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The calcimimetic AMG 641 abrogates parathyroid hyperplasia, bone and vascular calcification abnormalities in uremic rats

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Abstract

Calcimimetics and vitamin D sterols reduce serum parathyroid hormone (PTH) in patients with secondary hyperparathyroidism receiving dialysis, a disease state associated with parathyroid hyperplasia, vascular calcification, bone disease, and increased mortality. The aim of this study was to determine the effects of the research calcimimetic AMG 641 (Amgen, Inc., Thousand Oaks, CA) or calcitriol (Sigma Aldrich Corporation, St. Louis, MO) on vascular calcification in a rodent model of progressive uremia with accompanying secondary hyperparathyroidism induced by dietary adenine. Treatment effects on parathyroid gland hyperplasia and bone loss were also investigated. Rats were treated daily with vehicle, calcitriol (10 ng), AMG 641 (3 mg/kg), or no treatment during the 4 week period the animals were fed adenine. The uremia-induced increases in serum PTH levels were significantly attenuated by both AMG 641 (>90%) and calcitriol (~50%). AMG 641 significantly reduced calcium–phosphorus product (Ca×P) and significantly attenuated the development of both parathyroid hyperplasia and vascular calcification. In addition, AMG 641 prevented the defects in trabecular bone volume, trabecular number, and bone mineralization, as well as increases in trabecular spacing in this rodent model of secondary hyperparathyroidism. Calcitriol (10 ng/rat) decreased osteoid surface/bone surface, but had no effects on other bone parameters, or parathyroid hyperplasia (likely due to the lower PTH suppressive effect of calcitriol at the dose used in this study). However, this dose of calcitriol significantly exacerbated vascular calcification. These results suggest that calcimimetics can reduce the development of vascular calcification, parathyroid hyperplasia and bone abnormalities associated with secondary hyperparathyroidism.

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Keywords

Bone; Calcimimetic; Calcium-sensing receptor; Parathyroid gland hyperplasia; Secondary hyperparathyroidism; Vascular calcification; Vitamin D sterol

1. Introduction

Patients with chronic kidney disease receiving dialysis often develop secondary hyperparathyroidism characterized by increased serum parathyroid hormone (PTH), increased serum phosphorous, decreased serum calcium and calcitriol (Goodman and Quarles, 2008; Drueke et al., 2007). Secondary hyperparathyroidism is accompanied by parathyroid hyperplasia and excessive synthesis and secretion of PTH, which can result in disproportionate bone resorption and other bone disorders, soft tissue and vascular calcification, and significant risk for cardiovascular morbidity and mortality (Block et al., 2004; de Francisco, 2004; Hebert, 2006; Kalantar-Zadeh et al., 2006; Young et al., 2005).

Evidence suggests that reductions in PTH and serum phosphorus may slow or prevent secondary hyperparathyroidism-associated parathyroid hyperplasia, vascular calcification, and renal osteodystrophy. Traditional therapeutic approaches rely on the actions of vitamin D sterols, which, while able to decrease PTH levels, have also been associated with hypercalcemia and vascular calcification in preclinical studies (Henley et al., 2005; Lopez et al., 2006). Calcimimetics (e.g., cinacalcet HCl), pharmacologic agents that act directly at the calcium-sensing receptor in the parathyroid gland to reduce PTH secretion, represent a relatively new therapeutic approach. Evidence suggests that calcimimetics may slow or prevent parathyroid hyperplasia in uremic animals (Colloton et al., 2005; Wada et al., 1997) without inducing vascular calcification (Henley et al., 2005; Lopez et al., 2006). Moreover, preclinical (Wada et al., 1998) and clinical (Lien et al., 2005; Cunningham et al., 2005) evidence suggests that calcimimetics improve bone health, including reducing the incidence of fractures. Some clinical data show that calcitriol may influence bone remodeling and ameliorate osteitis fibrosa (Slatopolsky et al., 1984; Andress et al., 1989), although other studies have shown either no or detrimental effects on bone remodeling (Costa et al., 2003; Pahl et al., 1995).

There are currently no reports of calcimimetic effects on the totality of biochemical (PTH, calcium, phosphorus, $\text{Ca} \times \text{P}$) and pathological parameters of secondary hyperparathyroidism (parathyroid hyperplasia, vascular calcification and renal osteodystrophy) in a rodent model of uremia with secondary hyperparathyroidism. Herein, we investigated whether the research calcimimetic AMG 641 (chemical name: (1R)-N-((6-(methyloxy)-4'-(trifluoromethyl)-3-biphenyl)methyl)-1-phenylethanamine) could abrogate these characteristic biochemical and pathological changes without exacerbating vascular calcification observed in the adenine-treated uremic rat. AMG 641 is an arylalkylamine with a molecular weight of approximately 400 g/mol, is more potent than cinacalcet, and has approximately a 3-fold longer half-life and a larger volume of distribution. We also investigated the effects of calcitriol on the above parameters utilizing a dose that would significantly lower PTH while avoiding the potentially deleterious effects of hypercalcemia.

2. Materials and methods

Male Sprague–Dawley rats (350–390 g) were purchased from Harlan (Indianapolis, IN). Rats were pair-housed under a 12 h/12 h light/dark cycle and given ad libitum access to standard rat chow (1.2% calcium, 0.9% phosphorus) and water. Experiments were performed under protocols approved by Amgen's Internal Animal Care and Use Committee.

2.1. Adenine-induced uremia and drug administration

Rats were randomly assigned into treatment groups based on the normal distribution of baseline body weights, then fed a diet containing 0.75% adenine (Adenine freebase A8626, Sigma Aldrich, St. Louis, MO) or a control diet with equivalent amounts of calcium (1.1%) and phosphorus (0.9%) (Dyets, Inc, Bethlehem, PA) for 4 weeks. Concurrent with adenine exposure, rats were divided into the following daily treatment groups: normal, non-adenine control ($n=8$), adenine control (no treatment; $n=9$), vehicle for AMG 641 (12% captisol in water p.o.; $n=9$), AMG 641 (3 mg/kg, p.o.; $n=9$), vehicle for calcitriol (0.19% ethanol in phosphate buffered saline, s.c.; $n=10$), or calcitriol (1 α , 25-dihydroxycholecalciferol, Sigma Aldrich Corp, St. Louis, MO, 10 ng/rat [~ 0.025 – 0.028 $\mu\text{g/kg}$], s.c.; $n=10$). The dose of calcitriol was chosen since higher doses resulted in significant vascular calcification in subtotal nephrectomized rats (Henley et al., 2005), and we wanted to reduce serum PTH significantly while attempting to avoid overwhelming vascular calcification in the rat uremia adenine model.

2.2. Aorta histopathology

Twenty-four hours following the last dose, rats were carbon dioxide-euthanized. The thoracic aorta was then excised and fixed in aqueous buffered zinc formalin (Z-fix; Anatech Ltd; Battle Creek, MI), paraffin embedded, sectioned longitudinally, and von Kossa stained. A board-certified pathologist blinded to treatment groups evaluated and scored the stained sections within a single session (to avoid drift in scoring outcome). One section per animal was scored on a 0–5 scale: 0=no calcification, 1=minimal, 2=mild, 3=moderate, 4=marked, and 5=severe calcification.

2.3. Blood biochemistries

PTH, total serum calcium and phosphorus, ionized calcium, blood urea nitrogen [BUN], and creatinine were determined from blood collected from the retro-orbital sinuses of isoflurane anesthetized rats. Ionized calcium was measured using a Ciba-Corning 634 ISE Ca^{++}/pH Analyzer (Ciba-Corning Diagnostics Corp, Medfield, MA) immediately after collection into heparinized capillary tubes. Blood for the remaining parameters was collected into SST (clot activator) brand blood tubes. Serum was removed and stored at -70 °C. A blood chemistry analyzer (AU 400; Olympus, Melville, NY) was used to determine calcium, phosphorus, BUN, and creatinine, and rat $\text{PTH}_{(1-34)}$ immunoradiometric assay kits (Immutopics, San Clemente, CA) were used to determine PTH levels.

2.4. Parathyroid hyperplasia

After sacrifice, the laryngo-tracheal complex was removed, stored in zinc-buffered formalin (2 to 3 days), transferred to 70% alcohol and trimmed. The thyroid and parathyroid glands

were dissected from each other, and parathyroid glands were blotted dry on a lint-free Kim wipe (KimberlyClark Corp., Roswell, GA, USA), weighed, and paraffin embedded. Sections (5 μ m) were placed onto charged slides (VWR Scientific, West Chester, PA, USA). Increases in the number of proliferating parathyroid cells were determined by immunostaining using a proliferating cell nuclear antigen (PCNA) staining kit (Zymed Laboratories, Inc., South San Francisco, CA, USA). Parathyroid area was determined as previously described (Colloton et al., 2005) using an area-measurement graticle with 0.01 mm² grids. Data is expressed as the number of PCNA-positive cells/mm².

Total gland weight is expressed as parathyroid gland weight/body weight.

2.5. Bone histopathology and histomorphometry

Approximately 1/3 of the distal femur and 1/3 midshaft femur was prepared using slow speed saw. Undecalcified segments were processed through defatting and infiltration and embedded in methylmethacrylate. Frontal sections of the distal femur were obtained near the middle of the bone using a Leica/Jung 2255 microtome set at 4 μ m. Distal femur sections were counter-stained with Von Kossa and tetrachrome bone stains. Histomorphometric evaluations (Bioquant Image Analysis Corporation, Nashville, TN) of the stained slides were performed in a blinded manner with the nomenclature and calculations based on standardized terms and formulae (Parfitt et al., 1987), such as percent bone volume per tissue volume (%BV/TV), percent osteoblast surface per bone surface (%ObS/BS), percent osteoclast surface per bone surface (%OcS/BS), trabecular spacing (TbSp, measured in μ m), trabecular number (TbN)/millimeter, and percent osteoid surface/bone surface (%OS/BS).

Analyses were performed in the secondary spongiosa of the distal femurs that included the trabecular area between 0.1 mm and 1.1 mm distal to the lowest point of the growth plate. Structural evaluation of the trabecular bone at the metaphysis included measurement of bone area, tissue area, and bone perimeter; measurement of cellular level activities included osteoid, osteoclastic, and osteoblastic perimeters.

2.6. Statistical analyses

The mean and standard error of the mean was determined and statistical analyses were used to compare differences between the treatment groups in body weight, serum chemistry and biochemical markers of bone turnover, and parathyroid gland weights. Analysis of variance (ANOVA; $\alpha=0.05$) was used to test for overall treatment effects and post-hoc analysis (Fisher's Protected Least Squares Difference tests) was used for between group comparisons ($\alpha=0.05$). Paired *t*-test ($\alpha=0.05$) were used to test for differences between predose and postdose values for a given treatment condition and day.

3. Results

3.1. Effects of AMG 641 or calcitriol on blood biochemical parameters

Adenine-induced uremia was confirmed by significant increases in serum creatinine and BUN compared with untreated controls ($P<0.05$; Table 1), and the uremic state was unaffected by any of the treatments (Table 1). While a slight decrease in creatinine and BUN

levels were observed in the AMG 641 group ($P=0.045$ compared with vehicle), they remained grossly elevated (6- to 10-fold) over the normal values. It is noteworthy that Piecha et al., have reported that the calcimimetic R-568 or calcitriol have beneficial effects on progression of renal damage in subtotal nephrectomized rats (Piecha et al., 2008). Since we did not assess the myriad of parameters that were assessed in their study we cannot definitively say that AMG 641 was nephroprotective in our study. Secondary hyperparathyroidism was confirmed by a significant rise in serum PTH in rats receiving dietary adenine compared with untreated controls ($P<0.05$). Uremic rats (on adenine diet) lost an average of ~32.6% body weight over the course of the study. The rats receiving standard chow were weight stable; gaining 20 g over the time course. Body weight in the uremic animals was unaffected by any treatment (i.e., AMG 641, calcitriol or their vehicles).

Both calcitriol and AMG 641 significantly reduced serum PTH (Table 1). Rats treated with AMG 641 exhibited greater than 90% reduction in PTH compared with animals treated with its vehicle (13 ± 3 vs 656 ± 76 pg/mL respectively, $P<0.001$). Rats in the calcitriol-treatment group exhibited a significant reduction in PTH compared with vehicle-treated controls (396 ± 57 vs 801 ± 70 pg/mL, respectively, $P<0.01$). AMG 641 prevented adenine-induced increase in PTH such that PTH levels were not statistically different than those observed in non-adenine controls. It is important to note that the magnitude of PTH suppression by calcitriol was 30-fold less than was observed for AMG 641. The calcitriol dose (10 ng/rat) was limited to avoid significant hypercalcemia and moderate to severe vascular calcification observed with higher doses in uremic rats (Henley et al., 2005; Miller et al., 2006a,b). Serum PTH levels in the corresponding vehicle-treated groups were not significantly different from the adenine-induced uremic rats receiving no treatment (Table 1).

Total serum calcium was unchanged in any treatment group compared with non-adenine controls, with the exception of AMG 641-treated rats (Table 1), which exhibited significantly reduced total serum calcium compared with all other groups ($P<0.05$, Table 1). Serum phosphorus was significantly increased in adenine-fed rats compared with rats receiving the adenine-free diet ($P<0.05$), regardless of treatment group.

3.2. Effects of AMG 641 or calcitriol on vascular calcification

Aortas were collected from all rats/group ($n=9-10$) per week at weeks 1, 2, 3, and 4. The degree of aortic calcification was determined in a blinded manner. Aortic calcification slowly progressed such that there was no significant difference between groups on weeks 1–3 compared to non-adenine controls; hence only 4 week data is reported. Adenine treatment alone or in the presence of vehicle produced minimal to mild aortic vascular calcification by the end of the 4-week treatment period, with calcification scores significantly higher than non-adenine-treated controls ($P<0.0001$), which exhibited no calcification. Calcification scores in adenine only (1.33 ± 0.02), and vehicle controls (adenine+vehicle for AMG 641 (1.77 ± 0.49 ; Fig. 1B), and adenine+vehicle for calcitriol (1.5 ± 0.5 ; Fig. 1D), were not significantly different from each other. Calcitriol exacerbated adenine-induced calcification, with moderate to severe calcification occurring in 40% of animals and an overall calcification score of 2.8 ± 0.49 ($P<0.0001$ vs adenine alone or its own vehicle) (Fig. 1C). Calcification was confined to the medial soft tissue layers (Fig. 1). Calcification was not

observed in any of the adenine animals treated with AMG 641 (calcification score 0.0 ± 0.0) (Fig. 1A).

3.3. Effect of AMG 641 or calcitriol on parathyroid gland weight, area, and hyperplasia

At week 4, adenine-fed animals receiving either vehicle or no treatments displayed significant ($P < 0.01$) time-dependent increases in PCNA-positive cells and parathyroid weight compared with animals on a standard non-adenine diet (Tables 2 and 3) (data on non-adenine diet animals were not collected at weeks 1, 2 or 3). There were no significant differences between adenine-fed rats with or without vehicle. In contrast, administration of AMG 641 at 3 mg/kg daily for 4 weeks resulted in a significant ($P < 0.001$) reduction in the number of PCNA-positive cells (40 ± 5) compared with vehicle-treated adenine-fed animals (157 ± 22). The number of PCNA-positive cells in AMG 641-treated animals (40 ± 5) were not significantly ($P > 0.05$) different from the number of PCNA-positive cells observed in animals on a standard non-adenine diet (33 ± 6). Similarly, adenine+AMG 641 resulted in a significant ($P < 0.01$) reduction in parathyroid gland weight/body weight (0.891 ± 0.087 mg/kg) compared with adenine+AMG 641 vehicle (2.501 ± 0.119 mg/kg; Table 3). Parathyroid gland area was increased in adenine and adenine plus vehicle-treated animals. The calcimimetic AMG 641 significantly ($P < 0.05$) reduced parathyroid gland area by approximately 50% compared with its own vehicle (Fig. 2).

In contrast to calcimimetic-treated animals, calcitriol at the dose utilized had no significant ($P > 0.05$) effect on reducing the adenine-induced increases in PCNA-positive cells (147 ± 19 vs 142 ± 19 , respectively; Table 2), parathyroid gland weight (2.665 ± 0.181 vs 2.408 ± 0.113 mg/kg; Table 3) or parathyroid gland area (0.725 ± 0.05 vs 0.69 ± 0.06 mm²; Fig. 2) compared with vehicle-treated animals. Previously, in a different chronic kidney disease model (5/6 nephrectomized rats), higher calcitriol doses (30 and 100 ng/day) led to PTH lowering equivalent to that observed with calcimimetic treatment and to significant reductions in parathyroid PCNA staining, however, calcitriol treatment was associated with extensive calcification. Thus, it is likely that a higher calcitriol dose than used here could decrease PTH as well as PCNA-positive cells to the same degree as AMG 641 in this model.

3.4. Effect of AMG 641 or calcitriol on bone

In line with the known effects of uremia on bone health, adenine exposure for 4 weeks led to loss of bony trabeculae at the distal femur. Trabeculae were preserved by AMG 641, but not calcitriol, treatment (Fig. 3), likely reflecting differences in the degree of PTH reduction. Histomorphometric analysis revealed loss of trabecular bone volume (% BV/TV) in adenine-fed rats receiving no treatments compared with rats fed a normal diet ($P < 0.001$; Table 4). Similarly, animals receiving adenine+calcitriol or adenine+vehicle for calcitriol ($P < 0.05$) or vehicle for AMG 641 ($P < 0.01$) showed decrease in the %BV/TV compared with rats fed a normal diet. Adenine-fed animals treated with AMG 641, however, did not show the decrease in %BV/TV, instead exhibited %BV/TV values similar to those in rats fed a normal diet (Table 4). Adenine-fed rats receiving no treatments exhibited a significant ($P < 0.001$) reduction in trabecular number (TbN) and a significant ($P < 0.01$) increase in trabecular spacing (TbSp) compared with rats fed a normal diet (Table 4). Similar results were seen in the vehicle-treated rats (Table 4). In contrast, AMG 641 treatment had a pronounced,

beneficial effect on TbN and TbSp. The adenine-mediated reduction in TbN ($P<0.001$) and increase in TbSp ($P<0.01$) was significantly prevented in animals treated with AMG 641 compared with vehicle (Table 4). Both TbN and TbSp were normalized in AMG 641-treated rats (Table 4). In contrast, calcitriol treatment did not prevent bone loss or changes in TbN and TbSp, again likely reflecting differences in the degree of PTH reduction associated with this dose of calcitriol used.

Osteoid surface/bone surface (%OS/BS) was significantly elevated in adenine-fed rats receiving no treatment ($P<0.01$), -vehicle for calcitriol ($P<0.001$) or -vehicle for AMG 641 ($P<0.05$) compared to non-adenine (control) rats. The increase in osteoid (unmineralized bone due to PTH driven increases in osteoblast activity) surface was inhibited by treatment with calcitriol ($P<0.05$) or AMG 641 ($P<0.01$) compared with their respected vehicle controls. The OS/BS for the AMG 641 group was similar to non-adenine controls. Calcitriol treatment decreased OS/BS to a lesser degree than AMG 641, likely reflecting less efficacious lowering of PTH with the dose employed.

Osteoblast surface/bone surface (%ObS/BS) was significantly increased in vehicle-treated controls for both calcitriol and AMG 641 vehicles ($P<0.001$ and <0.01 respectively). Compared with respective vehicle controls, treatment with either agent resulted in a significant decrease in %ObS/BS ($P<0.01$), consistent with the decrease in osteoid parameters.

Adenine had no significant effect on bone resorption parameters (Table 4). Neither osteoclast surface nor osteoclast surface/bone surface were different from normal (non-adenine control) animals after 4 weeks of treatment despite the significant decreases in PTH.

4. Discussion

Calcimimetics suppress PTH and prevent parathyroid hyperplasia without producing vascular calcification in subtotal 5/6 nephrectomized uremic rats (Henley et al., 2005; Lopez et al., 2006, 2008). While the 5/6 nephrectomized model exhibits the biochemical, bone, and parathyroid hyperplasia changes associated with secondary hyperparathyroidism, significant vascular calcification is not observed without concomitant administration of the vitamin D sterol, calcitriol (Henley et al., 2005). In the present study we investigated calcimimetic effects in the adenine-induced uremia model in which rats develop a more severe, rapidly progressing renal failure accompanied by secondary hyperparathyroidism and the clinical consequences or pathologies of vascular calcification and renal osteodystrophy along with the biochemical disturbances that are seen in patients with secondary hyperparathyroidism receiving dialysis (Yokozawa et al., 1986; Tamagaki et al., 2006). In addition, we investigated the effects of calcitriol in this model, at a lower dose than used in previous studies in 5/6 nephrectomized rats, in an attempt to obviate moderate to severe vascular calcification.

We demonstrated that the potent research calcimimetic AMG 641 prevents elevations in PTH, parathyroid gland hyperplasia, vascular calcification and renal osteodystrophy in adenine-treated uremic rats. AMG 641, like cinacalcet HCl, acts as an allosteric modulator

of the calcium-sensing receptor to reduce plasma PTH levels, providing beneficial effects on multiple pathologies associated with elevated PTH.

In contrast to AMG 641, which abrogated vascular calcification, calcitriol in a minimal effective PTH lowering dose (10 ng/day) exacerbated calcification of the aorta. Osteoid parameters were improved with calcitriol, but hyperplasia and bone loss were not; while AMG 641 prevented parathyroid cell proliferation, and was bone protective. Prevention of parathyroid hyperplasia and bone disease by AMG 641 may be secondary to serum lowering of PTH. The use of sufficiently high doses of calcitriol to reduce PTH to levels observed with AMG 641 would likely result in similar effects on bone and hyperplasia (Cozzolino et al., 2001), but at the expense of increased vascular calcification (Henley et al., 2005; Lopez et al., 2006, 2008). Vitamin D sterols exhibit varied effects, including increasing serum calcium and phosphorus levels (Tentori et al., 2006) and mediating expression of bone-associated genes in vascular smooth muscle cells (Shalhoub et al., 2006a,b).

An important question regarding calcimimetic or vitamin D sterol use in secondary hyperparathyroidism is whether treatment modifies parathyroid gland hyperplasia and reduces gland mass. We previously showed that cinacalcet prevented acute parathyroid hyperplasia in uremic rats (Colloton et al., 2005). The present study with AMG 641 confirms that calcimimetics attenuate progression of parathyroid hyperplasia and corroborates several reports indicating marked reduction in numbers of S-phase, PCNA-positive and overall parathyroid cell numbers in calcimimetic-treated, uremic rats (Wada et al., 2000; Chin et al., 2000; Mizobuchi et al., 2004; Colloton et al., 2005). Of note, AMG 641 was able to prevent parathyroid gland hyperplasia even in the presence of extremely high serum levels of phosphorus, a known direct stimulator of parathyroid hyperplasia (Almaden et al., 1996; Slatopolsky et al., 1996).

Several studies support the notion that calcium-sensing receptor signaling is a major determinant in controlling hyperplasia and disease progression (Brown and MacLeod, 2001; Drueke et al., 2007). Parathyroid hyperplasia is observed in patients with neonatal severe hyperparathyroidism, a disease attributed to calcium-sensing receptor loss of function mutations (Chattopadhyay et al., 1996). Furthermore, parathyroid hyperplasia is observed in calcium-sensing receptor null mice, further demonstrating that the absence of the calcium-sensing receptor can influence chief cell proliferation in non-uremic animals (Ho et al., 1995). Interestingly, vitamin D receptor knockout mice develop parathyroid hyperplasia that is mitigated by a high calcium diet (Li et al., 1998). As previously noted we did not use higher doses of calcitriol that would result in increased serum calcium to prevent hyperplasia in the current study because of excessive aortic calcification.

Studies have demonstrated that calcitriol can prevent parathyroid cell proliferation in uremic rats when given at the very start of chronic renal failure (Szabo et al., 1989). A decline in both calcium-sensing receptor and vitamin D receptor expression is characteristic of hyperplastic parathyroid cells (see review Drueke et al., 2007). Once parathyroid cell proliferation advances, traditional therapies such as calcium and calcitriol become ineffective (i.e., refractory to subsequent treatment). Recent studies have shown that the downregulation of both vitamin D receptor and calcium-sensing receptor mRNA and protein

in uremic animals can be reversed by calcimimetics (Mizobuchi et al., 2004; Rodríguez et al., 2007) and some, but not all, evidence suggests that vitamin D sterols may also increase calcium-sensing receptor expression in the parathyroid gland and kidney of the rat (Brown et al., 1996; Rogers et al., 1995). Vitamin D-elicited regulation of the parathyroid gland calcium-sensing receptor would tend to facilitate inhibition of parathyroid function in the uremic state by increasing sensitivity to serum calcium. Furthermore, vitamin D-induced increases in serum calcium could secondarily decrease PTH secretion via increased parathyroid calcium-sensing receptor expression and subsequent inhibition of parathyroid hyperplasia (see Rodríguez et al., 2005). Thus, upregulation of the calcium-sensing receptor may lead to reduced hyperplasia.

The precise molecular mechanism for decreased parathyroid cell proliferation by calcimimetics is unclear. Miller et al. (2006) demonstrated that cinacalcet reduced hyperplasia in long-term uremic rats with established secondary hyperparathyroidism, whereas discontinuation of cinacalcet resulted in a gradual return of parathyroid hyperplasia. The reduction in cellular proliferation as measured by PCNA staining was associated with a concomitant rise in the number of cells expressing the cyclin-dependent kinase inhibitor p21. The increase in p21 was not sustained upon discontinuation of treatment. These findings suggest that the calcimimetic-mediated increase in p21 in uremic rats may mediate, at least in part, the effects on hyperplasia. The direct mechanism(s) by which calcimimetics regulate hyperplasia has not been adequately investigated and is the focus of further study.

Increasing evidence suggests that disordered mineral metabolism and bone disease, common complications of chronic kidney disease, are associated with increased risk for cardiovascular calcification (Chertow et al., 2002; Block et al., 2005; Braun et al., 2004), morbidity, and mortality (Block and Cunningham, 2006; Block et al., 2007; Matsuoka et al., 2004). The underlying mechanisms for this linkage are not completely understood, but are likely related to vascular calcification leading to changes in cardiovascular structure and function (Ketteler et al., 2005; London et al., 2005).

The present data, coupled with previous findings (Tamagaki et al., 2006), demonstrate that rats fed a 0.75% adenine diet for four weeks exhibit vascular calcification. In agreement with Tamagaki et al. (2006), medial calcification, one of the characteristics of vascular calcification in hemodialysis patients, was confirmed in these studies. This type of calcification is independent of lipids and seems to be related to the expression of numerous bone-associated proteins (Jono et al., 2000; Tyson et al., 2003). It is generally accepted that elevated Ca $\times$ P predisposes patients with secondary hyperparathyroidism to vascular and tissue calcification and increased cardiovascular mortality risk (Goodman et al., 2004; Block et al., 1998). O'Neill (2007) recently downplayed the assumption that Ca $\times$ P product drives ectopic calcification and highlighted the fact that calcification is a complex biological process not governed completely by CaHPO₄ precipitation (O'Neill, 2007). In this study, serum calcium levels were reduced by AMG 641 without a pronounced effect on serum phosphorus. Reduction in blood calcium mediated by calcimimetics has been observed in both hemodialysis patients as well as acutely nephrectomized animals, which suggests that this phenomenon may be mediated in part through non-renal mechanisms (Fox et al., 1999). Calcimimetics in rats cause a transient increase in serum calcitonin, which may contribute to

the rate of onset of the observed decrease in serum calcium levels (Nemeth et al., 2004; Fox et al., 1999).

The importance of serum calcium in giving rise to vascular calcification independent of phosphorus levels was demonstrated by the ability of AMG 641 to prevent the development of vascular calcification despite the highly elevated phosphorus levels, which have been associated with the rapid development of vascular calcification (Tamagaki et al., 2006). The ability of calcimimetics to prevent vascular calcification is consistent with the effect of the calcimimetics R-568, AMG 641 or cinacalcet in 5/6 nephrectomized rats (Lopez et al., 2006, 2008; Kawata et al., 2008), and of R-568 in attenuating calcium-induced mineral disposition in cultured vascular smooth muscle cells (Alam et al., 2009). These authors suggested that the loss of functional vascular smooth muscle cell calcium-sensing receptor induced by disturbances in serum calcium and phosphorus in stage 5 chronic kidney disease exacerbates mineral deposition. Calcimimetics may prevent calcium-sensing receptor loss and/or improve calcium-sensing receptor functionality to attenuate the calcification process; similar to the calcimimetic-induced increase in parathyroid gland calcium-sensing receptor expression from rats with secondary hyperparathyroidism (Mizobuchi et al., 2004) and in the intima of uremic rats (Koleganova et al., 2009). Recently resolution of soft tissue calcification was observed in a hemodialysis patient following six months of treatment with cinacalcet (Zerbi et al., 2008).

Persistently elevated levels of PTH, as seen in secondary hyperparathyroidism, lead to high-turnover bone disease, the severity of which is directly proportional to the magnitude of the disease and overproduction of PTH. The calcimimetic R-568 reversed the development of osteitis fibrosa and restored cortical bone strength through normalization of serum PTH in uremic rats (Wada et al., 1998). Consistent with this observation, preclinical and clinical data have suggested that cinacalcet may reduce bone turnover, tissue fibrosis, bone loss and fractures (Lien et al., 2005; Cunningham et al., 2005). Likewise, some evidence suggests that calcitriol can ameliorate osteitis fibrosa (Slatopolsky et al., 1984; Andress et al., 1989), while other clinical studies show either no or detrimental effects on bone remodeling (Costa et al., 2003; Pahl et al., 1995).

Consistent with a previous study (Tamagaki et al., 2006), adenine-fed rats presented severe bone lesions within 4 weeks. AMG 641 prevented adenine-induced loss in bone volume and trabecular number, increase in trabecular spacing, and defects in bone mineralization. Although PTH levels were low in the AMG 641-treated animals (to 13 pg/ml), they were not significantly lower than normal non-uremic controls, and did not result in adynamic bone disease or significant alterations in bone resorption parameters.

In conclusion, the present study indicates that the calcimimetic AMG 641 reduced circulating PTH levels, and prevented parathyroid hyperplasia, vascular calcification and bone abnormalities associated with secondary hyperparathyroidism. The ability of calcimimetics to modify disease progression and morbidities related to secondary hyperparathyroidism as observed in preclinical models is currently being tested clinically in stage 5 chronic kidney disease patients with secondary hyperparathyroidism.

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Fig. 1.

Von Kossa-stained sections of aortas showing mineralization from animals fed adenine (0.75%) for 4 weeks and treated for 4 weeks with either (A) AMG-641 (3 mg/kg, p.o.); (B) 10% captisol-vehicle for AMG 641 (1 ml/rat, p.o.); (C) calcitriol (10 ng/rat, s.c.) or (D) PBS-vehicle for calcitriol (0.2 ml/rat s.c.).

Fig. 2. Rats fed 0.75% adenine leads to an increase in parathyroid gland area. Treatment with AMG-641 (3 mg/kg p.o. for 4 weeks), normalized parathyroid gland area.

Fig. 3. Adenine leads to loss of bony trabeculae at the distal femur, which is preserved in AMG 641-treated, but not calcitriol-treated rats. White arrows point to the trabecular bone (black) in the distal femoral metaphysis. Distal femurs from (A) normal control animals, (B) adenine no treatment, (C) adenine+calcitriol (10 ng/rat, s.c.) and (D) adenine+AMG 641 (3 mg/kg, p.o.).

Table 1

Biochemical markers.

Treatment	No adenine	Adenine	Adenine + AMG 641 vehicle	Adenine + AMG 641 (3 mg/kg)	Adenine + calcitriol vehicle	Adenine + calcitriol (10 ng/rat)
Creatinine mg/dL	0.3 ± 0.01	3.5 ± 0.2 ^a	3.5 ± 0.3 ^a	2.8 ± 0.1 ^a	3.6 ± 0.4 ^a	3.3 ± 0.4 ^a
BUN mg/dL	18 ± 1	160 ± 12 ^a	146 ± 9 ^a	125 ± 5 ^a	159 ± 10 ^a	139 ± 5 ^a
iPTH pg/ml	35 ± 3	565 ± 91 ^a	656 ± 76 ^a	13 ± 3 ^c	801 ± 70 ^a	396 ± 57 ^{a, b}
Ca mg/dL	10.6 ± 0.1	10.7 ± 0.1	10.8 ± 0.2	9.4 ± 0.3 ^{a, c}	10.8 ± 0.2	11.0 ± 0.2
P mg/dL	7.2 ± 0.2	23.0 ± 2.0 ^a	21.1 ± 0.9 ^a	21.8 ± 1.0 ^a	20.1 ± 1.7 ^a	18.7 ± 0.7 ^a

Measurements were taken 24 h after treatment administration on treatment day 28. Values are mean ± S.E.M. (*n* = 8–10).^a *P* < 0.05 significantly different from standard chow (no adenine, no treatment).^b *P* < 0.05 significantly different from adenine + calcitriol vehicle treated group.^c *P* < 0.05 significantly different from adenine + AMG 641 vehicle.

Table 2

Parathyroid PCNA-positive cells from adenine-induced uremic rats.

PCNA-positive cells (cells/mm²)						
Weeks	Treatments					
	No adenine	Adenine	Adenine + AMG 641 vehicle	Adenine + AMG 641	Adenine + calcitriol vehicle	Adenine + calcitriol
1	ND	111 ± 14 ^a	93 ± 9 ^a	72 ± 12	106 ± 18 ^a	97 ± 15 ^a
2	ND	116 ± 11 ^a	120 ± 18 ^a	50 ± 10 ^b	108 ± 8 ^a	107 ± 12 ^a
3	ND	144 ± 16 ^a	140 ± 16 ^a	52 ± 9 ^c	135 ± 16 ^a	141 ± 13 ^a
4	33 ± 6	167 ± 18 ^a	157 ± 22 ^a	40 ± 5 ^c	147 ± 19 ^a	142 ± 19 ^a

Parathyroid PCNA-positive cell numbers, values are mean ± S.E.M. (*n* = 16–20).^a*P* < 0.01 significantly different from standard chow (no adenine, no treatment values measured at week 4).^b*P* < 0.01.^c*P* < 0.001 significantly different from adenine + AMG 641 vehicle.

Table 3

Parathyroid gland weight/body weight in adenine-induced uremic rats.

Weeks	Treatments					
	No adenine	Adenine	Adenine + AMG 641 vehicle	Adenine + AMG 641	Adenine + calcitriol vehicle	Adenine + calcitriol
1	ND	1.087±0.044	0.959±0.062	0.907±0.086	1.083±0.092	0.979±0.068
2	ND	1.540 ± 0.096 ^a	1.534±0.144 ^a	1.154±0.085 ^b	1.683 ± 0.109 ^a	1.739±0.096 ^a
3	ND	1.805±0.109 ^a	1.908±0.107 ^a	1.275±0.077 ^b	1.939±0.091 ^a	1.731 ±0.151 ^a
4	1.096 ± 0.083	2.378±0.193 ^a	2.501 ±0.119 ^a	0.891 ± 0.087 ^b	2.665±0.181 ^a	2.408±0.113 ^a

Parathyroid weight/body weight values are mean ± S.E.M. (*n* = 16–20).

ND not determined for no adenine controls since parathyroid weight normalized to body weight does not change over time (unpublished observations).

^a *P*<0.01 significantly different from standard chow (no adenine, no treatment) values measured at week 4.

^b *P*<0.01 significantly different from adenine +AMG 641 vehicle.

Table 4

Static histomorphometry of cancellous bone in distal femur of rats treated with AMG 641 or calcitriol.

	Normal (no adenine)	Adenine + no treatment	Adenine + AMG 641 vehicle	Adenine + AMG 641	Adenine + calcitriol vehicle	Adenine + calcitriol
BV/TV (%)	21.91 ± 1.52	14.42 ± 2.06 ^b	13.85±1.69	23.43 ± 1.58 ^a	13.95±1.77	15.45±1.95
TbN (mm)	3.84 ± 0.27	2.58±0.31 ^b	2.38±0.22	3.87 ± 0.28 ^a	2.29±0.18	2.6±0.26
TbSp (pm)	214.3 ± 20.05	391.0±78.4 ^b	397.4±48.13	207.9 ± 18.1 ^a	412.4±52.87	339.2±26.10
OcS/BS (%)	9.63 ± 1.66	11.18 ± 1.44	10.56±2.02	6.34 ± 1.25	11.23±1.27	11.68±1.84
ObS/BS (%)	17.92±4.15	26.80±4.01	38.34±3.91	19.61 ±4.19 ^a	38.00±2.74	21.28±3.78 ^a
OS/BS (%)	8.98±3.12	32.71 ± 7.24 ^b	39.66±7.13	8.84±4.10 ^a	42.92±7.41	25.36±5.16 ^a

Bone histology measurements were from animals sacrificed after treatment administration on treatment day 28. Values are mean ± S.E.M. Comparisons were made for Adenine + No treatment vs Normal; Adenine + AMG641 vs its respective vehicle (Adenine + AMG641 vehicle); Adenine + calcitriol vs its respective vehicle (Adenine + Calcitriol Vehicle). ANOVA with Fisher's least protected LSD; alpha = 0.05.

^a $P < 0.05$ vs respective vehicles.

^b $P < 0.05$ vs normal (no adenine).