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Cinacalcet attenuates hypercalcemia observed in mice bearing either Rice H-500 Leydig cell or C26-DCT colon tumors

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ABSTRACT

Excessive secretion of parathyroid hormone-related protein (PTHrP) by tumors stimulates bone resorption and increases renal tubular reabsorption of calcium, resulting in hypercalcemia of malignancy. We investigated the ability of cinacalcet, an allosteric modulator of the calcium-sensing receptor, to attenuate hypercalcemia by assessing its effects on blood ionized calcium, serum PTHrP, and calcium-sensing receptor mRNA in mice bearing either Rice H-500 Leydig cell or C26-DCT colon tumors. Cinacalcet effectively decreased hypercalcemia in a dose- and enantiomer-dependent manner; furthermore, cinacalcet normalized phosphorus levels, but did not affect serum PTHrP. Ribonuclease protection assay results demonstrated presence of PTHrP receptor, but not calcium-sensing receptor mRNA in C26-DCT tumors. The mechanism by which cinacalcet lowered serum calcium was investigated in parathyroidectomized rats (i.e., without PTH) made hypercalcemic by PTHrP. Cinacalcet attenuated PTHrP-mediated elevations in blood ionized calcium, which were accompanied by increased plasma calcitonin. Taken together these results suggest that the cinacalcet-mediated decrease in serum calcium is not the result of a direct effect on tumor cells, but rather is the result of increased calcitonin release. In summary, cinacalcet effectively reduced tumor-mediated hypercalcemia and corrected hypophosphatemia in mice. Further investigation of cinacalcet for treatment of hypercalcemia of malignancy is warranted.

1. Introduction

Hypercalcemia and, frequently, hypophosphatemia are observed in patients with malignant tumors (Stewart, 2002, 2005). The condition occurs most frequently in patients with carcinomas of the lung, breast, head, neck, esophagus, ovary and kidney and is associated with poor prognosis (Grill and Martin, 2000; Strewler and Nissenson, 1990). A variety of symptoms accompany the disease that involves the gastrointestinal, neurological, psychiatric and renal systems (Grill and Martin, 2000). Most of these symptoms are mediated by elevated levels of parathyroid hormone-related protein (PTHrP) and, if left untreated, is associated with hypercalcemia. Typical treatment first includes rehydration and loop diuretics, bisphosphonates for longer term management and or calcitonin for short-term control of severe hypercalcemia (Camozzi et al., 2012); however, patients usually become refractory to such treatments. Refractoriness to bisphosphonates and calcitonin has also been observed in preclinical rodent models of hypercalcemia of malignancy (Onuma et al., 2005a).

While PTH is known to activate reabsorption of calcium and magnesium in the kidney (Brown and Hebert, 1997) PTHrP is the primary factor responsible for the biochemical abnormalities associated with hypercalcemia of malignancy (Stewart, 2005). Similar to PTH, PTHrP interacts with the type 1 PTH receptor and under normal conditions, acts locally in a paracrine or autocrine manner to regulate bone and cartilage formation (Karaplis, 2001; Mundy and Edwards, 2008). When secreted by tumors, PTHrP may also function as a circulating factor, exerting PTH-like activity (Karaplis, 2001).

The contribution of tumor-derived PTHrP to hypercalcemia has been supported in clinical studies utilizing quantitative bone histomorphometry that have demonstrated increased osteoclast activity and decreased osteoblast activity in patients with humoral hypercalcemia of malignancy (Martin, 2005; Miao et al., 2005; Mundy and Edwards, 2008; Stewart et al., 1982). Further, PTHrP increases renal tubular reabsorption of calcium in humans (Horwitz et al., 2003). Each of these biological processes contributes to elevated serum calcium concentrations observed in these patients (Onuma et al., 2005b). Nude rats with LC-6-JCK human

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lung cancer tumors demonstrated increased secretion of PTHrP into the blood, cancer-associated wasting syndrome, and humoral hypercalcemia of malignancy (Onuma et al., 2005b). In these studies an anti-PTHrP antibody effectively controlled serum calcium and phosphorous and improved locomotor activity and body weights. In contrast, the calcium lowering effects of the bisphosphonate, alendronate, were transient with no effect on locomotor activity or serum phosphorus (Onuma et al., 2005b).

A key regulator of calcium homeostasis is the extracellular calcium-sensing receptor, a G protein-coupled cell surface receptor that is expressed in a diverse array of mammalian tissues (Brown et al., 1993; Brown and MacLeod, 2001). The primary physiologic ligand of the calcium-sensing receptor is extracellular ionized calcium (Hammerland et al., 1999). In response to extracellular calcium, calcium-sensing receptor-mediated signaling inhibits the secretion and synthesis of PTH from the parathyroid gland (Nemeth et al., 1998, 2004) and stimulates secretion of calcitonin from the thyroid gland (Fudge and Kovacs, 2004).

Cinacalcet (Sensipar®, Mimpara®) is US Food and Drug Administration or FDA and European Medicines Agency or EMEA approved for the treatment of hypercalcemia in patients with parathyroid carcinoma and for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease who are on dialysis. Cinacalcet has proven effective in the treatment of hypercalcemia in patients with inoperable parathyroid carcinoma (Silverberg et al., 2007; Wei and Harari, 2012) and has been FDA approved and granted orphan status for treatment of severe hypercalcemia in patients with primary HPT who are unable to undergo parathyroidectomy. Cinacalcet is a type II calcimimetic that allosterically modulates the activity of the calcium-sensing receptor, increasing its sensitivity to extracellular calcium and thereby inhibiting PTH secretion from parathyroid cells (Nemeth et al., 2004). As such, cinacalcet effectively lowers serum PTH while also reducing serum calcium levels in patients with secondary hyperparathyroidism (Block et al., 2004) or primary hyperparathyroidism (Shoback et al., 2003).

The fundamental role of the calcium-sensing receptor in calcium homeostasis, together with the demonstrated safety and efficacy of cinacalcet in parathyroid carcinoma, suggest that modulation of the calcium-sensing receptor by cinacalcet may have the potential to reduce serum calcium concentrations in patients with hypercalcemia of malignancy. To test this hypothesis we evaluated the effects of cinacalcet in murine models of tumor-induced hypercalcemia. Calcimimetic effects independent of PTH lowering were studied using parathyroidectomized rats supplemented with PTHrP (to induce hypercalcemia) to determine if reduction in ionized calcium by cinacalcet is due, at least in part, to calcitonin secretion and/or drug effects on PTHrP.

2. Materials and methods

Experiments were performed under protocols approved by Amgen's Internal Animal Care and Use Committee. Female, 4–6 week-old CB17/SCID mice (*Mus musculus*) and male, 12–14 week-old CDF-1 mice (*Mus musculus*) were purchased from Charles River Laboratories (Wilmington, MA) and housed in groups of 10. Male, Sprague-Dawley rats (300 g) were purchased from Harlan Laboratories (Indianapolis, IN) and housed in pairs. All animals were cared for in accordance to the *Guide for the Care and Use of Laboratory Animals, 8th Edition* (NRC 2011) and were housed at an AAALAC, Internationally accredited facility in non-sterile ventilated microisolator housing on wood chip (aspen) bedding. All research protocols were approved by the Amgen, Inc., Thousand Oaks, CA Internal Animal Care and Use Committee. Animals had ad libitum access to pelleted feed (Harlan Teklad 22/5 Rodent Diet, Indianapolis, IN) and reverse osmosis-purified water via water bottle. Animals were maintained on a 12:12 h light: dark cycle in rooms at 72 ± 2°F, humidity range (30% and 70%) and had access to enrichment opportunities. All animals were determined specific pathogen free for specific mouse, rat viral and bacterial agents, enteric protozoa, arthropod ectoparasites and helminth endoparasites.

2.1. Tumor models

Rice H-500 Leydig tumor cells (National Cancer Institute, Frederick, MD) were grown in RPMI containing 10% FBS and harvested from subconfluent cultures washed in medium. The cells were resuspended in PBS at a density of 2.5×10^7 cells/ml. Tumor cells (5×10^6 cells/0.2 ml) were implanted subcutaneously into the proximal dorsal midline of 4–6 week old female CB17/SCID mice under aseptic conditions. In single dose studies, cinacalcet (3 mg/kg or 30 mg/kg) or vehicle was administered as a single oral dose on day 10 post inoculation. In multiple dose studies, cinacalcet (30 mg/kg), vehicle, or the less active enantiomer (Nemeth et al., 2004) of cinacalcet, S-AMG 073 (30 mg/kg), was administered orally once daily starting at day 1 post inoculation and continuing until sacrifice on day 10.

C26-DCT tumor cells (National Cancer Institute) were grown in Dulbecco's Modified Eagle's Medium (high glucose) containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 mM nonessential amino acids. Cells were harvested from subconfluent cultures in medium and resuspended in PBS at a density of 2.5×10^7 cells/ml. C26-DCT tumor cells were implanted subcutaneously into the flank of 12–14 week old male CDF-1 mice under aseptic conditions. Cinacalcet, vehicle, or S-AMG 073 (less potent enantiomer of AMG 073) was administered as a single oral dose on day 17 after inoculation.

2.2. Biochemical assays

On day 10 (CB17/SCID mice) or day 17 (CDF-1 mice), animals were euthanized 4 h after drug or vehicle treatment, and blood samples were taken from the dorsal aorta. Blood ionized calcium, total calcium, and phosphorus levels were determined using a Bayer Ciba-Corning 634 calcium analyzer or Roche Hitachi 717 blood chemistry analyzer. Plasma PTHrP levels were determined according to the vendor's instructions using a PTHrP 65T kit (catalog number 34478X; Nichols Institute, Madison, NJ).

2.3. Calcium-sensing receptor and parathyroid hormone-related protein ribonuclease protection assay

Expression of calcium-sensing receptor and PTHrP mRNA in tumor tissues was assessed using ribonuclease protection assays. C26-DCT tumor cells were implanted subcutaneously into the flank of 12–14 week old male CDF-1 mice under aseptic conditions (see above). Male CDF-1 mice received cinacalcet (30 mg/kg orally [p.o.]) or vehicle (0.2 ml p.o.) daily for 17 days starting from the day of inoculation. Animals were sacrificed by CO_2 inhalation on day 17, and tumors were excised and snap-frozen in liquid nitrogen. Total RNA was extracted from frozen tissues using Stat-60 reagent (Tel-Test "B," Friendswood, TX) following the manufacturer's instructions.

Probe templates for the ribonuclease protection assay were prepared by RT-PCR amplification of fragments corresponding to nucleotides 1938–2986 from the published mouse calcium-sensing receptor sequence, Gb:AF110178, and nucleotides 152–428 from the published mouse PTHrP sequence, Gb:M60056. Amplicons were cloned into pGEM-T (Promega) and verified by sequencing. Radiolabeled antisense RNA probes were transcribed using Sp6 RNA polymerase (Promega) and [α^{32}P]rUTP (> 3000 Ci/mol; Amersham,