Rapid Inhibition of Thyroxine-Induced Bone Resorption in the Rat by an Orally Active Vitronectin Receptor Antagonist

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ABSTRACT

An excess of thyroid hormone results in increased bone turnover and loss of bone mass in humans. Exogenous administration of thyroid hormone to rats has served as a model of human hyperthyroidism in which antiresorptive therapies have been tested. We have further refined this model of thyroxine (T4)-induced turnover in the rat. Daily administration of T4 to aged rats for as short as 1 week resulted in elevated bone resorption determined by significantly higher urinary deoxypyridinoline (Dpd) compared with vehicle controls or animals receiving T4 plus estradiol. In a follow-up study, a depot formulation of T4 caused an increase in Dpd identical to that achieved with a bolus dose. SB-273005 [(4S)-2,3,4,5-tetrahydro-8-[2-[6-(methylamino)-2-pyridinyl]-2,3-benzazepine-4-acetic acid] a potent antagonist of the integrins αvβ3 and αvβ5, has been shown previously to inhibit bone resorption in cultures of human osteoclasts and to protect bone in ovariectomized rats. The effect of SB-273005 by oral administration was evaluated in this thyroxine-induced turnover model. Dose-dependent inhibition of resorption was seen with SB-273005 after 7 days of dosing using Dpd as a measure of bone resorption. In summary, it has been demonstrated that the antiresorptive activity of a vitronectin receptor antagonist can be measured after only 7 days of treatment in this refined rat model of thyroxine-induced bone turnover. These data suggest that SB-273005 may be useful for the treatment of metabolic bone diseases, including those resulting from hyperthyroidism.

Studies have demonstrated that hyperthyroidism, whether endogenous or exogenous, is associated with decreased bone density at various skeletal sites and increased risk of bone fracture (Paul et al., 1988; Kung et al., 1993; Wejda et al., 1995). Using histomorphometry, it has been shown that the bone loss is attributed to an increase in bone turnover associated with a larger increase in bone resorption than bone formation (Meunier et al., 1972; Perry, 1989; Moskilde et al., 1990). Studies in the rat have supported this hypothesis (Eriksen et al., 1985; Yamamoto et al., 1993b). Biochemical markers of bone turnover have also been shown to be increased in the rat (Harvey et al., 1991; Kung and Ng, 1994; Taimela et al., 1994; Ishihara et al., 1997). Taken together, these results suggest that patients with hyperthyroidism could potentially benefit from therapy that prevents the increased bone turnover and bone loss.

For bone resorption to occur, osteoclasts must first adhere to the bone matrix. This adhesive event is mediated by the interaction of the osteoclast vitronectin receptor (αvβ3 integrin) with the RGD tripeptide sequence present in several bone matrix proteins (Clover et al., 1992; Helfrich et al., 1992; Nesbitt et al., 1993; Shinar et al., 1993). Disruption of this interaction results in inhibition of resorption both in vitro and in vivo (Fisher et al., 1993; Yamamoto et al., 1993b, 1998; Crippes et al., 1996). Unlike αvβ3, which is expressed on mature osteoclasts, αvβ5 is expressed on osteoclast precursors and has been proposed to play a role in osteoclast differentiation (Sago et al., 1999). SB-273005, a potent antagonist of both of these integrins, inhibits bone resorption in cultures of human osteoclasts with an IC50 of 11 nM (Lark et al., 2001). This compound also inhibits bone resorption after oral administration in the thyroparathyroidectomized rat and prevents bone loss in ovariectomized (OVX) rats (Lark et al., 2001).

The present study had two purposes: to establish a short-term model of thyroid-induced osteopenia in the rat and to evaluate the effect of a vitronectin receptor antagonist, SB-273005, in this high turnover model. For the latter, the model was shortened from the standard 21 days previously used by most investigators to 7 days through the use of biochemical

ABBREVIATIONS: SB-273005, (4S)-2,3,4,5-tetrahydro-8-[2-[6-(methylamino)-2-pyridinyl]ethoxy]-3-oxo-2-{2,2,2-trifluoroethyl}-1H-2-benzazepine-4-acetic acid; OVX, ovariectomized; BMD, bone mineral density; T4, thyroxine; T3, triiodothyronine; pQCT, peripheral quantitative computed tomography; Dpd, deoxypyridinoline; ANOVA, analysis of variance.
markers of bone turnover. Rats were made thyrotoxic by exogenous administration of l-thyroxine (T4), and bone turnover was elevated. A depot formulation of T4 was employed to eliminate daily dosing of T4. We then evaluated the effect of SB-273005 given concomitantly with T4 on biochemical markers of bone turnover.

Materials and Methods

Model Development. All procedures were approved by the Animal Care and Use Committee of GlaxoSmithKline, and animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-month-old virgin female Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were used for all three experimental protocols. The animals were randomly sorted into groups of eight based on body weight.

The first protocol was designed to investigate the effects of thyroid hormone on bone markers and bone mineral density (BMD) in rats. For this experiment, we followed a method published previously (Yamamoto et al., 1993b). All animals were dosed subcutaneously once daily for 24 days starting at day 0 with either vehicle (0.003 N NaOH in saline), T4 (Sigma-Aldrich, St. Louis, MO) bolus at 250 μg/kg in vehicle, or T4 plus estradiol (2.5 mg, 90 day-time-release subcutaneous pellet of 17β-estradiol was purchased from Innovative Research of America, Sarasota, FL). Blood was collected in the morning on days –4 and 18 for the analysis of osteocalcin. Additional blood samples were collected prior to dosing and at 0.5, 2, 8, and 24 h postadministration from the T4 control group for the determination of triiodothyronine (T3) and T4 levels on days 0 and 18. Peripheral quantitative computed tomography (pQCT) was performed on six rats per group, 1 week prior to treatment and just prior to termination of the dose period. Twenty-four-hour urine samples were collected on days –5, 7, 14, and 21. On day 24, the rats were euthanized by CO₂ inhalation.

A second experiment was performed to determine the appropriate dose of subcutaneous depot T4 that would be required to achieve the same elevation in bone turnover that was achieved with the T4 bolus. The animals were dosed subcutaneously for 17 days starting at day 0 with one of the following: vehicle (0.003 N NaOH in saline); T4 bolus at 250 μg/kg/day in vehicle; or T4 (21-day subcutaneously implanted time-release pellet at a dose of 25, 50, or 100 mg obtained from Innovative Research of America). T3 and T4 were measured in the proximal tibia of the left proximal tibia. Animals were anesthetized with isoflurane inhalant. A three-dimensional, 0.5-mm slice was taken through the proximal tibial metaphysis at a point 15% of the length between the tibia-fibula junctions and closer to the knee. Scans for the mask were as follows: object length, 200 mm; voxel size, 0.1 mm; diameter, 40 mm; speed, 3 mm/sec; number of blocks, 2; scout view speed, 30 mm/sec; and scout view distance between lines, 0.5 mm. BMD, bone mineral content, and cross-sectional area were determined for the total, trabecular, subcortical, and cortical regions. Analysis was as follows: Calcld (density and area calculations for the trabecular, total, and subcortical regions) was set at contour mode 2, peel mode 2, inner threshold of 800 mg/cm², and Cortbd (cortical bone density and area determinations) was set at separation mode 2 with a threshold of 800 mg/cm².

Statistical Analysis. Osteocalcin (except for protocol 3), Dpd, and BMD data were analyzed by repeated measures analysis of variance (ANOVA) (Statastica version 5.1; Statsoft Inc., Tulsa, OK). For these data, we compared the change from baseline to the final time point between treated groups and the appropriate control. In addition, two other statistical tests were performed on the urinary Dpd data from protocol 3: a linear trends test and a simple ANOVA using SAS (version 8.01; SAS Institute, Inc., Cary, NC). All other data were analyzed by Student’s t test (Microsoft Excel; Microsoft, Redmond, WA).

Results

Animal Health. The animals in all three experiments appeared in good general health. T4-treated animals, however, did lose a significant (p < 0.05) amount of body weight over the course of all three studies compared with vehicle controls (approximately 9, 7, and 5% for studies 1, 2, and 3, respectively). In addition, approximately 20% of the 50-mg T4 pellet group and nearly all of the 100-mg T4 pellet group exhibited edema around the site of the T4 pellet.

Model Development. To verify and extend previously reported results, we evaluated daily bolus injection of T4 in intact rats. Bolus administration of T4 on day 0 resulted in a rapid rise in serum T4 levels that remained elevated for at least 24 h (Fig. 1). Subsequently, a rise in T3 levels was observed, which remained elevated from at least 0.5 to 8 h after T4 administration. On day 18, levels for both T3 and T4 were similar to day 0 levels (data not shown).

In response to the increase in thyroid hormone, a robust increase in bone turnover was observed. Urinary Dpd levels in the T4-treated group were significantly elevated over vehicle controls as early as day 7 and remained elevated throughout the study (Fig. 2A). Estradiol administration prevented the increase in this marker of bone resorption. A significant increase in serum osteocalcin levels on day 18 was also observed with T4 treatment (Fig. 2B). The elevation was partially reduced by estradiol. The level of the increase in osteocalcin relative to vehicle-treated control animals was less than that observed in urinary Dpd. Because osteocalcin is a marker of osteoblast activity, this suggests that the
increase in bone resorption is greater than that of bone formation, resulting in a negative bone balance.

To determine whether the change in bone turnover had an effect on bone mass, the trabecular BMD of the proximal tibial metaphysis was measured using pQCT. In rats receiving T4, trabecular BMD decreased by 18% relative to the vehicle-treated control group after 24 days of exposure to T4 (Fig. 3). Treatment with estradiol completely prevented the bone loss. These data confirm a negative bone balance in the presence of T4. This also shows that significant increases of Dpd preceded bone mass changes at day 24.

Having demonstrated the significant impact of daily thyroid hormone administration on bone turnover, we tested a depot formulation of T4 to eliminate daily injections. Commercially available time-release pellets of T4, reported to give constant exposure of T4, were used. Because the depot formulation was expected to result in a different systemic exposure profile than subcutaneous injection, the effects of a variety of doses on bone turnover were examined and compared with once a day subcutaneous administration.

T4 treatment by time-release pellets resulted in increases in serum T4 and T3 levels (Fig. 4, A and B). Significant elevations in T4 were observed with all three doses of T4 pellet. There was a large variation on day 7 in T4 and T3 levels in the high dose group due to one outlier. Only the 50- and 100-mg T4 pellets elevated T3 levels consistently and significantly.

T4 bolus administration increased Dpd levels over vehicle control at days 7 and 14 (Fig. 5A). In addition, T4 pellets dose dependently increased Dpd levels over vehicle control. The Dpd response of the 50-mg T4 pellet was most comparable to the T4 bolus.

On day 17, levels of serum osteocalcin were raised over that of vehicle control by T4 bolus administration (Fig. 5B). A dose-dependent increase in osteocalcin was also observed in the T4 pellet groups. Both the 50- and 100-mg pellets caused increases in osteocalcin comparable to those seen with the T4 bolus. The approximately 20% decline of osteocalcin levels in the vehicle and 25-mg pellet groups could possibly be due to the day-to-day variation in the assay. As in experiment 1, the change in osteocalcin was less than the change in Dpd, indicating that there was a negative bone balance between bone resorption and formation.

The increase in bone turnover from the T4 bolus led to a 7% decrease by day 16 in trabecular BMD of the proximal tibia (Fig. 6). The bone turnover changes from the T4 pellets resulted in a dose-dependent decrease in cancellous bone with the 100-mg dose losing 17%. Both the 25- and 50-mg T4 pellets resulted in a bone loss similar to that with the bolus dose.

Effect of Vitronectin Receptor Antagonist, SB-273005, on Bone Resorption in T4-Induced Turnover Model in the Rat.

As shown above, the increased bone turnover and level of bone loss resulting from treatment with the 50-mg pellet of T4 were comparable to those following T4 daily bolus injection. Furthermore, in both experiments, the elevation of bone resorption after 7 days of treatment resulted in measurable loss of trabecular BMD at later time points. Based on these data, the effect of a vitronectin recep-
tor antagonist, SB-273005, on bone turnover was evaluated in this system. To do this, SB-273005 was coadministered with T4 for a period of 8 days, and biochemical markers of bone turnover were evaluated.

Baseline Dpd levels were not significantly different from one another (ANOVA). On day 7, a doubling of Dpd levels over vehicle controls was observed with a 50-mg T4 pellet (Fig. 7A). Coadministration of SB-273005 with T4 decreased Dpd levels dose dependently with reductions of 26, 40, and 68% for the 3, 10, and 30 mg/kg oral doses, respectively. This dose dependence was verified through a linear trends analysis of day 7 raw data. A probability value of 0.0007 for all three doses and a probability value of 0.0277 for the two lowest doses were observed. In addition, repeated measures analysis of the baseline and day 7 data confirmed significant inhibition by SB-273005 at the 30 mg/kg dose ($p < 0.01$). The 50-mg T4 pellet increased serum osteocalcin levels over vehicle controls on day 8 (Fig. 7B). SB-273005 had no significant effect on serum osteocalcin levels.

**Discussion**

Treatment of rats with excess thyroid hormone for 3 weeks produced increased T3 levels. This led to increases in urinary Dpd (resorption) and serum osteocalcin (osteoblast activity) and, subsequently, a loss of trabecular bone in the proximal tibia. Cotreatment of T4 and estradiol prevented the increased turnover and bone loss associated with levothyroxine therapy. The marker changes were observed as early as 7 days and were predictive of bone mass changes at 3 weeks. Taken together, these data show that bone markers are an excellent predictor of changes in bone mass. These data are in agreement with clinical data showing the predictive value of markers in hyperthyroidism (Siddiqi et al., 1997) and in osteoporosis following treatment (Ravin et al., 1999; Delmas et al., 2000).

A second study demonstrated that a 50-mg T4 pellet produced a response in bone over 2 weeks similar to that with the T4 bolus. The use of the pellets is a convenience that eliminates daily dosing of the T4. The two lower dose pellets were very consistent in the day-to-day systemic levels of T3 that were produced.

Having confirmed that excess thyroid hormone in rats induces high bone turnover and cancellous bone loss, the antiresorptive effect of the osteoclast vitronectin receptor antagonist, SB-273005, on T4-induced turnover changes was examined. The observation that SB-273005 inhibited the increase in Dpd associated with T4 administration suggests that the compound inhibited bone resorption. No difference was seen in serum osteocalcin levels by SB-273005 at the 30 mg/kg dose ($p < 0.01$). The 50-mg T4 pellet increased serum osteocalcin levels over vehicle controls on day 8 (Fig. 7B).
tase, pyridinoline, and Dpd. Ongphiphadhanakul et al. (1993) have shown that after 3 weeks of treatment in male hyperthyroid rats with a bisphosphonate, femoral mRNA levels of tartrate-resistant acid phosphatase and alkaline phosphatase were reduced compared with T4 controls. The current study is, however, the first report of inhibition in as little as 7 days of a bone resorption marker by an antiresorptive agent. Such a short-term model is an advance over previous study designs with respect to evaluation of antiresorptive therapy.

The efficacy of SB-273005 in the prevention of bone loss has been observed in several animal models. SB-273005 reduced resorption by inhibiting the parathyroid hormone-stimulated calcemic response of hypocalcemic thyroparathyroidectomized rats, and in the OVX rat SB-273005 reduced the bone resorption marker, Dpd, and prevented bone loss in the lumbar vertebrae (Lark et al., 2001). In a study by Engleman et al. (1997), a peptide mimetic of the vitronectin receptor was administered intravenously in the rat, and inhibited the OVX-induced increase in urinary pyridinyl cross-links and prevented trabecular bone loss. In both of these studies, an inhibition of OVX-induced bone loss was preceded by an inhibition of a marker of osteoclastic bone resorption. Therefore, if the current study was extended beyond 8 days, it is expected that the reduction in Dpd would lead to a reduction in the loss of bone mass.

Treatment of thyrotoxicosis in humans restores bone metabolism to normal (Macleod et al., 1993) and leads to an increase in BMD (Rosen and Adler, 1992). However, several clinical studies have shown that despite effective treatment for hyperthyroidism, bone loss recovery may be incomplete (Toh et al., 1985; Mosekilde et al., 1990; Franklyn et al., 1994; Lupoli et al., 1996). Since reduction in BMD is a risk factor for subsequent bone fracture, antiresorptive therapy may prove beneficial.

Other antiresorptive therapies have been tested in hyperthyroid patients with mixed success. In a study that supports the effect of estradiol observed here, Franklyn et al. (1995) reported that estrogen replacement therapy abolished the reduction in femoral and vertebral BMD in postmenopausal women with previous thyrotoxicosis and subsequent T4 therapy. In studies by Kung and Yeung (1996) and Jodar et al. (1997), intranasal calcitonin provided no additional benefit over calcium or attainment of a euthyroid state, respectively. However, alendronate, a bisphosphonate, increased BMD...
and decreased serum osteocalcin levels in pre- and postmeno-
gastral hyperthyroid women treated with methimazole, an
antithyroid agent (Lupoli et al., 1996).

In conclusion, we have established a short-term model of
thyroid hormone-induced osteopenia. The resorption inhibi-
tors, estradiol and the αvβ3 and αvβ5 antagonist SB-273005,
were able to inhibit T4-induced turnover. Although the
present study did not examine whether the vitronectin re-
cptor antagonist, SB-273005, could restore the established
bone loss in a rat model of hyperthyroidism, the findings
indicate that a vitronectin receptor antagonist may be of

potential benefit for thyrotoxic patients with high bone turn-
over osteoporosis.

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Fig. 7. Comparison of the effect of vehicle, T4 time-release pellet, or T4
time-release pellet and SB-273005 at 3, 10, or 30 mg/kg on urinary Dpd
(A) on day 7 and serum osteocalcin (B) on day 8 in rats. The animals in the
group received vehicle once daily by subcutaneous injection for 8
days. Data are presented as mean ± S.E. of eight animals per group. *,
***, p < 0.01; ***, p < 0.001 versus T4 group as determined by repeated
measures analysis. #, p < 0.001 for linear trends test.

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