Discovery of a Calcimimetic with Differential Effects on Parathyroid Hormone and Calcitonin Secretion

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ABSTRACT
Calcimimetics are positive allosteric modulators to the calcium-sensing receptor (CaSR). Activation of the CaSR inhibits the secretion of parathyroid hormone (PTH), stimulates the secretion of calcitonin, and decreases serum calcium (Ca2+). Cinacalcet, a second-generation calcimimetic, is used therapeutically to control PTH in patients with chronic kidney disease who are on dialysis with secondary hyperparathyroidism. A calcimimetic that displays increased separation of PTH versus Ca2+ lowering in patients would potentially allow the use of calcimetics to treat patients in earlier stages of renal disease because hypocalcemia can develop in this population. Toward this end, we developed a third-generation calcimimetic, determined the molecular pharmacological properties of it using an operation model of allosteric modulation/agonism, and measured the compound effects on PTH, serum ionized Ca2+, and calcitonin levels in 5/6 nephrectomized rats. We found the new molecule effectively reduced PTH levels without promoting calcitonin secretion or hypocalcemia. Furthermore, our third-generation molecule was less efficacious at promoting calcitonin secretion from human thyroid carcinoma cells compared with 3-[(2-chlorophenyl)-N-((1R)-1-(3-methoxyphenyl)ethyl]-1-propanamine (R-568), a first-generation calcimimetic. These data provide evidence that calcimimetics with increased potency can be used to lower PTH without production of significant hypocalcemia because the threshold for inhibition of PTH secretion is much lower than the threshold for calcitonin secretion.

Introduction
The calcium-sensing receptor (CaSR) is a class C G protein-coupled receptor originally identified from bovine parathyroid cells and isolated by an expression cloning strategy in Xenopus laevis oocytes (Brown et al., 1993). The highest level of expression of the CaSR is found in parathyroid tissue where it plays a crucial role in calcium and phosphate homeostasis. The receptor is also expressed in other tissues including the thyroid, kidney, brain, stomach, and gastrointestinal tract (Brown, 2007). CaSR activation by millimolar concentrations of calcium in the parathyroid gland inhibits parathyroid hormone (PTH) secretion, thereby reducing bone resorption (Brown, 1991). In addition, CaSR activation in C cells of the thyroid stimulates secretion of calcitonin, an inhibitor of bone resorption (Fox et al., 1999a). Stimulation of the receptor by millimolar concentrations of Ca2+ can activate both Gq and Gi signaling pathways, leading to inositol phosphate (IP) production, intracellular Ca2+ flux, and ERK phosphorylation (Chen et al., 1989; Hawkins et al., 1989; Kifor et al., 1997). In the kidney, the CaSR and the PTH receptor regulate calcium reabsorption (Chang and Shoback, 2004). Activation of the receptor in the stomach is associated with increased acid secretion, whereas activation of the CaSR in the gastrointestinal tract has been shown to inhibit fluid secretion from colonic crypt cells (Dufner et al., 2004; Geibel et al., 2006).

ABBREVIATIONS: CaSR, calcium-sensing receptor; hCaSR, human CaSR; CKD, chronic kidney disease; ESRD, end-stage renal disease; FLIPR, fluorescent imaging plate reader; IP, inositol phosphate; PTH, parathyroid hormone; Nx, nephrectomized; SHPT, secondary hyperparathyroidism; R-568, 3-[(2-chlorophenyl)-N-((1R)-1-(3-methoxyphenyl)ethyl]-1-propanamine; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase.

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Disorders of Ca$^{2+}$ and phosphate homeostasis develop in end-stage renal disease (ESRD) because of reduced serum calcitriol and elevated serum PTH levels. Calcimimetics are positive allosteric modulators that bind in the transmembrane domain on the CaSR. They increase the potency and/or affinity of Ca$^{2+}$ to the receptor (Hammerland et al., 1998; Petrel et al., 2004). Modulation of the CaSR with calcimimetics effectively reduced serum PTH in rodent models of ESRD (Fox et al., 1999b). Cinacalcet is a second-generation calcimimetic indicated for the treatment of secondary hyperparathyroidism (SHPT) in patients with chronic kidney disease (CKD) on dialysis (Goodman et al., 2002; Block et al., 2004). In addition, cinacalcet is indicated for the treatment of hypercalcemia in patients with parathyroid carcinoma and in some countries for the treatment of primary hyperparathyroidism. Furthermore, three reports demonstrate the effectiveness of cinacalcet in treating familial hypocalciuric hypercalcemia (Timmers et al., 2006; Festen-Spanjer et al., 2008; Alon and Vandervoorde, 2010).

Treatment of SHPT in CKD stages 3 and 4 with calcimimetics has not been approved by the Food and Drug Administration and requires further evaluation. Patients with stages 3 and 4 CKD also have elevated PTH but at lower levels than patients with ESRD. In a randomized, double-blind, placebo-controlled study to assess efficacy and safety of cinacalcet in nondialysis patients, cinacalcet effectively decreased plasma intact PTH. However, hypocalcemia (generally asymptomatic) was observed (Chonchol et al., 2009). Hence, it is desirable to develop a molecule that is able to suppress PTH secretion without causing hypocalcemia for use in this group of patients. Toward this end, we developed N-R-((1R)-1-(6-methoxy-4′-(trifluoromethyl)-3-biphenyl)-N'-((1R)-1-phenylethyl)ethanamine (calcimimetic B) that we compared with 3-(2-chlorophenyl)-N-(trifluoromethyl)-3-biphenyl(ethyl)-1-propanamine (R-568), a first-generation calcimimetic (Harrington et al., 2010). We determined the molecular pharmacological parameters of each calcimimetic and measured the compound effects on PTH, serum-ionized calcium (Ca$^{2+}$), and calcitonin levels in 5/6 nephrectomized (Nx) rats. Measurements were conducted up to 4 h postdose to observe the differential effects on Ca$^{2+}$ lowering of these two calcimetics. We found that calcimimetic B was more potent and had less Ca$^{2+}$-lowering effect relative to PTH lowering in 5/6 Nx rats compared with R-568.

**Materials and Methods**

**Chemicals, Plasmids, and Reagents.** R-568 and calcimimetic B were prepared at Amgen, Inc. (Thousand Oaks, CA). HEK293, Chinese hamster ovary, and TT cells were obtained from the American Type Culture Collection (Manassas, VA). HEK293 cells stably expressing the human CaSR were obtained from NPS Pharmaceuticals (Bedminster, NJ). Yttrium silicate SPA beads and tritiated inositol pressuring the human CaSR were obtained from NPS Pharmaceuticals (Manassas, VA). HEK293 cells stably expressing the human CaSR (hCaSR) were dispersed in a 96-well, tissue culture-treated plate (Corning Life Sciences, Lowell, MA). One hundred microliters of cells was then added to the plate on a MicroLumat luminometer (Berthold Technologies USA, Oak Ridge, TN), and the luminescence was recorded. The lowest concentration of calcium tested was 0.25 mM.

**Fluorescent Imaging Plate Reader Assay.** Ca$^{2+}$ flux was measured using a fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Twenty thousand HEK cells stably expressing the hCaSR were placed in a 384-well, poly-D-lysine-coated plate with a clear bottom and black sides in 50 μl of cell media (DMEM/10% fetal bovine serum). The next day, the media were removed and the cells were incubated in 50 μl of dye buffer (BD Biosciences Discovery Labware, Bedford, MA) and allowed to incubate for 1.5 h at room temperature. Compounds were dissolved in 100% DMSO at 100× concentration. The compounds were then diluted 100-fold in HEPES-buffered saline containing 1.0 mM CaCl$_2$. Twenty-five microliters of compound was then added to the cells, and Ca$^{2+}$ flux was monitored for 90 s on FLIPR. Maximum observed fluorescence values were used in the calculation of LogEC$_{50}$.

**Inositol Phosphate Accumulation Assay.** HEK293 cells were dispensed into a poly-L-lysine tissue culture-treated, 96-well plate at a density of 25,000 cells per well. The next day, the cells (80–90% confluent) were transfected with 100 ng of hCaSR plasmid per well using Lipofectamine 2000 according to the manufacturer’s instructions. Six hours after transfection the medium was replaced with DMEM/10% dialyzed fetal calf serum supplemented with 10% dialyzed inositol. After incubation overnight, the cells were washed once in calcium-free HBSS and then treated with the 100 μM of calcium-free HBSS/0.1% BSA containing various concentrations of CaCl$_2$, R-568 (prepared as above in DMSO), and 10 mM LiCl and incubated at 37°C for 1 h. The media were aspirated and the cells were lysed with ice-cold 20 mM formic acid. ERK phosphorylation was induced by 5 min incubation with serum-free medium followed by 5 min incubation with serum-free medium containing 100 ng/mL EGF. After incubation, the cells were washed once in calcium-free HBSS and then treated with the 100 μM of calcium-free HBSS/0.1% BSA containing various concentrations of CaCl$_2$, R-568 (prepared as above in DMSO), and 10 mM LiCl and incubated at 37°C for 1 h. The media were aspirated and the cells were lysed with ice-cold 20 mM formic acid. After incubation at 4°C for 5 h, the lysate was added to yttrium silicate SPA beads, allowed to settle overnight, and read on a Beckman TopCount scintillation counter (Beckman Coulter, Fullerton, CA).

**Aequorin Assay.** Six million Chinese hamster ovary cells were seeded in a 150-cm$^2$ dish in DMEM/F12/10% fetal calf serum without antibiotics. One hour after plating, the cells were transfected with 10 μg of hCaSR plasmid and 10 μg of aequorin plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cells were incubated at 37°C with 5% CO$_2$ for 6 h, and the medium was replaced. One day after transfection, the cells were detached by brief treatment with trypsin/EDTA, washed, and resuspended in 10 ml of HBSS containing 0.5 mM CaCl$_2$ and 0.1% BSA. Coelenterazine F was added to a final concentration of 1 μM/μl. The cells were gently agitated every 15 min for a total of 2 h. The cells were then diluted 3-fold in the same buffer lacking coelenterazine F. R-568 was prepared as a 100× stock in DMSO. Just before the assay, the R-568 was diluted in 100 μl of calcium-free HBSS with 0.1% BSA and placed in a 96-well white plate (Corning Life Sciences, Lowell, MA). One hundred microliters of cells was then added to the plate on a MicroLumat luminometer (Berthold Technologies USA, Oak Ridge, TN), and the luminescence was recorded.

**ERK Phosphorylation Assay.** HEK293 cells stably expressing the hCaSR were dispersed in a 96-well, tissue culture-treated plate at a density of 45,000 cells per well for 36 h. The cells were then starved overnight in serum-free medium and then washed with phosphate-free buffer without Ca$^{2+}$. ERK phosphorylation was induced by compound addition for 5 min, and the media were aspirated. The cells were lysed by quick-freezing in liquid nitrogen. Fifty microliters of Laemmli sample buffer (Bio-Rud Laboratories, Hercules, CA) was added to each well and denatured by heating to 100°C for 5 min, and then 10 μl of the cell suspension was loaded on a 4 to 12% gradient SDS-polyacrylamide gel electrophoresis gel. The gel was transferred to a polyvinylidene difluoride membrane and probed with primary phospho-ERK antibody (Cell Signaling Technology, Danvers, MA) at 1:10,000 dilution at 4°C overnight. The membrane was then probed with a goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, Waltham, MA) at a dilution of 1:5000 for 1 h at room temperature. The membrane signal was detected using SuperSignal Substrate (Thermo Fisher Scientific).
**Experimental Subjects.** Subtotal Nx (5/6) male Sprague-Dawley rats were used to evaluate calcimimetic effects on mediators of serum Ca\(^{2+}\), including PTH and calcitonin secretion. All studies and procedures were performed in accordance with policies and procedures approved by the Internal Animal Care and Use Committee of Amgen Inc.

**Surgical Procedures for 5/6 Nx Rats.** A two-step procedure was performed starting with ligation of renal artery (left kidney; uppermost bifurcation) in anesthetized rats [intramuscular cocktail: two parts ketamine (100 mg/ml), one part xylazine (100 mg/ml), 0.3 parts acepromazine (10 mg/ml)]. After a 1-week recovery period after ligation of the left kidney, the right kidney was removed in anesthetized animals. This procedure reduces the original functional renal mass by five-sixths and induces CKD accompanied by SHPT. After the administration of the left kidney, the right kidney was removed in anesthetized rats [intramuscular cocktail: two parts ketamine (100 mg/ml), one part xylazine (100 mg/ml)]. After a 1-week recovery period after ligation of the left kidney, the right kidney was removed in anesthetized animals. This procedure reduces the original functional renal mass by five-sixths and induces CKD accompanied by SHPT. After the animal's recovery for 6 weeks before the initiation of studies.

**Drug Treatment.** Six weeks after 5/6 Nx rats (n = 7–8/group) were fasted overnight (14–16 h) and then treated with single oral doses of the research calcimetics, R-568 (1, 10, 30, or 100 mg/kg) or calcimimetic B (0.03, 0.1, or 0.3 mg/kg). Vehicle-treated control rats (n = 8) received 2% hydroxypropyl methylcellulose, 1% pluronic F68, and 10% Captisol in water in equimolar amounts (5 ml/kg) as the calcimimetic-treated groups.

**Biomarker Analysis.** Blood was collected from the retro-orbital sinus from anesthetized rats (2% isofoam in O\(_2\)). Whole blood was centrifuged at 1,500 g for 10 min, and the serum was stored frozen at −70°C until analysis. At the end of the 6-week treatment period, serum PTH and calcitonin were determined by an ELISA rat PTH[1-34] and rat calcitonin assay kits (Immutopics, San Clemente, CA). Tissues were promptly removed from the animal at sacrifice and placed in 70% alcohol. Tissues were washed in phosphate-buffered saline, pH 7.4, at room temperature and stored at −20°C until analysis. Tissues were then dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin.

**In Situ Hybridization.** A standard in situ hybridization protocol was performed to examine the expression of the rat CaSR in normal rat parathyroid and thyroid tissue derived from archived blocks of immersion-fixed, paraffin-embedded material (Wilcox, 1993). In brief, a 282-base pair 33P-labeled RNA antisense probe corresponded to nucleotides 2154 to 2435 of the rat CaSR gene (GenBank accession number U10354) was used as a probe. Hybridization was conducted at 60°C in a hybridization solution containing 1 × 10\(^{6}\) cpm of 32P-labeled antisense riboprobe, followed by RNase digestion, and a series of high-stringency washes at 55°C for 30 min. The slides were coated with Kodak NTB emulsion (Eastman Kodak, Rochester, NY), exposed for 3 weeks in the dark at 4°C, developed, and then counterstained with hematoxylin and eosin.

**Calcitonin Secretion Assay.** TT cells were seeded in 96-well plates at a density of 30,000 cells per well and grown for 48 h until they reached 80% confluence, washed in phosphate-free buffer without Ca\(^{2+}\) and serum, and Ca\(^{2+}\)-starved for 2 h. Cells were subsequently treated with 1 mM CaCl\(_2\) or 1 mM CaCl\(_2\) plus different concentrations of calcimimetic (stock prepared at 100× in DMSO) for 12 h. The medium from treated or untreated cells was centrifuged at 1,500 g for 5 min, and the concentration of calcitonin in the culture medium was determined using a commercial ELISA method per the manufacturer's instructions (Immutopics) and normalized to cell protein content. Control medium, which was not exposed to the cells, had no detectable levels of calcitonin. Absorbance at 450 nm was measured using an Envision instrument (PerkinElmer Life and Analytical Sciences, Waltham, MA) and normalized to cell protein content. Values were plotted against a calcitonin standard curve, and each treatment dose was conducted in triplicate.

**Data Analysis.** Individual dose-response curves were fitted to the variable slope Hill equation by nonlinear regression analysis using Prism 4.01 (GraphPad Software Inc., San Diego, CA). Fitting of data from the inositol phosphate accumulation assay in Fig. 4 was performed using the following form of an operational model of allosterism/agonism (see Fig. 3; also see Lu et al., 2009):

\[
E = E_m \left[ \frac{[A][K_B] + \alpha[B]}{[K_B] + [B]^n} + \frac{[A][K_B] + \alpha[B]}{[K_B] + [B]^n} \right]^{[\alpha][B]} \left( EC_{50A} \right)^{[\alpha][B]} \left( EC_{50B} \right)^{[\alpha][B]}
\]

where \(A\) and \(B\) denote the molar concentrations of the orthosteric ligand and allosteric ligand, respectively; \(E\) denotes response; \(E_m\) denotes the maximum response; \(K_B\) denotes the dissociation constant of allosteric ligand \(B\); \(EC_{50A}\) denotes the half-maximal response of orthosteric full agonist \(A\); \(\alpha\) denotes the efficacy of ligand \(B\); \(\alpha\) denotes a cooperativity factor that governs the magnitude and extent of the allosteric effect on the binding affinity of each ligand; \(\beta\) is a measure of the allosteric effect of the modulator on the signaling efficacy of the orthosteric agonist; and \(n\) denotes a logistic slope factor. \(K_B\), \(EC_{50A}\), \(\alpha\), and \(\beta\) (determined as a composite parameter) were estimated as base 10 logarithms (Christopoulos, 1998). Experimental error is reported as S.D. unless otherwise noted.

**Results**

We accessed the molecular pharmacological properties of R-568 and calcimimetic B using Ca\(^{2+}\) flux, IP accumulation, and ERK phosphorylation assays. Because calcimetics are positive allosteric modulators to the CaSR, we performed allosteric titration experiments in 1 to 1.2 mM CaCl\(_2\), a concentration that is similar to the level of ionizable Ca\(^{2+}\) in human serum. Shown in Table I are the LogEC\(_{50}\) values for R-568 and calcimimetic B against the hCaSR. R-568 displayed an average LogEC\(_{50}\) of −7.2, −6.4, and −6.4 in the FLIPR, aequorin, and IP assays, respectively (Fig. 1, A–C). Calcimimetic B displayed an average LogEC\(_{50}\) of −8.1, −7.8, and −7.7 in the FLIPR, aequorin, and IP assays, respectively. Similar potency values were obtained against the rat CaSR (data not shown). Calcimimetic B was significantly more potent than R-568 in all three assay formats. A typical dose-response curve of CaCl\(_2\) compared with R-568 and calcimimetic B in Ca\(^{2+}\)-free buffer is shown in Fig. 1D. Both compounds displayed partial agonism in addition to their allosteric modulatory properties.

Shown in Fig. 2 are the effects of R-568 and calcimimetic B on ERK phosphorylation in the presence and absence of extracellular Ca\(^{2+}\). As observed in the IP accumulation assay, both R-568 and calcimimetic B promoted ERK phosphorylation in the absence of extracellular Ca\(^{2+}\), indicating the compounds are agonists as well as allosteric modulators to the CaSR. Potency values for R-568 and calcimimetic B in the absence of extracellular Ca\(^{2+}\) were LogEC\(_{50}\) = −5.4 ± 0.15 (n = 3) and −7.2 ± 0.46 (n = 3), respectively (Fig. 2, A and C). In the presence of 1 mM extracellular Ca\(^{2+}\), the potency of R-568 and calcimimetic B was increased approximately 10-fold to LogEC\(_{50}\) = −6.8 ± 0.14 (n = 3) and −8.2 ± 0.44 (n = 3), respectively (Fig. 2, B and D).

**TABLE 1**

<table>
<thead>
<tr>
<th>Log EC(_{50})</th>
<th>R-568</th>
<th>Calcimimetic B</th>
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<tbody>
<tr>
<td>Ca(^{2+}) flux (FLIPR)</td>
<td>−7.2 ± 0.14 (n = 9)</td>
<td>−8.1 ± 0.35 (n = 37)*</td>
</tr>
<tr>
<td>Ca(^{2+}) flux (aequorin)</td>
<td>−6.4 ± 0.10 (n = 3)</td>
<td>−7.8 ± 0.14 (n = 9)*</td>
</tr>
<tr>
<td>IP accumulation</td>
<td>−6.4 ± 0.13 (n = 33)</td>
<td>−7.7 ± 0.15 (n = 32)*</td>
</tr>
</tbody>
</table>

* Significantly different compared with R-568, P < 0.001 (t test).
Activation and modulation of the CaSR by R-568 and calcimimetic B is a composite of 1) the affinity of the compound at the receptor denoted $K_B$, 2) the intrinsic efficacy denoted $\tau_B$, 3) the ability of the compound to modulate the affinity of Ca$^{2+}$ to the CaSR denoted $\alpha$ (cooperativity), and 4) the ability of the compound to modulate the coupling of the Ca$^{2+}$-occupied CaSR to downstream signaling pathways denoted $\beta$ (efficacy modulation). These parameters were quantitated using an operational model of allosteric modulation and allosteric agonism (Leach et al., 2007; Aurelio et al., 2009; Lu et al., 2009).

Shown in Fig. 3A is a diagram depicting the parameters of the model along with three graphs showing a simulation that demonstrated the effect of the parameters (Fig. 3, B–D). Shown in red is the agonist (Ca$^{2+}$) dose-response curve and shown in red is the agonist dose-response curve in the presence of a positive allosteric modulator. In the case of positive allosteric modulators such as calcimimetics ($\alpha > 1$), affinity modulation has the effect of left-shifting the agonist dose-response curve (Fig. 3B). If the calcimimetic also possesses intrinsic efficacy, as is the case of R-568 and calcimimetic B, the agonist dose-response curve is again left-shifted with an increase in basal response caused by modulator efficacy (Fig. 3C). Allosteric modulators that possess efficacy modulation ($\beta > 1$) and intrinsic efficacy also left-shift the agonist dose-response curve in addition to increasing the basal response (Fig. 3D). It is noteworthy that affinity modulation ($\alpha$) cannot be distinguished from efficacy modulation ($\beta$) when the orthosteric agonist (Ca$^{2+}$ in this case) is a full agonist (Lu et al., 2009).

Using this model, we performed a series of Ca$^{2+}$ dose-response curves in the presence of increasing doses of calcimimetic (Fig. 4). The hCaSR displayed a robust response to Ca$^{2+}$ in the inositol phosphate accumulation assay. The effect of R-568 and calcimimetic B was characterized by an increase in basal responsiveness and an enhancement of the potency of extracellular Ca$^{2+}$. This result is consistent with both compounds being positive allosteric modulators as well as partial agonists. There was no significant effect on maximum orthosteric agonist response, $E_{max}$, consistent with extracellular Ca$^{2+}$ being a full agonist at the hCaSR. Global fitting of the entire family of curves to an operational model of allosterism/agonism yielded the estimates of orthosteric agonist potency ($\log EC_{50}$), modulator affinity ($\log K_B$), modulator efficacy ($\log\gamma_B$), and cooperativity ($\log\alpha$) shown in Table 2. Because of the large number of parameters associated with the model, it was necessary to estimate the cooperativity ($\alpha$) and efficacy modulation ($\beta$) as a composite parameter, $\alpha\beta$ (Lu et al., 2009). We found that both R-568 and calcimimetic B are allosteric modulators as well as partial agonists because their $\tau_B$ values were greater than 0. Calcimimetic B showed a significant improvement in affinity compared with R-568 by 0.6 log units. In addition, calcimimetic B displayed greater efficacy than R-568 ($\tau_B = 1.2$ versus 0.8; $p = 0.001$) as evidenced by the greater basal responsiveness observed in the absence of Ca$^{2+}$ (Fig. 4). As a reference, a $\tau$ value of 1 indicates a partial agonist that achieves 50% of the maximum full-agonist response at saturating concentrations. A full agonist typically has $\tau$ values of 10 or more. Both compounds displayed positive cooperativity in the IP assay. However, the cooperativity observed for R-568 and calcimimetic B was not statistically different. Because the $\alpha$ and $\beta$ values were obtained as one composite $\alpha\beta$ value, we were unable to ascertain the relative contribution of affinity versus efficacy modulation. The use of eq. 1 requires that the value of $\tau$ for Ca$^{2+}$ be large, indicating receptor reserve. This could be a borderline case for the CaSR. Hence, we also examined simulations using the expanded operational model...
Fig. 2. hCaSR mediated ERK phosphorylation. Dose-response curves of R-568 (A and B) and calcimimetic B (C and D) on HEK293 cells stably expressing hCaSR in the presence or absence of 1 mM CaCl2. Cells were treated with the indicated concentrations of compound, and samples were analyzed for ERK phosphorylation by Western blot as described under Materials and Methods. Results from a representative experiment performed in duplicate are shown. Similar results were obtained from at least two additional experiments. Error bars represent S.E.M.
Fig. 3. Operational model of allosteric modulation/agonism. A, schematic illustration of the CaSR indicating location of the orthosteric (Ca\(^{2+}\) binding) site, putative allosteric modulator binding site (green ellipse) regions, and the molecular parameters governing the allosteric interaction between the orthosteric, allosteric, and G protein-coupling sites (blue). Receptor occupancy is governed by the dissociation constant of each ligand to its site on the receptor (\(K_A\) for orthosteric ligand, \(A\); \(K_B\) for allosteric modulator) and the binding cooperativity factor, \(\alpha\), which defines the magnitude of the allosteric effect that each ligand exerts on the binding affinity of the other. Positive cooperativity (\(\alpha > 1\)) results in an increase in affinity of the orthosteric ligand when the receptor is occupied by the allosteric ligand and vice versa. We define \(\beta\) as a measure of the allosteric effect of the modulator on the signaling efficacy of the orthosteric agonist over and above any effects the modulator has on the binding affinity of the agonist (see Leach et al., 2007; Aurelio et al., 2009; Lu et al., 2009). For \(\beta > 1\), cellular response increases in the presence of modulator and orthosteric ligand relative to the orthosteric ligand alone, whereas \(\beta < 1\) results in reduced responsiveness. \(\tau_A\) and \(\tau_B\) are parameters that incorporate the influence of receptor density and stimulus-response amplification (Leach et al., 2007); the larger the value, the greater the cellular response elicited by the agonist. B to D, simulations of the effect of \(\alpha\), \(\beta\), and \(\tau_B\) on the orthosteric agonist dose-response curve. \(\tau_A = 63\) (full agonist) in all three simulations.
Allosteric model parameters of affinity, potency, efficacy, and cooperativity for R-568, calcimimetic B, and extracellular Ca\(^{2+}\). Cellular response was monitored by inositol phosphate accumulation assay.

**Materials and Methods**

An allosteric titration experiment was conducted using HEK293 cells stably expressing hCaSR. Each curve corresponds to a fixed concentration of calcimimetic B or R-568 with increasing concentrations of extracellular Ca\(^{2+}\). Cellular response was monitored by inositol phosphate accumulation assay, and data were globally fit to eq. 1 as described under *Materials and Methods*. Similar results were obtained from at least two additional experiments. Results from a representative experiment performed in duplicate are shown. Error bars represent S.E.M.

**Table 2**

Allosteric model parameters of affinity, potency, efficacy, and cooperativity for R-568, calcimimetic B, and extracellular Ca\(^{2+}\) to the hCaSR in the inositol phosphate accumulation assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogEC(_{50})</th>
<th>LogK(_{d})</th>
<th>Logβ</th>
<th>Logαβ</th>
<th>αβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-568</td>
<td>−2.6 ± 0.047</td>
<td>−6.2 ± 0.12</td>
<td>−0.073 ± 0.062</td>
<td>0.58 ± 0.20</td>
<td>3.8 (2.4–5.9)</td>
</tr>
<tr>
<td>Calcimimetic B</td>
<td>−2.7 ± 0.021</td>
<td>−6.8 ± 0.082(^*)</td>
<td>0.086 ± 0.039(^*)</td>
<td>0.82 ± 0.17</td>
<td>6.6 (4.5–9.8)</td>
</tr>
</tbody>
</table>

\(^*\) Significantly different compared with R-568, *P* ≤ 0.05 (t test).
R-568 or calcimimetic B. This result is consistent with Ca\(^{2+}\) acting as a full agonist. In allosteric titration experiments involving the IP, Ca\(^{2+}\) flux, and ERK phosphorylation as-

says, we found that calcimimetic B was significantly more potent than R-568. We showed that R-568 and calcimimetic B promoted IP accumulation and ERK phosphorylation in the

![Graphs and images showing calcium and PTH levels over time for different treatments.]

**Fig. 5.** In vivo effects of 5/6 Nx rats treated with calcimimetic B or R-568. Expression of CaSR mRNA in rat parathyroid and thyroid tissue. A, calcimimetic B reduces serum PTH over a lower dose range than R-568. B, calcimimetic B has less effect on ionized Ca\(^{2+}\)-lowering compared with R-568. C, calcitonin secretion is promoted by R-568 but not by pharmacologic doses of calcimimetic B. 5/6 Nx rats were treated with increasing doses of compounds and blood was drawn at the indicated times. Levels of Ca\(^{2+}\), intact PTH, and calcitonin were measured as described under Materials and Methods. Results are from a representative experiment repeated at least three times. Error bars represent S.E.M. D, in situ hybridization (right) with rat CaSR antisense probe showing strong expression of CaSR mRNA in the parathyroid tissue relative to the surrounding thyroid tissue. Hematoxylin and eosin stain is shown on the left (magnification, 4X).
calcimimetic B was less than that for R-568. It is plausible to hypothesize that this observation was caused by a lack of an effect of calcimimetic B on thyroid-mediated secretion of calcitonin. This notion is supported by the observation that in R-568-treated, thyroidectomized rats (i.e., no calcitonin-releasing target) with intact parathyroids the rate of onset of calcium lowering was slower than in normal rats with intact thyroid glands (Fox et al., 1999a). In addition, it has been shown that R-568 rapidly reduced Ca\(^{2+}\) levels in parathyroid-intact rats that were given PTH infusions to restore normocalcemia after removal of the parathyroids (Fox et al., 1999a), supporting the notion that decreased PTH secretion was not solely responsible for calcimimetic-induced hypocalcemia. The ability of calcimimetic B to differentially affect PTH suppression compared with calcitonin secretion may in part be explained by its pharmacokinetic properties. A 30 mg/kg dose of R-568 administered to uremic rats produced R-568 exposure levels that were up to 100-fold higher than a 0.1 mg/kg dose of calcimimetic B (Supplemental Table 1). It has been shown in rodents that calcimimetics (R-568 and R-467) depress serum PTH at doses much lower than doses that increase plasma calcitonin (Nemeth and Fox, 1999). R-568 has been shown to be approximately 40 times more potent in reducing PTH levels than in increasing calcitonin levels (Fox et al., 1999a). Similar findings have been reported for cinacalcet HCl (Nemeth et al., 2004). In addition, R-568 in doses that suppressed PTH and minimally decreased serum calcium have been shown to increase serum calcitonin in dialysis patients (Antonsen et al., 1998).

It is plausible to hypothesize that the lower exposure levels of calcimimetic B translate into lower tissue levels that may be below the threshold for calcitonin secretion, yet sufficient for inhibition of PTH secretion. These data suggest that calcitonin secretion plays a role in the hypocalcemia observed in this CKD model. Calcitonin is a potent stimulator of bone formation in rodents, resulting in deceased serum Ca\(^{2+}\) levels (Baylink et al., 1969; Sørensen et al., 1970). Hence, calcimimetic B was able to achieve a desirable PTH suppression while minimizing hypocalcemia.

The functional selectivity of calcimimetic B to lower PTH while having minimal effect on calcitonin secretion may be a result of selective activation of CaSR pathways in the chief cells of the parathyroid compared with the C cells of the thyroid. The expression of the CaSR in parathyroid tissue is much higher than in thyroid tissue as demonstrated by the in situ hybridization experiment shown in Fig. 5D. Because compound efficacy is proportional to receptor density, this may in part explain the differential effects seen with calcimimetic B. Consistent with our in vivo data, we found that human thyroid carcinoma TT cells secrete calcitonin in response to R-568, whereas calcimimetic B showed reduced efficacy. It is noteworthy that no functional selectivity was observed for calcimimetic B over R-568 in our second-messenger and ERK phosphorylation assays. There are examples of a compound promoting activation of a receptor in one cell type but not another. For example, binding of nicotinic acid to the nicotinic acid receptor inhibits lipolysis in adipocytes and stimulates prostaglandin release from Langerhans cells in

**Fig. 6.** A, Western blot analysis of various cell lines for CaSR expression. Cell membrane proteins prepared from HT-29, SW480, HCT116, CaCo-2, HEK293-CaSR, and TT cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The membrane was probed with an hCaSR antibody and developed with a chemiluminescence reagent as described previously (Lu et al., 2009). Molecular mass of the protein markers is measured in kDa. B, R-568 stimulates calcitonin secretion from TT cells with greater efficacy than calcimimetic B. TT cells were seeded in 96-well plates and washed in phosphate-free medium was determined by ELISA. Error bars represent S.E.M. Results are from a representative experiment repeated two additional times.

absence of Ca\(^{2+}\), indicating that both compounds have inherent CaSR agonist activity in addition to being positive allosteric modulators. We used an operational model of allosteric modulation/agonism to demonstrate that calcimimetic B displayed higher affinity and greater intrinsic efficacy for the CaSR compared with R-568. However, we did not observe a statistically significant difference in cooperativity values between R-568 and calcimimetic B. The serum concentration of ionizable Ca\(^{2+}\) is approximately 1.4 mM in rodents. Serum Ca\(^{2+}\) cannot be lowered significantly without resulting in serious adverse effects on experimental animals. Hence, the effect of improved intrinsic efficacy of calcimimetic B (the activity present in the absence of Ca\(^{2+}\)) was not possible to examine in vivo. The greater in vivo potency we observed with calcimimetic B in terms of its ability to suppress PTH secretion is likely a combination of factors, including higher potency, higher affinity and intrinsic efficacy, longer half-life, and higher oral bioavailability compared with R-568.

We found that calcimimetic B showed a greater separation of PTH versus ionized Ca\(^{2+}\) lowering compared with R-568. It is noteworthy that whereas R-568 was both an inhibitor of PTH secretion and stimulator of calcitonin secretion, calcimimetic B did not stimulate calcitonin secretion at doses sufficient to inhibit PTH secretion. This may explain, at least
the skin. However, some synthetic nicotinic acid receptor agonists inhibit lypolysis in adipocytes but show no stimulation of prostaglandin secretion from Langerhans cells (Richman et al., 2007). This ligand bias was found to be a result of differential activation of δ-arrestin pathways in Langerhans cells over G protein pathways. It remains to be determined whether calcimimetic B exhibits a signaling bias in chief cells over C cells via a similar mechanism. It is noteworthy that it has been reported that an autoantibody to the CaSR was found to selectively activate the Gq pathway while inhibiting ERK phosphorylation in CaSR expressing HEK293 cells (Makita et al., 2007). Whether or not these dual acting pathways exist in C cells remains to be determined. Of course, it is also plausible that a combination of the improved potency and pharmacokinetic parameters of calcimimetic B are responsible for the observed selective in vivo effects.

In conclusion, we have developed a calcimimetic that has the unique ability to increase the separation of PTH lowering versus Ca2+ lowering in 5/6 Nx rats compared with R-568, the first calcimimetic introduced into clinical trials. Our data support the hypothesis that calcimimetic B is able to accomplish this effect, in part, because of the lack of calcitonin secretion at doses sufficient to inhibit PTH secretion. We presented an allosteric model that demonstrates that calcimimetic B has higher affinity and greater intrinsic efficacy and improved pharmacokinetic properties compared with R-568. These properties taken together may explain how calcimimetic B attenuates PTH secretion at much lower exposure (concentration) levels than R-568. The amount of R-568 needed to maintain adequate PTH suppression seems to be sufficient enough to also target calcitonin secretion. The data we provide suggests that it is plausible that calcimimetics with specific properties could be developed for use in patients with renal disease where it is desirable to lower PTH without causing significant reduction in serum Ca2+ levels (Levin et al., 2007).

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Participated in research design: Henley and Reagan.
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References
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