Pharmacology of AMG 416 (Velcalcetide), a Novel Peptide Agonist of the Calcium-Sensing Receptor, for the Treatment of Secondary Hyperparathyroidism in Hemodialysis Patients

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ABSTRACT

A novel peptide, AMG 416 (formerly KAI-4169, and with a United States Adopted Name: velcalcetide), has been identified that acts as an agonist of the calcium-sensing receptor (CaSR). This article summarizes the in vitro and in vivo characterization of AMG 416 activity and the potential clinical utility of this novel compound. AMG 416 activates the human CaSR in vitro, acting by a mechanism distinct from that of cinacalcet, the only approved calcimimetic, since it can activate the CaSR both in the presence or the absence of physiologic levels of extracellular calcium. Administration of AMG 416 in vivo into either normal or renally compromised rats results in dose-dependent reductions in parathyroid hormone (PTH) levels and corresponding decreases in serum calcium, regardless of the baseline level of PTH. Treatment of 5/6 nephrectomized rats with AMG 416 resulted in dramatic improvements in their metabolic profile, including lower PTH and serum creatinine levels, reduced amounts of vascular calcification, attenuated parathyroid hyperplasia, and greater expression of the parathyroid gland regulators CaSR, vitamin D receptor, and FGFR23 receptor compared with vehicle-treated animals. No drug accumulation was observed under this dosing regimen, and the terminal half-life of AMG 416 was estimated to be 2–4.5 hours. As a long-acting CaSR agonist, AMG 416 is an innovative new therapy for the treatment of hemodialysis patients with secondary hyperparathyroidism.

Introduction

Secondary hyperparathyroidism (SHPT) can develop in patients with chronic kidney disease (CKD) as a result of impaired calcium homeostasis when renal failure disturbs the complex interactions among parathyroid hormone (PTH), calcium, phosphorus, and vitamin D (Cunningham et al., 2011). Elevated PTH secretion and parathyroid gland hyperplasia, which are the hallmarks of SHPT, are driven by hypocalcemia and hyperphosphatemia, which develop in CKD as a result of low calcitriol levels and decreased kidney function (Cunningham et al., 2011). As SHPT progresses, there is a reduction in calcium-sensing receptor (CaSR) and vitamin D receptor (VDR) expression in the parathyroid glands, which further reduces gland responsiveness to normal calcium and calcitriol regulation of PTH secretion (Fukuda et al., 1993; Kifor et al., 1996) and may contribute to the overall pathogenesis of parathyroid hyperplasia (Arnold et al., 1995; Ritter et al., 2001; Canalejo et al., 2010). Traditional therapies, such as calcium-containing phosphate binders and vitamin D supplementation, can reduce PTH levels; however, their propensity to raise serum calcium and phosphorus levels can potentially increase the risk of vascular calcification (Cunningham et al., 2011). Vascular calcification is a common occurrence in this patient population, and it contributes to the increased risk of morbidity and mortality (Block et al., 2004a).

PTH production and secretion in response to serum calcium are tightly controlled by the CaSR. The CaSR is a G protein–coupled receptor expressed on the surface of the chief cells in the parathyroid glands, finely tuned to respond to subtle variations in serum calcium levels. The principal function of the CaSR in the parathyroid gland is to regulate PTH secretion to maintain blood and bone mineral homeostasis (Brown et al., 1993). Activation of the CaSR by normal physiologic calcium levels inhibits PTH production and secretion, whereas hypocalcemia leads to reduced activation...
of the CaSR and increased PTH secretion. One therapeutic approach to treat SHPT is the use of drugs that target the CaSR and thereby reduce PTH levels, such as cinacalcet (Nemeth et al., 2004).

Conceptually, drugs that act on the CaSR could inhibit PTH secretion by either lowering the threshold of receptor activation in response to extracellular calcium or by directly activating the receptor through binding to the extracellular domain (Nemeth et al., 2004). Cinacalcet (Sensipar; Amgen Inc., Thousand Oaks, CA) is an approved calcimimetic that has been shown to act as an allosteric modulator of the CaSR, potentiating the action of extracellular calcium by lowering the threshold for receptor activation, resulting in an inhibition of PTH secretion and production (Nemeth et al., 2004; Levi et al., 2006). Cinacalcet is approved as a once-daily oral treatment of SHPT in end-stage renal disease (ESRD) hemodialysis patients, is effective at decreasing serum intact parathyroid hormone, and is associated with corresponding reductions in serum calcium, phosphorus, and the calcium-phosphorus product in dialysis patients (Block et al., 2004b). Although patients receiving cinacalcet show significant improvements in PTH levels and bone and mineral metabolism, the clinical utility of cinacalcet can be limited by gastrointestinal (GI) intolerability (nausea, vomiting, and diarrhea), fixed-dose titration, cytochrome P450–mediated drug-drug interactions, and poor patient compliance (Padhi and Harris, 2009; Gincherman et al., 2010). Consequently, there is a need for an improved therapeutic agent for the treatment of SHPT.

In this article, we characterize the activity of AMG 416 (velcalcetide), a novel peptide agonist of the CaSR (Fig. 1), to demonstrate its potential clinical utility for patients with SHPT. We used a combination of in vitro and in vivo techniques, including the 5/6 nephrectomized (5/6 Nx) rat model. The 5/6 Nx model is a commonly used uremic animal model that displays biochemical and pathologic similarities to the human disorder of SHPT in ESRD patients (Drueke et al., 2007) and has been used to evaluate the pharmacology of various treatments, including cinacalcet (Nemeth et al., 2004).

### Materials and Methods

**Chemicals and Peptides.** AMG 416 was synthesized by solid-phase synthesis using the Fmoc strategy, purified in the acetate, trifluoroacetate, or hydrochloride salt form by high-performance liquid chromatography/mass spectroscopy (LC/MS). Peptides were synthesized at KAI Pharmaceuticals (South San Francisco, CA) or Bachem (Torrance, CA).

**HEK-293T/CaSR In Vitro Studies.** Human embryonic kidney cells (HEK-293T) (untransfected) or human CaSR (hCaSR) cells [a clone of HEK-293T stably transfected with the human CaSR (Multispan Inc., Hayward, CA)] were seeded into 384-well plates. Cell culture medium was aspirated from the wells and replaced with 20 μl of base stimulation buffer [10 mM Hepes (pH 7.4), 0.5 mM MgCl2, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl] containing peptides. Calcium was added to a final concentration of 1.2 mM CaCl2 as described. To determine whether AMG 416 activity was dependent on calcium, transfected HEK-293T cells were washed with Ca2+/Mg2+-free phosphate-buffered saline either with or without 2 mM EGTA. CaSR stimulation and inositol monophosphate (IP1) accumulation were induced at 37°C for 2 hours, and IP1 was quantified in cell lysates using the IP-One Tb Kit (#621PAPEC; Cisbio, Bedford, MA) according to the manufacturer’s instructions. Concentrations of IP1 in each sample were calculated from a standard curve using GraphPad Prism (Version 4.03; GraphPad Software, La Jolla, CA). EC50 values were reported from the IP1 data using curve-fitting software in GraphPad Prism. In cell-washing experiments, 384 well plates from each plating were washed as described, and cell numbers were quantified using CellTiter-Blue (Promega, Madison, WI) according to the manufacturer’s recommendation. Each experiment was performed in quadruplicate.

**Evaluation of AMG 416 in Normal Rats.** As part of a 4-week, repeat-dose i.v. toxicity study of AMG 416 in male and female SD rats (~250 g) at Pharmanor (Beijing, People’s Republic of China), biochemical endpoints of plasma PTH, serum calcium, and phosphorus were evaluated. Animals were dosed with vehicle or AMG 416 by i.v. bolus every day at doses of 0.3, 1, or 3 mg/kg. Free access to food and water was provided during the course of the study. Blood samples (0.5 ml) were taken for total serum calcium/phosphorus evaluations before dosing and at 8 hours after dose on day 1. K2EDTA blood samples (0.6 ml) were taken for PTH analysis before dosing and at 5 minutes, 2, 5, and 8 hours after dose on day 1. All blood samples were taken by retro-orbital plexus puncture while the animals were anesthetized with 70% CO2 plus 30% O2 inhalation. All animal protocols were approved by Pharmanor’s Institutional Animal Care and Use Committee (IACUC) in accordance with their IACUC policies and procedures.

**5/6 Nephrectomized and Double-Nephrectomized Rats.** We purchased 5/6 nephrectomized (5/6 Nx) male SD rats from Charles River Laboratories (Wilmington, MA); average body weight was ~320 g. Animals were allowed to recover for approximately 2 weeks after their second surgery before dosing. AMG 416 (0.3, 1, or 3 mg/kg) or vehicle (18 mg/ml glycine, 9 mg/ml trehalose, 2.4 mg/ml succinate, pH 5.0) was administered as an s.c. bolus injection (~0.67 ml/kg) three times weekly (Fig. 5). The s.c. injections were performed because of the length of the study and challenges associated with repeated i.v. dosing. Animals were allowed free access to water and chow. Blood samples (0.45 ml) were taken for PTH and calcium analysis before and after each dose. All animal protocols were approved by KAI Pharmaceuticals’ IACUC and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and regulated by the National Institutes of Health.

Double-Nx SD rats were generated at KAI Pharmaceuticals. Three days before surgery, animals were treated with antibiotics as previously described (Levine and Saltzman, 1997). General anesthesia was induced and maintained by i.p. injection of sodium...
pentobarbital (5.2%, 0.4 ml/rat). After making a 3–3.5 cm incision, the two kidneys were exposed and unencapsulated. The bilateral renal pedicles were clamped and ligated with 2-0 silk, and the kidneys were removed. The ligated pedicles were returned to a neutral anatomic position, and the abdomen and skin incisions were closed with 2-0 silk. After the surgical procedure, the diet was changed to a mixture of standard rat chow/sugar cubes in a 50:50 ratio (Teklad; Harlan Laboratories).

**Bioanalytical and Pharmacokinetic Analysis.** Normal, 5/6 Nx, and double-Nx rats were administered a single i.v. bolus of AMG 416 (1 mg/kg), and K₂EDTA blood samples were removed at 5 minutes, 1, 2 (normal and 5/6 Nx), 3, 6, 8, 11 (normal and double Nx), 12/56 Nx), and 24 hours and processed to plasma for measurement of AMG 416 concentration.

A protein precipitation method was used for extraction of AMG 416 from rat plasma followed by LC-tandem MS analysis. The lower limit of quantitation of this method was 1 ng/ml in K₂EDTA plasma samples. Samples were analyzed at KAI Pharmaceuticals.

Pharmacokinetic (PK) analyses for the rat studies were performed using the WinNonlin (Version 5.3; Pharsight Corp., Sunnyvale, CA) sparse data analysis function [linear trapezoidal rule for area under the curve (AUC) calculation]. Nominal dose values and sampling times were used for calculations.

**Serum/Plasma Analysis.** Plasma PTH levels were quantified according to the manufacturer’s protocol using rat (#1-84) bioactive intact PTH enzyme-linked immunosorbent assay kits from Immunotopics International (Rat: #60-2700; San Clemente, CA). Raw data were graphed using GraphPad Prism. Blood samples for creatinine, calcium, and phosphorus (0.2 ml) were taken and allowed to clot for approximately 30–60 minutes before centrifugation. Serum samples were analyzed for creatinine concentration using the Quantichrom kit (DICT-500; BioAssay Systems, Hayward, CA). The assay was performed according to the manufacturer’s protocol. Serum samples were analyzed for total calcium and phosphorus content at SRI (Menlo Park, CA) using the Roche Cobas C-501 autoanalyzer.

**Immunohistochemistry.** To evaluate the effect of AMG 416 on parathyroid cell proliferation, parathyroid gland receptor regulation, and kidney vascular calcification, the following methods were used.

Animals were dosed with bromodeoxyuridine (BrdU; 20 mg/ml solution in normal saline) at 50 mg/kg by i.p. injection at 2 and 24 hours before euthanization.

Rats were sacrificed and parathyroid glands removed for fixation in 4% paraformaldehyde for 24 hours. The parathyroid glands were removed from the fixation solution, blotted, dried, and weighed. The sums of weights from two glands per rat were recorded. After fixation, 3-μm-thick sections were prepared for immunohistochemistry staining. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol, and nonspecific binding sites were blocked with 10% normal serum (Dako Inc., Carpinteria, CA). Sections were incubated at 4°C overnight in primary antibody solution: mouse anti-BrdU monoclonal antibody, 125 μg/ml (Becton Dickinson Immunocytochemistry Systems, #347580; BD, Franklin Lakes, NJ).

A similar process was used for immunohistochemistry on sections from parathyroid glands to detect the CaSR, VDR, and FGFR1. After blocking, sections were incubated at 4°C overnight in the primary antibody solutions; mouse anti-CaSR (5C10, ADD) monoclonal antibody, 2 μg/ml (#NB120-19347; Novus Biologicals, Littleton, CO); rabbit anti-fibroblast growth factor receptor-1 (FGFR1) polyclonal antibody, 4 μg/ml (Abcam #71928; Abcam, Cambridge, MA); rabbit anti-rat vitamin D receptor, 1 μg/ml (Abcam #3508).

After staining with primary antibodies, tissue sections were incubated with biotinylated secondary antibodies (Dako, Inc.) for 30 minutes at room temperature. Color development was performed with DAB color development system (Dako Inc.).

When the rats were sacrificed, the remaining kidney remnant was removed and processed histologically. Sections (3 μm) were stained for calcification and the presence of collagen by von Kossa and Sirius Red methods, respectively (Sheehan and Hrapchak, 1980; Whittaker et al., 1994).

**Imaging and Quantification.** Images of whole sections (10× magnification) were captured using a microscope (DM 1000; Leica Microsystems, Wetzlar, Germany) coupled to a camera (Leica DFC 320).

Positive BrdU-stained parathyroid cells were counted by an observer blinded to treatment groups. The total number of positive parathyroid cells was expressed as number of cells per section.

Immunostained sections of the CaSR, FGFR1, and VitDR were analyzed using the optical density function of Image-Pro Plus software (Media Cybernetics, Rockville, MD). Threshold limits were set for color intensity, and the number of pixels meeting that threshold value was then quantified. The average optical density per section of gland tissue was calculated by dividing the sum integrated optical density by the total gland area. A single value per animal from the average of two glands (only one gland in some cases) was used.

**Data and Statistical Analysis.** Data were analyzed and graphed using GraphPad Prism. When appropriate, analysis of variance was used to determine statistical significance using Bonferroni post-test analysis.

**Results**

**AMG 416 Is an Agonist of the Calcium Sensing Receptor.** The potency of AMG 416 (structure shown in Fig. 1), was determined by constructing the dose-response relationships using IP₁ as an endpoint. Under appropriate cell culture conditions, IP₁, a downstream metabolite of the second messenger inositol 1,4,5-trisphosphate, accumulates after CaSR activation. Changes in IP₁ accumulation provide a quantitative and functional assessment of CaSR activity in these cells. Treatment of HEK-293T cells expressing the hCaSR with increasing concentrations of AMG 416 resulted in a dose-dependent increase in IP₁ accumulation with an apparent EC₅₀ of 25 μM. AMG 416 had no effect on untransfected HEK-293T cells, indicating that IP₁ accumulation in response to AMG 416 was dependent on the expression of the hCaSR (Fig. 2A). The calculated Hill coefficient was 1.1, suggesting that AMG 416 acts by a single binding site on the receptor. The EC₅₀ for calcium activation of hCaSR cells was measured as 5 mM (unpublished data), which is similar to other published results (Conigrave et al., 2000; Breitwieser and Gama, 2001).

The dependence of AMG 416 activity on extracellular calcium was explored by incubating hCaSR cells with increasing concentrations of AMG 416 in the presence or absence of 1.2 mM calcium. As shown in Fig. 2B, treatment with AMG 416 under calcium-free conditions resulted in activation of the hCaSR, albeit requiring approximately 30 times as much ligand to generate the magnitude of response observed in the presence of calcium. Furthermore, a single wash with phosphate-buffered saline resulted in a similar level of activation as the more stringent calcium removal with extra washing and calcium chelation.

**Pharmacokinetics and Tissue Distribution of AMG 416.** Given the proposed patient population for this compound, the pharmacokinetics of AMG 416 were assessed in animals with differing levels of renal function. Normal rats, 5/6 Nx rats, and double-Nx rats were given a single i.v. bolus of 1 mg/kg AMG 416; blood samples were taken and standard pharmacokinetic calculations were made (Table 1). Compared
with normal animals, in rats with no renal function there was a threefold increase in the AUC (3713 versus 11412 hour × ng/ml) and a 5-fold increase in terminal half-life (\( t_{1/2} \) of 1.32 hours and 6.68 hours) (Fig. 3A; Table 1).

To understand the tissue distribution profile of AMG 416, normal rats were administered a single i.v. bolus of AMG 416 (0.3 mg/kg) and sacrificed approximately 6 hours later, and tissues were processed for LC/MS. Liver and kidney showed the highest exposure to AMG 416, with little to no detectable amounts in the brain (Fig. 3B).

Effect of AMG 416 on Plasma PTH Levels in Normal Animals. To evaluate the activity of AMG 416 on PTH levels, male and female SD rats were dosed with AMG 416 at 0.3, 1, and 3 mg/kg by i.v. bolus administration. A single i.v. bolus of AMG 416 resulted in rapid and reversible reductions in plasma PTH, the duration of which was dose-dependent (Fig. 4A). Plasma PTH levels were suppressed to a similar extent 2 hours after administration at all three dose levels; however, by 5 hours, the mean plasma PTH levels in the 0.3 and 1.0 mg/kg animals had partially recovered (~50% of baseline levels). In contrast, mean plasma PTH levels remained markedly suppressed for at least 8 hours in animals dosed at 3 mg/kg.

As expected, when plasma PTH levels were reduced, corresponding reductions in serum calcium and increases in serum phosphorus were observed (Fig. 4, B and C).

Evaluation of AMG 416 in a Uremic Model of Kidney Dysfunction: Acute Treatment. Two weeks after 5/6 Nx surgery, a pharmacokinetic assessment of AMG 416 was performed in 5/6 Nx rats (see Table 2 for baseline biochemical characteristics) after a single s.c. dose (on day 1) and after 1 week of dosing s.c. three times weekly (on day 8; see Fig. 5A), a dosing regimen commonly used in chronic studies. Mean pharmacokinetic parameters for each dose group on day 1 and day 8 are presented in Table 3. Increasing doses of AMG 416 resulted in dose-proportional increases in exposure as reflected by AUCall and \( C_{\text{max}} \) values. Maximal plasma concentrations of AMG 416 were seen 2 hours after dosing by s.c. bolus (Fig. 6A; closed symbols). Mean clearance and half-life values were generally dose-independent, with values ranging between 213 and 294 ml/kg per hour and 2.0 and 4.6 hours for clearance and half-life, respectively. After 1 week of dosing, samples were taken again (on day 8) for AMG 416 plasma

![Fig. 2. AMG 416 activates the human CaSR in vitro in the presence and absence of added calcium. CaSR activation is reported as IP1 accumulation subsequent to inositol 1,4,5-trisphosphate induction. (A) Parental HEK-293T cells (□) or HEK-293T/hCaSR cells (■) were incubated with the indicated concentrations of AMG 416 in the presence of 1.2 mM CaCl2 (n = 6 for CaSR-expressing cells, n = 5 for untransfected cells). Data are mean ± S.E. of the mean. (B) HEK-293T/hCaSR cells were washed either once in phosphate-buffered saline (PBS; circles) or three times (diamonds) (once in PBS, once with EGTA, and a final wash with PBS) before incubation with AMG 416. As cell washing resulted in a loss of cell number from the plate, all data in this experiment were normalized to actual cell number rather than absolute IP1 concentrations. ○, no calcium plus AMG 416; ●, 1.2 mM CaCl2 plus AMG 416 (n = 2). Data are mean ± S.D.]

**TABLE 1**

<table>
<thead>
<tr>
<th>Model</th>
<th>Cmax (ng/ml)</th>
<th>AUCall (h-ng/ml)</th>
<th>Clive (ml/h/kg)</th>
<th>t1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1662 ± 187</td>
<td>3713 ± 346</td>
<td>271 ± 25</td>
<td>1.32 ± 0.19</td>
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<tr>
<td>5/6 Nx</td>
<td>1580 ± 170</td>
<td>5063 ± 781</td>
<td>201 ± 26*</td>
<td>2.00 ± 0.57</td>
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<tr>
<td>Double Nx</td>
<td>1428 ± 171</td>
<td>11,412 ± 1008**</td>
<td>81 ± 5b</td>
<td>6.68 ± 1.18**</td>
</tr>
</tbody>
</table>

AUCall, area under the curve from the time of dosing to the time of last observation; Clive, observed clearance from plasma.

* P < 0.01 vs. normal; **P < 0.001 vs. normal.
AMG 416 (Velcalcetide) Activates the Calcium Sensing Receptor

To characterize the acute PTH response in 5/6 Nx rats after dosing, animals were allowed a 1-week drug holiday after the PK assessment and were then dosed with a single s.c. bolus of AMG 416. After a single administration of 0.3, 1, or 3 mg/kg, AMG 416 was undetectable in the blood 48 hours after dosing, this prolonged suppression of PTH does not appear to be due to circulating levels of AMG 416 in the blood, but it may be due rather to a cumulative effect of AMG 416 treatment on the gland.

Serum calcium and phosphorus levels were also measured at the end of each 2-week period. Minimal changes were noted in either calcium or phosphorus levels over the 6-week study. Data from the 3 mg/kg group and vehicle groups are shown (Fig. 7, B and C).

Serum creatinine was assessed as a marker of renal function. As shown in Fig. 8A, treatment with AMG 416 had a significant effect on serum creatinine changes. At all the time points measured, animals treated with 3 mg/kg AMG 416 showed a larger reduction in creatinine levels from baseline compared with vehicle-treated animals. This effect was most pronounced at 6 weeks, when creatinine levels in control animals increased substantially from their baseline values. In contrast, at 6 weeks, AMG 416-treated animals showed a dose-dependent trend downward (P < 0.01 vehicle versus 3 mg/kg AMG 416).

After 6 weeks of dosing, the animals were sacrificed. At this time, the remaining kidney tissue was removed for histologic and morphologic evaluation. Figure 8B (upper panels) shows representative images of von Kossa-stained kidney tissue showing deposits of brown-black material, indicative of tubular calcification. AMG 416 treatment reduced the number of positively stained tubules relative to that seen in kidney tissue from vehicle-treated rats (Fig. 8C). The lower panels in Fig. 8B illustrate the increase in collagen deposition (red stain) in control versus AMG 416-treated animals.

Parathyroid glands were also removed for analysis at sacrifice. After fixation, the glands were weighed, and the combined weight of the two recovered glands was recorded. Parathyroid gland weights were normalized to body weight,
and mean data for all four treatment groups are shown in Fig.
9A, along with data from age-matched normal animals. There
was a trend toward dose-dependent reduction in parathyroid
gland weight with AMG 416, although it did not reach
statistical significance. Treatment with AMG 416 resulted in
a reduction in parathyroid cell proliferation as assessed by
BrdU staining (Fig. 9B). AMG 416-treated animals (3 mg/kg)
had a significantly lower number of BrdU-positive para-
thyroid gland cells compared with vehicle-treated rats (group
mean of 52 positive cells/gland in the treated group versus 127
positive cells in the vehicle group; \( P < 0.01 \) vehicle versus
3 mg/kg AMG 416). The two lower doses of AMG 416 also
appeared to have a dose-dependent trend on this proliferative
marker (14 and 42% reduction at 0.3 and 1 mg/kg, re-
spectively, versus controls), but this trend did not reach
statistical significance.

Consistent with the published literature, vehicle-treated
5/6 Nx rats displayed a decreased expression of the CaSR,
VDR, and FGFR1 compared with age-matched normal
animals (Fig. 10). Animals treated with 3 mg/kg AMG 416
showed a significantly higher level of expression of all three
receptors with levels close to those seen in normal control
animals and significantly higher compared with levels seen in
5/6 Nx vehicle-treated animals.

**Discussion**

Several lines of evidence in this study demonstrate that
AMG 416 is an agonist of the CaSR both in vitro and in vivo.
First, AMG 416 activates the CaSR in vitro; whereas
downstream signaling is evident in the absence of added
extracellular calcium, the signaling is increased substantially
in the presence of calcium, in contrast to cinacalcet, which acts
as an allosteric modulator of the CaSR and requires the
presence of extracellular calcium for activity (Nemeth et al.,

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.3 mg/kg (n = 5)</th>
<th>1 mg/kg (n = 5)</th>
<th>3 mg/kg (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH (pg/ml)</td>
<td>808 ± 199</td>
<td>793 ± 163</td>
<td>724 ± 73</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>0.87 ± 0.03</td>
<td>0.88 ± 0.08</td>
<td>0.88 ± 0.07</td>
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<tr>
<td>Body weight (g)</td>
<td>328 ± 11</td>
<td>340 ± 13</td>
<td>321 ± 5</td>
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</table>
AMG 416 (Velcalcetide) Activates the Calcium Sensing Receptor

Differences not significant for any equivalent dose between days 1 and 8.

<table>
<thead>
<tr>
<th>Day</th>
<th>1/2</th>
<th>AUC&lt;sub&gt;all&lt;/sub&gt;</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Cl&lt;sub&gt;obs&lt;/sub&gt;</th>
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<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>h</td>
<td>hg/ml</td>
<td>ml/h/kg</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.3</td>
<td>2.0 ± 0.1</td>
<td>1101 ± 158</td>
<td>265 ± 37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1101 ± 158</td>
<td>265 ± 37</td>
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<tr>
<td></td>
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<td>3</td>
<td>1101 ± 158</td>
<td>265 ± 37</td>
</tr>
<tr>
<td>Day 8</td>
<td>0.3</td>
<td>2.3 ± 0.3</td>
<td>1438 ± 253</td>
<td>314 ± 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1438 ± 253</td>
<td>314 ± 48</td>
</tr>
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<td></td>
<td></td>
<td>3</td>
<td>1438 ± 253</td>
<td>314 ± 48</td>
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</table>

AUC<sub>all</sub>, area under the curve from the time of dosing to the time of last observation; Cl<sub>obs</sub>, observed clearance from plasma.
CaSR (Kifor et al., 1996; Gogusev et al., 1997), FGFR1 (Canalejo et al., 2010), and VDR (Fukuda et al., 1993; Tokumoto et al., 2002) contribute to reduced responsiveness of the parathyroid gland to normal mineral and hormonal control, which helps explain the observation that parathyroid glands of SHPT patients frequently become progressively resistant to regulation by calcium and 1,25-dihydroxyvitamin D analogs (Drueke, 1995; Galitzer et al., 2009; Canalejo et al., 2010; Komaba et al., 2010). Thus, downregulation of these receptors seems to be an important contributing factor to the pathogenic progression of SHPT and further contributes to the mineral and endocrine derangements seen in these patients (Drueke et al., 2007; Komaba et al., 2010).

Consistent with previous reports, we observed that expression of the CaSR and VDR in untreated uremic rats decreased dramatically in the first several weeks after the onset of renal insufficiency. Activation of the CaSR by AMG 416 in 5/6 Nx rats resulted in higher expression levels of the CaSR, VDR, and FGFR1 compared with uremic control animals. The higher level of expression after 6 weeks of treatment with AMG 416 (which may be due to prevention of receptor downregulation since treatment was initiated soon after uremia) resulted in expression levels similar to levels observed in normal rats. The CaSR, VDR, and the FGF23 receptors have been implicated in either directly or indirectly controlling the production and secretion of PTH from the gland.

### Table 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (n = 12)</th>
<th>0.3 mg/kg (n = 12)</th>
<th>1 mg/kg (n = 12)</th>
<th>3 mg/kg (n = 12)</th>
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<tr>
<td>PTH (pg/ml)</td>
<td>1089 ± 75</td>
<td>1074 ± 101</td>
<td>1117 ± 131</td>
<td>1101 ± 88</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.09 ± 0.04</td>
<td>1.07 ± 0.03</td>
<td>1.06 ± 0.03</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>316 ± 3</td>
<td>317 ± 4</td>
<td>325 ± 5</td>
<td>324 ± 6</td>
</tr>
</tbody>
</table>
parathyroid gland (Ben-Dov et al., 2007; Druke et al., 2007; Krajisnik et al., 2007). FGF23 signals with highest efficacy through FGFR1 when bound by the transmembrane protein Klotho as a coreceptor (Ben-Dov et al., 2007). Paradoxically, FGF23 expression levels begin to rise early in CKD in stage 3 and 4 patients coincident with increases in serum phosphorus levels and become significantly elevated in ESRD patients, yet these elevated FGF23 levels are associated with increased PTH levels and decreased parathyroid gland responsiveness (Shigematsu et al., 2004; Gutierrez et al., 2005). The results presented here support prior reports that FGFR1 is downregulated in hyperplastic parathyroid glands of uremic rats (Komaba et al., 2010). Of note, we showed that activation of the CaSR by AMG 416 increases expression or prevents downregulation of FGFR1.

As noted already, three times weekly dosing of AMG 416 at 3 mg/kg did not generate detectable drug levels in the blood of uremic rats 48 hours after dosing; however, PTH levels 48 hours after dosing for several weeks were reduced by 51% below baseline. This prolonged suppression of PTH does not seem to be due to accumulation of circulating levels of AMG 416 in the blood but instead may be due to a cumulative effect of AMG 416, such as the result of increased expression of three key receptors in the parathyroid gland. The intriguing implication is that AMG 416, with its potent and prolonged activity, might have the ability to modify SHPT disease or disease progression.

In addition to the changes in the parathyroid gland, advanced CKD-MBD is associated with osteodystrophy and increased vascular and soft tissue calcification, which in turn lead to a substantial increase in cardiovascular morbidity and mortality (Block et al., 2004a; London et al., 2005; Demer and Tintut, 2008). Expression of the CaSR has been shown to decrease at sites of calcification both in vivo and in vitro, and calcium is a potent modulator of the vascular smooth muscle cell CaSR (Alam et al., 2009). Furthermore, a decreased mineral deposition in vascular smooth muscle cells in the presence of a calcimimetic has been demonstrated, suggesting that improvement in CaSR signaling may help prevent mineral deposition (calcification). Preclinical studies have

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Fig. 7. Chronic treatment with AMG 416 reduces PTH with minimal change to calcium and phosphorus in 5/6 Nx rats. AMG 416 was administered as a s.c. bolus three times weekly at doses of 0.3, 1, and 3 mg/kg, and samples were removed every 2 weeks, 48 hours after dosing, for PTH (A), calcium (B), and phosphorus (P) (C) analysis. Shown are group mean ± S.E.M. (*n* = 10–12). *P < 0.05 (vs. vehicle); **P < 0.001 (vs. vehicle, vs. 0.3 mg/kg); ***P < 0.01 (vs. vehicle).
previously demonstrated that calcimimetics (such cinacalcet or homologs thereof) can delay or slow calcification in uremic rats (Lopez et al., 2006; Moe et al., 2009). In line with these observations, treatment of uremic rats with AMG 416 decreased renal mineralization compared with vehicle-treated uremic rats. An important finding was that uremic rats treated with the highest dose of AMG 416 had significantly reduced serum creatinine levels, which coincided with significant reductions in kidney tubular calcification.

Fig. 8. Chronic treatment with AMG 416 reduces serum creatinine and vascular calcification in 5/6 Nx rats. Serum samples were removed every 2 weeks, 48 hours after dosing for serum creatinine measurements (A). Shown are group mean ± S.E.M. (n = 10–12). At sacrifice, remaining kidney tissue was stained for calcium (Von Kossa) (B and C) and collagen deposition (B) 10× magnification. *P < 0.05 vs. 0.3 mg/kg; #P < 0.01 vs. vehicle; ^P < 0.05 vs. vehicle.

Fig. 9. Chronic treatment with AMG 416 reduces parathyroid gland hyperplasia as measured by gland weight (A) and cell proliferation (B) in 5/6 Nx rats. At sacrifice, the rats’ parathyroid glands were removed, weighed in pairs, and then subjected to immunohistochemistry to assess cell proliferation through BrdU incorporation. Shown are group mean ± S.E.M. (n = 10–12). *P < 0.01 vs. vehicle.
One of the most common side effects seen with cinacalcet is elevated GI intolerability (most notably, increased incidence of nausea, vomiting, diarrhea), which can lead to patient noncompliance and discontinuation of treatment (Gincherman et al., 2010). Two possible explanations for the poor GI tolerability with cinacalcet have been suggested: high local exposure in the gut (particularly the GI lumen) and the potential of activating the CaSR located in the circumventricular regions of the brain (Mudo et al., 2009). As a small peptide agonist of the CaSR that is administered i.v., AMG 416 is expected to result in fewer GI adverse events since the i.v. route of administration should not result in significant exposure to the GI lumen. Moreover, the distribution studies presented here demonstrate that AMG 416 lacks significant exposure across the blood-brain barrier, which mitigates risk of CNS-mediated GI tolerability effects. The i.v. route of administration is potentially beneficial for the ESRD patient in terms of ease of use and compliance. For patients on three times weekly hemodialysis, i.v. treatment with AMG 416 at the end of each dialysis session offers a convenient method of controlling SHPT without adding to the pill burden.

For the reasons outlined herein, we anticipate that AMG 416 will have improved effectiveness (more prolonged and consistent PTH reduction), better tolerability, and better safety compared with cinacalcet, which should translate into improved treatment and outcomes for hemodialysis patients with SHPT.

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