. In vivo, SB 265123 completely blocks bone resorption in a thyroparathyroidectomy-induced rat model of acute bone resorption when dosed at 2.5 mg/kg/h by continuous i.v. infusion. When dosed orally with 3 to 30 mg/kg b.i.d., in the ovariectomy-induced rat model of osteoporosis, SB 265123 prevents bone resorption in a dose-dependent fashion. This is the first report of an orally active αvβ3 antagonist that is effective at inhibiting bone resorption when dosed in a pharmacologically acceptable fashion. Such a molecule may provide a novel therapeutic agent for the treatment of postmenopausal osteoporosis.

Osteoporosis is a chronic bone disease characterized by a decrease in bone mass resulting from accelerated osteoclast-mediated bone resorption relative to formation (Baron, 1996). Therefore, inhibition of osteoclast-driven bone resorption should prove beneficial in the treatment of this disease. For resorption to occur, osteoclasts must first attach to the bone. They then form a tightly sealed extracellular compartment beneath the cell (Baron, 1996) into which they secrete protons and matrix-degrading proteinases. Tissue distribution studies (Clover et al., 1992; Helfrich et al., 1992; Nesbitt et al., 1993; Shinar et al., 1993) have shown that αvβ3 is the predominate integrin on the osteoclast cell surface. Neutralizing antibodies directed against this receptor (Davies et al., 1989; Horton et al., 1991; Crippes et al., 1996) as well as Arg-Gly-Asp (RGD)-containing peptides (Davies et al., 1989; Fisher et al., 1993) inhibit osteoclast adhesion. In addition, osteoclast-mediated bone resorption in vitro as well as in the acute thyroparathyroidectomy (TPTX) rat model of bone resorption also is inhibited by several of these molecules (Fisher et al., 1993; Yamamoto et al., 1993; Crippes et al., 1996). Together, these data indicate that αvβ3 is the functionally important integrin on the osteoclast surface.

Recently, a nonorally bioavailable, RGD-peptide mimetic, SC56631, which binds to αvβ3, αvβ5 and αvβ8, was reported to inhibit osteoclast-mediated bone resorption in vitro as well as in vivo in the TPTX rat model (Engleman et al., 1997). In addition, SC56631 also appeared to prevent the estrogen deficiency-induced loss in trabecular structure in the ovariectomized (ovx) rat model after 6 weeks of continuous, high-dose i.v. infusion. However, these studies still leave open the question as to whether an αvβ3 antagonist could be developed for oral administration in a reasonable dosing regime. This is highly desirable for a compound to treat chronic bone loss in osteoporosis.

In this article, we report the pharmacological characterization of SB 265123, a potent, orally bioavailable, nonpeptide αvβ3 antagonist that inhibits αvβ3-mediated cell adhesion as well as osteoclast-mediated bone resorption in vitro. SB 265123 is efficacious at inhibiting acute bone resorption in vivo in the TPTX rat model. When dosed twice a day at 30

ABBREVIATIONS: TPTX, thyroparathyroidectomized; ovx, ovariectomized; PTH, parathyroid hormone; BMD, bone mineral density.
mg/kg orally, SB 265123 also is efficacious at preventing ovariectomy-induced bone loss. These data support the hypothesis that a nonpeptide $\alpha_\beta_3$ antagonist can be developed for pharmacologically acceptable dosing for the treatment of postmenopausal osteoporosis.

Materials and Methods

Integrin-Binding Assays. To determine the affinity of compounds for various integrins, binding assays were established as described previously (Wong et al., 1996). Integrins $\alpha_\beta_3$, $\alpha_\beta_5$, and $\alpha_\beta_3$ were purified from human placenta and $\alpha_\beta_3$ was purified from human platelets (Wong et al., 1996). Receptor-binding assays were established with the RGD-containing cyclic peptide $\alpha_\beta$HISKF&P 107260 as the ligand. The $K_i$ values represent the means of values determined in two or three separate experiments.

Inhibition of $\alpha_\beta_3$-Mediated Cell Adhesion. Inhibition of $\alpha_\beta_3$-mediated cell adhesion was monitored with human embryonic kidney (HEK)-293 cells cotransfected with recombinant human $\alpha_\beta_3$ (Kumar et al., 1997). Ninety-six-well plates were coated overnight with 0.15 $\mu$g of human vitronectin in 0.1 ml of RPMI 1640 medium. The plates were then washed once with RPMI 1640 medium and blocked with 3.5% BSA for 1 h at room temperature. Transfected cells were suspended in RPMI 1640 supplemented with 20 mM HEPES (pH 7.4), 0.1 M MnCl$_2$, and 0.1% BSA at a density of 0.5 × 10$^3$ cells/ml. A 0.1-ml aliquot of cells was added to each well and incubated for 1 h at 37°C in the presence or absence of inhibitor. After the incubation, 0.025 ml of 10% formaldehyde solution (pH 7.4) was added, and the cells were fixed at room temperature for 10 min. The plates were then washed three times with RPMI 1640, and the adherent cells were stained with 0.1 ml of 0.5% toluidine blue for 20 min at room temperature. Excess stain was removed by extensive washing with deionized water and the cell-associated toluidine blue was eluted by the addition of 0.1 ml of 50% ethanol containing 50 mM HCl. Toluidine blue, as a readout of cell number (Wong et al., 1996), was quantitated at an optical density of 600 nm on a microtitrator plate reader (Tulereck Multiskan, Sterling, VA). The potency of the compounds was evaluated with multidoze titrations to determine $IC_{50}$ values.

Human Platelet Aggregation Assay. Effects of the $\alpha_\beta_3$ antagonist on human platelet aggregation were determined as described previously (Nichols et al., 1994). Briefly, human platelet-rich plasma was stimulated with 10 $\mu$M ADP and aggregation monitored with impedance aggregometry in the presence of various concentrations of compound.

Pharmacokinetics in Rat. All animal procedures reported in this study were reviewed and approved by the Animal Care and Use Committee at SmithKline Beecham Pharmaceuticals. Compounds and pharmacokinetics were evaluated in adult male rats with a crossover design on two separate study days. The animals had femoral vein catheters surgically implanted for infusion of compound. On day 1, the animals received 2 $\mu$mol/kg target dose as a 30-min i.v. infusion in 5% polyethylene glycol 300 containing 0.5% dimethyl sulfoxide, pH = 3.5 to 4.0. On day 2, the animals received 2 $\mu$mol/kg target dose by oral gavage in 5% polyethylene glycol 300 containing 0.5% dimethyl sulfoxide, pH = 4.0. Blood samples were collected from a lateral tail vein. The concentrations of compound in the plasma were quantified by liquid chromatography tandem mass spectrometry (limit of detection = 10 ng/ml). Noncompartmental methods were used for pharmacokinetic analysis of plasma concentrations versus time data.

Human Osteoclast-Mediated Bone Resorption Assay. The isolation of disaggregated human osteoclasts from fresh osteoclastoma tissue (James et al., 1996) and the in vitro human osteoclast resorption assay have been described (James et al., 1999). Briefly, human osteoclasts were seeded onto bovine cortical bone slices with compound or vehicle for 48 h at 37°C. The culture media were then removed and the levels of the carboxyl-terminal peptide of the $\alpha_1$ chain of human type I collagen were quantitated as a biochemical readout of resorption, with a competitive binding enzyme-linked immunosorbant assay (Foged et al., 1996) (Osteometer A/S, Rodovre, Denmark). The results are expressed as percentage of inhibition of resorption compared with supernatants derived from osteoclasts cultured in vehicle without inhibitor. $IC_{50}$ values are determined from the resultant dose-response curves.

TPTX Rat Model of Bone Resorption. TPTX male Sprague-Dawley rats were received from the vendor (Taconic Farms, Inc., Germantown, NY) and blood-ionized calcium levels were quantitated to confirm the surgery (Chiron Diagnostics Cat# / pH Analyzer model 634). All animals received deionized water and normal chow ad libitum and were given a maintenance injection of thyroid hormone (2 $\mu$g/animal s.c.), three times weekly. Rats, anesthetized with isoflurane, were surgically implanted with a femoral i.v. polyurethane-anchored catheter and a femoral intra-arterial polyethylene catheter. Both catheters were exteriorized via the nape of the neck. Animals were individually housed in standard wire rack cages and tubing was protected with a commercially available tether/swivel/jacket system.

To establish a hypocalcemic baseline, the animals were fed a low-calcium diet 24 h before infusion of parathormone (PTH). Following confirmation of a hypocalcemic state, the animals were segregated into groups such that there was no significant difference in mean blood-ionized calcium level. One group ($n = 8$) received a continuous infusion of human PTH (PTH 1–34, 1.25 $\mu$g/kg/h), a second group ($n = 7$) received a continuous infusion of vehicle (saline, pH 9.0–9.2). A third group ($n = 7$) received a loading dose of SB 265123 (8.33 mg/kg i.v. bolus) followed by infusion of SB 265123 (2.53 mg/kg/h) and PTH. Blood-ionized calcium was determined at 2, 4, and 6 h. At the concentrations of PTH used in this model, there are significant increases in bone resorption with no effects on the kidney (Carney and Thompson, 1982). These data indicate that the increased serum calcium reflects elevated bone resorption and not a change in renal clearance. Data are reported as percentage of change relative to baseline calcium levels at time zero. Significance was calculated with a standard two-tailed $t$ test.

Ovariectomized Rat Model. Virgin female Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used at the age of 7 months following an acclimation period of at least 1 month. Immediately before either sham operation or ovariectomy, proximal tibial bone mineral density (BMD) was determined. BMD was determined by dual energy X-ray absorptiometry with a Hologic QDR-4500 (Hologic Inc., Waltham, MA) equipped with high-resolution scanning software. In regional high-resolution mode, the spacing and point resolution were each 0.31 mm. Animals were maintained anesthetized with isoflurane while placed prone on the scan surface. BMD was calculated by dividing the bone mineral content by the projected bone area. The rats were then segregated into groups ($n = 10$) that did not differ in their mean values for proximal tibial BMD. After surgery, groups of oxt rats received, by oral gavage, a twice-daily dose of either dosing vehicle [1% aqueous solution (w/v) of carboxymethyl cellulose], or compound suspended in vehicle. A group of sham-operated rats was dosed with vehicle as a control. Proximal tibial BMDs were determined 4 weeks after the initiation of the study and analyzed as percentage of change from baseline. The differences between the four oxt-treated groups were analyzed with ANOVA (Milliken and Johnson, 1984) followed by Dunnett’s test (Dunnett, 1965). In addition, the dose-related trend in the percentage of change was assessed with a trend test. This was done by with pairwise one-sided $t$ tests on the mean percentage of change values for the four groups. To adjust for multiple testing, bootstrap $P$ values (Westfall and Young, 1993) are reported.

Compounds. SB 223245 (Keenan et al., 1997) and SB 265123 (Miller et al., 1999) were synthesized in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA.
Results

Identification of α₃β₃ Antagonists for In Vivo Evaluation. Previous studies led to the identification of SB 223245, a potent, nonpeptide α₃β₃ antagonist (Keenan et al., 1997). Unfortunately, SB 223245 has low oral bioavailability (<10%) and a short circulating half-life (9–16 min); therefore, it was inappropriate for in vivo evaluation. Subsequent lead optimization studies led to the identification of SB 265123 (Miller et al., 1999) (Table 1), a potent α₃β₃ antagonist with improved pharmacokinetics. SB 265123 maintained its ability to potently bind (Kᵢ = 4 nM) α₃β₃ (Fig. 1A) and improved its potency (IC₅₀ = 60 nM versus 145 nM for SB 223245) at inhibiting α₃β₃-mediated cell adhesion (Fig. 1B). Furthermore, SB 265123 maintained its selectivity relative to other RGD-binding integrins. SB 265123 binds α₃β₃ with a Kᵢ = 4 nM, but is >1000-fold less potent at binding both α₁β₃ (Kᵢ = 9 μM) and α₅β₃ (Kᵢ = 18 μM). Consistent with its poor activity at α₁β₃, SB 265123 has an IC₅₀ >200 μM at inhibiting human platelet aggregation. Neither SB 223245 nor SB 265123 is completely selective for α₃β₃ because they also bind to the closely related α₅ integrin α₅β₃ with high affinity (Kᵢ values = 0.4 and 1.3 nM, respectively).

SB 265123 Inhibits Human Osteoclast-Mediated Bone Resorption In Vitro. To directly address whether SB 265123 would be effective at inhibiting osteoclast-mediated bone resorption, an in vitro assay was used in which human osteoclast-mediated bone resorption was monitored (James et al., 1999). Bone resorption was quantified biochemically with C-telopeptide fragments of type I collagen released into the culture medium as a readout. Previous studies have shown that there is a strong correlation between both osteoclast pit number (Foged et al., 1996) and pit volume relative to this C-telopeptide biochemical readout. In this assay, both SB 223245 and SB 265123 inhibit bone resorption in a concentration-dependent fashion (Fig. 2). However, SB 265123 (IC₅₀ = 48 ± 3 nM) is significantly more potent than SB 223245 (IC₅₀ = 300 ± 3 nM). Both compounds can completely inhibit bone resorption in vitro at concentrations >0.5 μM. These data indicate that both α₃β₃ antagonists can inhibit human osteoclast-mediated bone resorption in the same concentration range that they inhibit α₅β₃-mediated cell adhesion; however, consistent with its potency in the cell adhesion assay, SB 265123 is more potent in the resorption assay.

SB 265123 Inhibits Bone Resorption in the TPTX Rat Model. Because of its high affinity for α₃β₃, its favorable pharmacokinetic characteristics in the rat, and potency in the in vitro bone resorption assays, SB 265123 was evaluated in vivo in the TPTX rat model of bone resorption. In this model, TPTX rats are rendered hypocalcemic and infused with PTH to stimulate an osteoclast-mediated calcemic response. These data indicated that the increased serum calcium had no effect on kidney PTH receptors so SB 265123 was coinfused i.v. at a rate of 2.53 mg/kg/h with PTH and the effect on serum calcium was measured. Under these conditions, SB 265123 inhibits the PTH-induced calcemic response by >80% at all time points (Fig. 3). At the end of the experiment (6 h), 85% inhibition was observed. These data clearly indicate that SB 265123 is active in vivo in an acute model of PTH-stimulated bone resorption.

SB 265123 Inhibits Bone Resorption in the ovx Rat. The ovx rat provides an excellent model for the study of estrogen-deficiency induced osteopenia. In this model, aged female rats are surgically ovx and within 4 weeks a significant reduction in BMD is apparent (Fig. 4). To determine whether SB 265123 prevents this ovx-induced bone loss, animals were dosed from the time of surgery and BMD was measured 4 weeks after surgery in the proximal tibia. Because SB 265123 has high oral bioavailability and has a half-life of 3 to 6 h in the rat, compound was administered at 3, 10, and 30 mg/kg b.i.d. orally for the duration of the study. Under these conditions, SB 265123 significantly inhibited ovariectomy-induced bone loss in a dose-dependent fashion. ANOVA showed a significant difference between the four groups (p = .0196) with the SB 265123 30-mg/kg dose group being significantly different (p = .0171) from the ovx control group based on Dunnett’s test. In addition, the dose-related trend seen in the mean values was confirmed by the trend test (p = .0027). No overt signs of toxicity were noted in the animals, even in the high-dose group (30 mg/kg). These results suggest that an orally bioavailable α₃β₃ antagonist can safely and effectively prevent estrogen deficiency-induced bone loss.

TABLE 1
In vitro activity of nonpeptide α₃β₃ antagonists

<table>
<thead>
<tr>
<th>Entry</th>
<th>Structure</th>
<th>α₃β₃ Ki (nM)</th>
<th>α₃β₃-mediated cell adhesion IC₅₀ (nM)</th>
<th>t½ (min)</th>
<th>Plasma Clearance (mL/min/kg)</th>
<th>Oral Bioavailability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB 223245</td>
<td><img src="image" alt="Structure" /></td>
<td>2 ±/– 0.1</td>
<td>145</td>
<td>9–16</td>
<td>35 ± 2</td>
<td>3–7</td>
</tr>
<tr>
<td>SB 265123</td>
<td><img src="image" alt="Structure" /></td>
<td>4 ±/– 1</td>
<td>60</td>
<td>181–378</td>
<td>2.6</td>
<td>≈ 100</td>
</tr>
</tbody>
</table>
bone loss in vivo when administered at a pharmacologically acceptable dose and route of administration.

Discussion

Normal bone balance is controlled through a tightly integrated balance of osteoclast-mediated bone resorption and osteoblast-mediated bone formation. In an estrogen-depleted setting, such as is seen in postmenopausal osteoporosis, there appears to be an imbalance in this process resulting from a relative deficit in bone formation along with an increase in the rate of bone turnover. The resulting bone loss causes a significant reduction in bone strength, ultimately leading to an increased incidence of fracture. Several therapeutic approaches have been taken to try to regulate this imbalance, including estrogens and bisphosphonates. Both of these approaches have limitations, and the precise molecular mechanisms of action for both estrogen and bisphosphonates are unknown. Identification of specific molecular targets for pharmacological intervention provides opportunities for design of therapeutic modalities with improved safety and/or efficacy.

In this study, we report that the potent \(\alpha_v\beta_3\) antagonist SB 265123 is efficacious at inhibiting bone resorption both in vitro and in vivo in several bone resorption models. This compound inhibits both matrix degradation (type I collagen readout in the human osteoclast resorption assay) as well as demineralization (TPTX rat model). These results are consistent with the mechanism of action of SB 265123. Inhibition of the attachment and adhesion of osteoclasts, or osteoclast precursors, to the bone would be expected to prevent the formation of an acidic resorption lacuna in which mineral
twice a day. Interestingly, SB 265123 does bind $\alpha_\beta_3$ with high affinity, similar to SC56631. Although the biological role of $\alpha_\beta_3$ is not clear at this time, studies have shown that $\alpha_\beta_3$ is expressed on osteoclast precursors (Inoue et al., 1995; Teitelbaum et al. 1997) and is subsequently replaced by $\alpha_\beta_3$ as the cells mature into functionally active osteoclasts. In addition, studies suggest that $\alpha_\beta_3$ may mediate the attachment of these osteoclast precursors to matrix (Inoue et al., 1995; Teitelbaum et al. 1997). Therefore, in principle, inhibition of both $\alpha_\beta_3$ and $\alpha_\beta_3$ could have an even more profound effect at blocking bone resorption in vivo than inhibition of only one of these receptors.

Recently, the potent $\beta_3$ ligand echistatin also was reported to have efficacy in both the ovx rat and mouse models when dosed by continuous i.v. infusion (Yamamoto et al., 1997). Like SB 265123, echistatin inhibits bone resorption in both the TPTX (Fisher et al., 1993) and ovx rat models in vivo (Yamamoto et al., 1997). In the ovx rat, echistatin-inhibited resorption as measured by BMD, was $-26$ and $37\%$ in the femur. In our study, we demonstrated $\sim65\%$ prevention of bone loss with SB 265123 in the proximal tibia. Thus, SB 265123 appears as active as the extremely potent peptide inhibitor echistatin in this aggressive model of bone resorption.

Net bone loss is the result of limited bone formation as well as accelerated bone resorption. Thus, the effects of antiresorptive compounds such as SB 265123 could possibly be improved if administered in combination with an anabolic molecule, such as intermittent PTH (Reeve, 1996), which has been shown to stimulate bone formation in vivo.

In conclusion, this is the first demonstration of an orally bioavailable $\alpha_\beta_3$ antagonist that inhibits bone resorption both in vitro and in vivo. This molecule inhibits ovariectomy-induced bone resorption in a dose-dependent fashion as assessed with BMD. Such a molecule could be an efficacious and safe treatment for postmenopausal osteoporosis, which appears to be driven through increased osteoclastic activity resulting from estrogen deficiency.

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**SB 265123 mg/kg**

Fig. 4. SB 265123 inhibits ovariectomy-induced bone loss at 4 weeks in the ovx rat model in vivo. SB 265123 was dosed at 3, 10, or 30 mg/kg b.i.d. orally for 4 weeks from the time of initiation of ovariectomy. BMD was measured in the proximal tibia and is reported as percentage of change relative to time zero for each group. The BMD in compound-treated animals was compared with that quantified for sham-operated animals (Sham) and ovx animals dosed with only vehicle (ovx). Mean BMD (g/cm²) at time zero for each group was 0.2573 ± 0.006 for the Sham group; 0.2532 ± 0.005 for the ovx group; 0.2538 ± 0.006 for the 3 mg/kg SB 265123 group; 0.2609 ± 0.005 for the 10 mg/kg SB 265123 group; and 0.2544 ± 0.004 for the 30 mg/kg SB 265123 group. Ten animals per group were used in this study.

**dissolution and matrix degradation occur. This significant effect on osteoclast function appears to translate into a biologically relevant response in vivo as measured with BMD.**

It has recently been reported (McHugh et al., 1998) that knockout of $\alpha_\text{v}$ in mice results in elimination of $\alpha_\beta_3$. Osteoclasts isolated from the $\alpha_\beta_3$-deficient mice have matrix attachment defects and have only 15% of the bone-resorbing capabilities of cells from the wild-type littermates. It is currently unclear what effects on bone resorption in a high-turnover state the knockout will have; however, elimination of $\alpha_\beta_3$ clearly compromises osteoclastic activity and supports a role for therapeutic intervention with an $\alpha_\beta_3$ antagonist.

Previously, the small RGD-peptide mimetic SC56631 (Engleman et al., 1997) was reported to inhibit bone resorption when dosed by continuous i.v. infusion at a dose of 0.5 mg/kg/min (720 mg/kg/day). Both the route of administration (continuous i.v. infusion) and the amount of compound required for efficacy are unrealistic for treatment of postmenopausal osteoporosis. Although these initial studies were encouraging, the question remained as to whether an $\alpha_\beta_3$ antagonist could realistically be developed to control bone resorption in this disease. SB 265123 demonstrates that such a goal may be feasible.

SB 265123 is significantly more selective than SC56631 in that it binds weakly to the related RGD-binding integrin $\alpha_\alpha_\beta_3$ and minimally inhibits human platelet aggregation. Thus, extended chronic dosing with a compound like SB 265123 is not expected to result in complications due to inhibition of platelet aggregation. Consistent with its selectivity, no overt signs of toxicity were noted in the 4-week ovx rat study, even when the compound was dosed at 30 mg/kg

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**References**


