JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681

JPET #178681

Discovery of a Calcimimetic With Differential Effects on PTH and Calcitonin Secretion

Charles Henley III, Yuhua Yang, James Davis, Jenny Ying Lin Lu, Sean Morony, Wei Fan, Monica Florio, Banghua Sun, Edward Shatzen, James K. Pretorius, William G Richards, David J. St. Jean, Jr., Christopher Fotsch, Jeff D. Reagan

Department of Metabolic Disorders, Amgen San Francisco, 1120 Veterans Boulevard, South San Francisco, California 94080 (Y.Y., J.Y.L.L., J.D.R.)

Department of Metabolic Disorders, Amgen Thousand Oaks, One Amgen Center Drive, Thousand Oaks, CA 91320 (C.H., J.D., M.F., S.M., B.S., W.F., E.S., J.K.P., W.G.R.)

Department of Small Molecule Chemistry, Amgen Thousand Oaks, One Amgen Center Drive, Thousand Oaks, CA 91320 (D.St.J., C.F.)

Running Title: Calcimimetic Effects on PTH and Calcitonin Secretion

Corresponding author: Jeff D. Reagan, Amgen San Francisco, 1120 Veterans Boulevard, South San Francisco, California 94080, U.S.A, phone: (650)244-2887, fax: (650)837-9423, E-mail: jreagan@amgen.com

Number of text pages: 21

Number of tables: 2

Number of Supplementary tables: 1

Number of figures: 6

Number of references: 37

Number of words in abstract: 206

Number of words in introduction: 561

Number of words in discussion: 1195

The abbreviations used are: CaSR, calcium sensing receptor; CKD, chronic kidney disease; E_{max}, maximum agonist response; ESRD, end stage renal disease; FLIPR, Fluorescent Imaging Plate Reader; hCaSR, human calcium sensing receptor; IP, inositol phosphates; iPTH, intact parathyroid hormone; nephrectomized Nx; PTH, parathyroid hormone; SHPT, secondary hyperparathyroidism

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Ca^{2+}). Cinacalcet, a second generation calcimimetic, is used therapeutically to control PTH in chronic kidney disease (CKD) patients on dialysis with secondary hyperparathyroidism (SHPT). A calcimimetic that displays increased separation of PTH vs. Ca^{2+} lowering in patients would potentially allow the use of calcimimetics to treat patients in earlier stages of renal disease since hypocalcaemia can develop in this population. Towards 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 compound effects upon PTH, serum ionized Ca^{2+} and calcitonin levels in 5/6 nephrectomized rats. We found the new molecule effectively reduced PTH levels without promoting calcitonin secretion or hypocalcaemia. Furthermore, our third generation molecule was less efficacious at promoting calcitonin secretion from human thyroid carcinoma cells as compared to 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 hypocalcaemia since the threshold for inhibition of PTH secretion is much lower than the threshold for calcitonin secretion.

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

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 GI 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 Ca^{2+} can activate both G_q and G_i signaling pathways leading to inositol phosphate production, intracellular Ca^{2+} flux and ERK phosphorylation (Hawkins, et al., 1989; Kifor, et al. 1997; Chen, et al. 1989). 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 while activation of the CaSR in the GI tract has been shown to inhibit fluid secretion from colonic crypt cells (Dufner, et al. 2004; Geibel, et al. 2005).

Disorders of Ca^{2+} and phosphate homeostasis develop in end-stage renal disease (ESRD) due to 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 hypocalcaemia 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 hypercalcaemia (Timmers, et al. 2006; Festen-Spanjer, et al., 2007; Alon, et al. 2010).

Treatment of SHPT in CKD stage 3 and 4 with calcimimetics has not been approved by the FDA and requires further evaluation. CKD stage 3 and 4 subjects also have elevated PTH but at lower levels than ESRD patients. In a randomized, double-blind, placebo-controlled study to assess efficacy and safety of cinacalcet in non-dialysis patients, cinacalcet effectively decreased plasma iPTH. However, hypocalcaemia (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 hypocalcaemia for use is this group of patients. Towards this end, we developed Calcimimetic B that we compared to R-568, a first generation calcimimetic (Harrington, et al. 2010). We determined the molecular pharmacological parameters of each calcimimetic and measured compound effects upon PTH, serum ionized calcium (Ca^{2+}) and calcitonin levels in 5/6 nephrectomized (Nx) rats. Measurements were conducted up to 4 hours

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

post-dose in order to explore the differential effects on Ca^{2+} lowering of these two calcimimetics. We found that calcimimetic B was more potent and had less Ca^{2+} lowering effect relative to PTH lowering in 5/6 Nx rats as compared to R-568.

Methods

Chemicals, Plasmids and Reagents- R-568 [3-(2-chlorophenyl)-N-((1R)-1-(3-methoxyphenyl)ethyl)-1-propanamine] and Calcimimetic B [(1*R*)-1-(6-methoxy-4'-(trifluoromethyl)-3-biphenylyl)-*N*-((1*R*)-1-phenylethyl)ethanamine] were prepared at Amgen, Inc. (Thousand Oaks, CA), HEK293, CHO, and TT cells were obtained from ATCC (Manassas, VA). HEK293 cells stably expressing the human CaSR were obtained from NPS Pharmaceuticals (Bedminster, NJ). Yttrium silicate SPA beads and tritiated inositol (50-80 Ci/mmol) were obtained from Amersham Biosciences (Pittsburgh, PA). pcDNA3.1-Aequorin and pcDNA3.1- hCaSR (human calcium sensing receptor) were prepared by standard cloning techniques. Plasmids were prepared with a QIAGEN plasmid mega DNA preparation kit (QIAGEN, Inc., Chatsworth, CA).

Experimental animals- Male, Sprague-Dawley rats (249-424 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.

Aequorin Assay- Six million CHO cells were seeded in a 150 cm^2 dish in DMEM/F12/10% FCS without antibiotics. One hour after plating, the cells were transfected with 10 μg hCaSR plasmid and 10 μg aequorin plasmid with Lipofectamine 2000 (Invitrogen Technologies, Carlsbad, CA) according to the manufacturer's instructions. The cells were incubated at 37^{o}C, 5% CO_2 for 6 hours and the medium

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 μg/mL. The cells were gently agitated every 15 minutes for a total of 2 hours. The cells were then diluted 3- fold in the same buffer lacking coelenterazine F. R-568 was prepared as a 100X stock in DMSO. Just prior to 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, Lowell, MA). One hundred microliters of cells were then added to the plate on a MicroLumat luminometer (Berthold Technologies, Oak Ridge, TN) and the luminescence recorded. The lowest concentration of calcium tested was 0.25 mM.

FLIPR (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 uL of cell media (DMEM/10% fetal bovine serum). The next day, the media was removed and the cells were incubated in 50 uL of dye buffer (BD Biosciences, Bedford, MA) and allowed to incubate for 1.5 hours at room temperature. Compounds were dissolved in 100% DMSO at 100X 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 seconds on FLIPR. Maximum observed fluorescence values were used in calculation of Log EC_{50}.

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

Inositol Phosphate Accumulation Assay- HEK293 cells were dispensed into a poly-D-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 hCaSR plasmid per well using Lipofectamine2000 according the manufacturer's instructions. Six hours after transfection the medium was replaced with inositol free DMEM/10% dialyzed FCS supplemented with 1μCi/mL tritiated inositol. After incubation overnight, the cells were washed once in calcium free HBSS and then treated with the 100 μL calcium free HBSS/0.01% BSA containing various concentrations of $CaCl_2$, R-568 (prepared as above in DMSO), 10 mM LiCl and incubated at 37^{o}C for 1 hour. The media was aspirated and the cells were lysed with ice cold 20 mM formic acid. After incubation at 4^{o}C for 5 hours, the lysate were added to yttrium silicate SPA beads, allowed to settle overnight and read on a Beckman TopCount scintillation counter.

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 36h. 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 minutes and the media aspirated. The cells were lysed by quick freezing in liquid nitrogen. Fifty uL of Laemmli sample buffer (Bio-Rad, Hercules, CA) was added to each well, denatured by heating to 100^{0}C for 5 minutes and then 10 uL of the cell suspension was loaded on a 4-12% gradient SDS-PAGE gel. The gel was transferred to a PVDF membrane and probed with primary phospho ERK antibody (Cell Signaling Technology, Danvers, MA) at a 1:1000 dilution at 4^{0}C overnight. The membrane was

then probed with a goat anti- rabbit IgG secondary antibody (Pierce, Rockford, IL) at a dilution of 1:5000 for 1 hour at room temperature. The membrane signal was detected using Supersignal Substrate (Pierce, Rockford, IL).

Experimental Subjects- Subtotal nephrectomized (5/6 Nx) 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 Investigation Animal Care and Use Committee of Amgen Inc.

Surgical Procedures for 5/6Nx- A two step procedure was performed starting with ligation of renal artery (left kidney; uppermost bifurcation) in anesthetized rats (IM cocktail: two parts ketamine (100 mg/mL), one part xylazine (100 mg/mL), 0.3 parts acepromazine (10 mg/m). After a one week recovery period following 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. Following the surgical procedures, animals were recovered for 6 weeks prior to the initiation of studies.

Drug Treatment- Six weeks after 5/6 Nx rats (n=7-8/group) animals were fasted overnight (14-16h) and then treated with single oral doses of the research calcimimetics, R-568 (1, 10, 30 or 100 mg/kg) or Calcimimetic B (0.03, 0.1, 0.3 mg/kg. Vehicle-treated

control rats (n=8) received HPMC 2%, pluronic F68 1%, Captisol 10% in water in equivolume amounts (5ml/kg) as the calcimimetic treated groups.

Biomarker analysis- Blood was collected from the retro-orbital sinus from anesthetized rats (2% isofluorane in O_2). Whole blood ionized Ca^{2+} and serum PTH and calcitonin were determined at 0, 0.25, 0.5, 1, 2, 4 hrs post dose. Immediately after the animals recovered from the last blood sample collection animals were given free access to food. For ionized Ca^{2+}, a Ciba-Corning 634 ISE Ca^{2+} /pH Analyzer (Ciba-Corning Diagnostics Corp., Medfield, MA, USA). Serum PTH and calcitonin levels were determined using commercial ELISA rat PTH[1–34] and rat calcitonin assay kits (Immutopics, San Clemente, CA, USA).

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). Briefly, a 282 base pair ^{33}P labeled RNA antisense probe corresponding to nucleotides 2154-2435 of the rat CaSR gene (Genbank #U10354) was used as a probe. Hybridization was conducted at 60^{o} C in a hybridization solution containing 1 x 10^{6} cpm of ^{33}P-labeled antisense riboprobe, followed by RNase digestion, and a series of high stringency washes at 55^{o} C for 30 minutes. The slides were coated with Kodak NTB emulsion and exposed for 3 weeks in the dark at 4^{o} 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 48h 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 1mM $CaCl_2$ or 1mM $CaCl_2$ plus different concentrations of calcimimetic (stock prepared at 100x in DMSO) for 12h. The medium from treated or untreated cells was centrifuged (1500 x g for 3 min), and the concentration of calcitonin in the culture medium was determined using a commercial ELISA method per the manufacturer's instructions (Immutopics International, San Clemente, CA) 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 utilizing an Envision instrument (Perkin Elmer, Waltham, MA) and normalized to cell protein content. Values we 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 Figure 4 was performed using the following form of an operational model of allosterism/agonism (Figure 3; also see Lu, et al. 2009):

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

(Equation 1)

where A and B denote the molar concentrations 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; τ_B denotes the efficacy of ligand B; α denotes a cooperativity factor that governs the magnitude and extent of the allosteric effect on the binding affinity of each ligand; β 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_{50}, τ_B, and $\alpha\beta$ (determined as a composite parameter) were estimated as base 10 logarithms (Christopoulos, 1998). Experimental error is reported as standard deviation unless otherwise noted.

Results

We accessed the molecular pharmacological properties of R-568 and Calcimimetic B using Ca^{2+} flux, inositol phosphate (IP) accumulation and ERK phosphorylation assays. Since calcimimetics are positive allosteric modulators to the CaSR, we performed allosteric titration experiments in 1-1.2 mM $CaCl_2$, a concentration that is similar to the level of ionizable Ca^{2+} in human serum. Shown in Table 1 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 (Fluorescent Imaging Plate Reader), aequorin and IP assays, respectively (Figure 1A-C). Calcimimetic B displayed an average $LogEC_{50}$ of -8.1, -7.8 and -7.7, 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 $CaCl_2$ as compared to R-568 and Calcimimetic B in Ca^{2+} free buffer is shown in Figure 1D. Both compounds displayed partial agonism in addition to their allosteric modulatory properties.

Shown in Figure 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 $LogEC_{50} = -5.4 \pm 0.15$ (n = 3) and $LogEC_{50} = -7.2 \pm 0.46$ (n = 3), respectively (Figure 2A and C). In the presence of 1 mM extracellular Ca^{2+}, the potency

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

of R-568 and Calcimimetic B was increased approximately 10 fold to $LogEC_{50}$ = -6.8 ± 0.14 (n = 3) and $LogEC_{50}$ = -8.2 ± 0.44 (n = 3), respectively (Figure 2B and D).

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 τ_B, (3) the ability of the compound to modulate the affinity of Ca^{2+} to the CaSR denoted α (cooperativity) and (4) the ability of the compound to modulate the coupling of the Ca^{2+} occupied CaSR to downstream signaling pathways denoted β (efficacy modulation). These parameters were quantitated utilizing an operational model of allosteric modulation and allosteric agonism (Leach, et al. 2007; Aurelio, et al. 2009; Lu, et al. 2009).

Shown in Figure 3A is a diagram depicting the parameters of the model along with three graphs showing a simulation which demonstrates the effect of the parameters (Figure 3B-D). Shown in black 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 (Figure 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 due to modulator efficacy (Figure 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 (Figure 3D). It is noteworthy that affinity

modulation (α) cannot be distinguished from efficacy modulation (β) when the orthosteric agonist (Ca^{2+} in this case) is a full agonist (Lu, et al. 2009).

Utilizing this model, we performed a series of Ca^{2+} dose response curves in the presence of increasing doses of calcimimetic (Figure 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 upon 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 estimates of orthosteric agonist potency ($\log EC_{50}$), modulator affinity ($\log K_B$), modulator efficacy ($\log \tau_B$) and cooperativity ($\log \alpha\beta$) shown in Table 2. Due to the large number of parameters associated with the model, it was necessary to estimate the cooperativity (α) and efficacy modulation (β) as a composite parameter, $\alpha\beta$ (Lu, et al. 2009). We found that both R-568 and Calcimimetic B are both allosteric modulators as well as partial agonists as their τ_B values were greater than 0. Calcimimetic B showed a significant improvement in affinity compared to R-568 by 0.6 log units. In addition, Calcimimetic B displayed greater efficacy than R-568 (τ_B=1.2 vs. 0.8, p=0.001) as evidenced by the greater basal responsiveness observed in the absence of Ca^{2+} (Figure 4). As a reference, a τ value of 1 indicates a partial agonist that achieves 50% of the maximum full agonist response at saturating concentrations. A full agonist typically has τ values of 10 or greater. Both

compounds displayed positive cooperativity in the IP assay. However, the cooperativity observed for R-568 and Calcimimetic B were not statistically different. As the α and β values are obtained as one composite $\alpha\beta$ value, we were unable ascertain the relative contribution of affinity vs. efficacy modulation. The use of Equation 1 (Methods) requires that the value of τ 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 of allosterism and agonism in which no assumptions about receptor reserve were made (Leach et al., 2007; Lu, et al. 2009). The simulations revealed minor differences in the α and β values obtained as compared to Equation 1 (data not shown). However, one is not able to fit the experimental data presented in Figure 4 to the expanded operational model as there are too many parameters to be determined. In either case, both R-568 and Calcimimetic B possessed composite cooperativity/efficacy values that were not statistically different.

To assess the activity of the calcimimetics *in vivo*, we utilized the 5/6 Nx rat as an animal model of SHPT (Henley, et al. 2005). Rats were treated with single oral doses of Calcimimetic B (0.03, 0.1, 0.3 mg/kg) or R-568 (10, 30, 100 mg/kg). Higher doses of R-568 versus Calcimimetic B were selected based upon pharmacokinetic data in rats: R-568 has a half-life of 2.3 hr and an oral bioavailability of 4.6% while Calcimimetic B has a half-life of 9.3 hr and an oral bioavailability of 60%. Serum Ca^{2+}, PTH and calcitonin were measured at 0, 0.25, 0.5, 1, 2, and 4 hours post-dose (Figure 5). R-568 elicited a rapid, significant decrease (0.25 hours after drug administration) in serum PTH (Figure 5A). In contrast, Calcimimetic B effects on PTH lowering during the first 30 minutes

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

after drug administration were less pronounced than R-568. However, by 1 hour after treatment, PTH was lowered to approximately the same level by the highest dose (0.3 mg/kg) of Calcimimetic B (PTH=37±17 pg/ml; n=8) compared to R-568 (PTH=35±19; n=8 and 37±10; n=7 pg/ml for the 30 and 100mg/kg doses, respectively). By 2 hours after treatment a lower dose of Calcimimetic B (0.1 mg/kg) lowered PTH (102±37 pg/ml; n=8) to comparable levels achieved by R-568 (PTH=114±85 and 72±44 pm/ml for the 30 and 100 mg/kg doses, respectively). Serum PTH measured 4 hours after drug administration was lowest in the Calcimimetic B (0.3mg/kg) group (PTH=14±6 pg/ml) and the R-568 (100mg/kg) group (PTH=44±13), followed by the Calcimimetic B (0.1mg/kg; PTH=107±47) and the R-568 (30mg/kg; PTH 204±84) groups. Effects of R-568 on Ca^{2+} lowering were greater and more immediate than effects of Calcimimetic B (Figure 5B). In addition, doses of Calcimimetic B that produced similar PTH lowering compared to R-568 at a given time after drug administration had lesser effects on blood Ca^{2+} levels (Figure 5A). R-568 elicited a rapid and dose-dependent increase in calcitonin secretion which correlated with decreases in Ca^{2+} (Figure 5C). In contrast, Calcimimetic B had no effect on calcitonin at any dose including the highest dose (0.3 mg/kg) that profoundly suppressed PTH. Shown in Figure 5D is an situ hybridization experiment demonstrating high expression of the CaSR in parathyroid relative to the surrounding thyroid tissue.

The differential effects of R-568 and Calcimimetic B on calcitonin secretion were further explored utilizing the thyroid carcinoma cell line (TT) as an established *in vitro* model of calcitonin secretion (Leong, et al. 1981). Western blot analysis revealed TT cells

express the CaSR with the major band migrating at ~160 kilodaltons (Figure 6A). This contrasts with an earlier report that found TT cells devoid of CaSR expression (Garrett, et al. 1995). An allosteric titration experiment was performed in which cells were treated for 12 h with increasing concentrations of calcimimetics and a fixed concentration of Ca^{+2} (1mM). The cells showed a dose dependent secretion of calcitonin in response to increasing concentrations of R-568. In contrast, cells treated with Calcimimetic B secreted much lower levels of calcitonin (Figure 6B).

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Calcimimetics are positive allosteric modulators that increase the potency of Ca^{2+} to the CaSR resulting in a left shift of the calcium dose response curve. However, the overall signaling efficacy of the CaSR is not affected by either 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 assays, 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 absence of Ca^{2+} indicating that both compounds have inherent CaSR agonist activity in addition to being positive allosteric modulators. We utilized an operational model of allosteric modulation/agonism to demonstrate that Calcimimetic B displayed higher affinity and greater intrinsic efficacy for the CaSR as compared to 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 upon 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 observe with Calcimimetic B in terms of its ability to suppress PTH secretion is likely to be a combination of factors. These include higher potency, higher affinity and intrinsic efficacy, longer half-life, and higher oral bioavailability as compared to R-568.

We found that Calcimimetic B showed a greater separation of PTH vs. ionized Ca^{2+} lowering as compared to R-568. It is noteworthy that while 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 in part, why Calcimimetic B has less effect upon the reduction in ionized Ca^{2+} as compared to R-568 as calcitonin administration in rodents lowers serum ionized Ca^{2+} levels (Boron and Boulpaep 2004). The rate of onset of calcium lowering by Calcimimetic B was less than that for R-568. It is plausible to hypothesize that this observation was due to lack of an effect of Calcimimetic B on thyroid mediated secretion of calcitonin. This notion is supported by the observation 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 parathyroidectomized thyroid-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 30mg/kg dose of R-568 administered to uremic rats produced R-568 exposure levels that were up to 100-fold higher than a 0.1mg/kg dose of Calcimimetic B (Supplementary Material 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). NPS R-568 has been shown to

be about 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 which 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 hypocalcaemia observed in this CKD model. Calcitonin is a potent stimulator of bone formation in rodents resulting in deceased serum Ca^{2+} levels (Sørensen, et al. 1970; Baylink, et al. 1969). Hence, calcimimetic B was able to achieve a desirable PTH suppression while minimizing hypocalcaemia.

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 as compared to the C-cells of the thyroid. The expression of the CaSR in parathyroid tissue is much higher than in thyroid tissue as demonstrated by our in situ hybridization experiment shown in Figure 5D. As 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. Interestingly, no functional selectivity was observed for

Calcimimetic B over R-568 in our second messenger and ERK phoshphorylations 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 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 β-arrestin pathways in Langerhans cells over G-protein pathways. It remains to be determined if Calcimimetic B exhibits a signaling bias in chief cells over C-cells via a similar mechanism. Interestingly, it has been reported that an autoantibody to the CaSR was found to selectively activate the G_q 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 vs. Ca^{2+} lowering in 5/6 Nx rats as compared to 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, due to 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 to R-568. These properties, taken

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

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 appears 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 Ca^{2+} levels (Levin, et al. 2007).

Acknowledgements

We thank Arthur Christopoulos (Monash University, Melbourne, Australia) for his contribution to the artwork in Figure 3.

Authorship Contributions

Participated in research design: Henley, Reagan

Conducted experiments: Davis, Fan, Lu, Morony, Pretorius, Reagan, Shatzen, Sun, Yang

Contributed new reagents: Fan, Florio, Fotsch, St. Jean,

Performed data analysis: Florio, Henley, Pretorius, Richards, Reagan

Wrote or contributed to the writing of the manuscript: Henley, Reagan, Richards

References

Alon US, Vandervoorde RG (2010) Beneficial effect of cinacalcet in a child with familial hypocalciuric hypocalcaemia. *Pediatr Nephrol* 25: 1747-1750.

Antonsen JE, Sherrard DJ, Andress DL (1998) A calcimimetic agent acutely suppresses parathyroid hormone levels in patients with chronic renal failure. *Kidney Int* 53: 223-227.

Aurelio L, Valant C, Flynn BL, Sexton PM, Christopoulos A, Scammells PJ (2009) Allosteric modulators of the adenosine A1 receptor: synthesis and pharmacological evaluation of 4-substituted 2-amino-3-benzoylthiophenes. *J Med Chem* 52: 4543-4547.

Baylink D, Morey E, Rich C (1969) Effect of calcitonin on the rates of bone formation and resorption in the rat. *Endocrinology* 84: 261-269.

Block GA, Martin KJ, de Francisco AL, Turner SA, Avram MM, Suranyi MG, Hercz G, Cunningham J, Abu-Alfa AK, Messa P, Coyne DW, Locatelli F, Cohen RM, Evanepoel P, Moe SM, Fournier A, Braun J, McCary LC, Zani VJ, Olson KA, Drüeke TB, and Goodman WG (2004) Cinacalcet for secondary hyperparathyroidism in patients receiving hemodialysis, *N Engl J Med* 350: 1516-1525.

Boron WF, Boulpaep EL (2004). Endocrine system chapter. *Medical Physiology: A Cellular And Molecular Approach*. Elsevier/Saunders.

Brown EM (1991) Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. *Physiol Rev* 71: 371-411.

Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifori O, Sun A, Hediger M, Lytton J, and Herbert SC (1993) Cloning, expression, and characterization of an extracellular Ca^{2+} sensing receptor from bovine parathyroid. *Nature* 366: 575-580.

Brown, EM (2007) Clinical lessons from the calcium-sensing receptor. *Nat Clin Pract Endoc* 3:122-133

Chang W and Shoback D (2004) Extracellular Ca^{2+}-sensing receptors--an overview. *Cell Calcium* 35: 183-196.

Chen CJ, Barnett JV, Congo DA, and Brown EM (1989) Divalent cations suppress 3', 5'-adenosine monophosphate accumulation by stimulating a pertussis toxin-sensitive guanine nucleotide-binding protein in cultured bovine parathyroid cells. *Endocrinology* 124: 233-239.

Chonchol M, Locatelli F, Abboud HE, Charytan C, de Francisco ALM, Jolly S, Kaplan M, Roger SD, Sarkar S, Albizem MB, Mix CH, Kubo Y, Block GA (2009) A randomized,double-blind, placebo-controlled study to assess the efficacy and safety of cinacalcet HCl in participants with CKD not receiving dialysis. *Am J Kidney Dis* 53:197 207

Christopoulos A (1998) Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends Pharm Sci* 19: 351-357.

Dufner MM, Kirchhoff P, Remy C, Hafner P, Müller MK, Cheng SX, Tang LQ, Hebert SC, Geibel JP, and Wagner CA (2004) The calcium-sensing receptor acts as a modulator of gastric acid secretion in freshly isolated human gastric glands. *Am J Physiol Gastrointest Liver Physiol* 289: 1084-1090.

Festen-Spanjer, B, Haring, CM, Koster, JB, Mudde, AH (2007) Correction of hypercalcaemia by cinacalcet in familial hypocalciuric hypercalcaemia. *Clinical Endocrinology* 68, 321-325.

Fox J, Lowe SH, Conklin RL, Petty BA, and Nemeth EF (1999A) Calcimimetic compound NPS R-568 stimulates calcitonin secretion but selectively targets parathyroid gland Ca^{2+} receptor in rats. *J Pharm Exp Ther* 290: 480-486.

Fox, J, Lowe, SH, Petty, BA, Nemeth, EF (1999B) NPS R-568: A type II calcimimetic compound that acts on parathyroid cell calcium receptor of rats to reduce plasma levels of parathyroid hormone and calcium. *J Pharm Exp Ther* 290: 473-479.

Garrett, JE, Tamir, H, Kifor, O, Simin, RT, Rogers, KV, Mithal, A, Gagel, RF, Brown, EM (1995) Calcitonin-secreting cells of the thyroid express an extracellular calcium receptor gene. *Endocrinology* 136 (11): 5202-5211.

Geibel J, Sritharan K, Geibel R, Geibel P, Persing JS, Seeger A, Roepke TK, Deichstetter M, Prinz C, Cheng SX, Martin D, and Hebert SC (2005) Calcium-sensing receptor abrogates secretagogue- induced increases in intestinal net fluid secretion by enhancing cyclic nucleotide destruction. *Proc Natl Acad Sci* 103: 9390-9397.

Goodman WG, Hladik GA, Turner SA, Blaisdell PW, Goodkin DA, Liu W, Barri YM, Cohen RM, and Coburn JW (2002) The calcimimetic agent AMG 073 lowers plasma parathyroid hormone levels in hemodialysis patients with secondary hyperparathyroidism. *J Am Soc Neprol* 13: 1017-1024

Hammerland LG, Garrett JE, Hung BC, Levinthal C, and Nemeth EF (1998) Allosteric activation of the Ca^{2+} receptor expressed in *Xenopus laevis* oocytes by NPS 467 or NPS 568 *Mol Pharmacol* 53: 1083-1088.

Harrington, PE, St Jean, DJ Jr, Croghan, M, Kelly, MG, Poon, S.F, Yao, G, Zeng, Q, Davis, J, Lott, F, Morony, S, Lu, JY, Reagan, JD, Yang, Y, Coulter, TS, Davenport, A, Ghiron, C, Hutchinson, J, Portero-Larragueta, E, Wang, M, Martin, D, Henley, C III, Tasker, A, Fotsch, C (2010) The Discovery of Orally Efficacious Positive Allosteric Modulators of the Calcium Sensing Receptor. *Bioorg. Med. Chem. Lett.* 20(18): 5544-5557.

Hawkins D, Enyedi P, and Brown EM (1989) The effects of high extracellular Ca^{2+} and Mg^{2+} concentrations on the levels of inositol 1,3,4,5-tetrakisphosphate in bovine parathyroid cells. *Endocrinology* 124: 838-844.

Henley C, Colloton M, Cattley RC, Shatzen E, Towler, DA, Lacey D, Martin D (2005) 1,25-Dihydroxyvitamin D3 but not cinacalcet HCl (Sensipar/Mimpara) treatment mediates aortic calcification in a rat model of secondary hyperparathyroidism. *Nephrol Dial Transplant* 20: 1370-1377.

Kifor O, Diaz R, Butters R, and Brown EM (1997) The Ca^{2+}-sensing receptor (CaR) activates phospholipases C, A2, and D in bovine parathyroid and CaR-transfected, human embryonic kidney (HEK293) cells. *J Bone Miner Re.* 12: 715-725.

Leach KL, Sexton PM, and Christopoulos A (2007) Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends in Pharm Sci* 28: 382-389.

Leong, SS, Horoszewicz, JS, Shimaoka, K, Friedman, M, Kawinski, E, Song, MJ, Zeigel, R Chu,TM Baylin S, Mirand, EA (1981) A new cell line for study of human medullary thyroid carcinoma, *Adv in Thyroid Neoplasia*. Rome: Field Education 95–108.

Levin A, Bakris GL, Molitch M, Smulders M, Tian J, Williams LA, Andress DL (2007) Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: Results of the study to evaluate early kidney disease. *Kidney Int* 71: 31-38.

Lu, J.Y., Yang, Y., Gnacadja, G., Christopoulos, A., Reagan, J.D. (2009) Effect of the Calcimimetic R-568 [3-(2-Chlorophenyl)-N-((1R)-1-(3-methoxyphenyl)ethyl)-1-propanamine] on Correcting Inactivating Mutations in the Human Calcium Sensing Receptor. *J. Pharmacol. Exp. Ther*. 331, 775-786.

Makita, N, Sato, J, Manaka, K, Shoji, Y, Oishi, A, Hashimoto, M, Fujita, T, Iiri, T (2007) An acquired hypocalciuric hypercalcemia autoantibody induces allosteric transition among active human Ca-sensing receptor conformations, *Proc Natl Acad Sci* 104 (13): 5443-5448.

Nemeth EF and Fox J (1999) Calcimimetic compounds: A direct approach to controlling plasma levels of parathyroid hormone in hyperparathyroidism. *Trends in Endocrinology & Metabolism* 10:66-71.

Nemeth EF, Heaton WH, Miller M, Fox J, Balandrin MF, Van Wagenen BC, Colloton M, Karbon W, Scherrer J, Shatzen E, Rishton G, Scully S, Qi M, HarrisR, Lacey D, Martin D (2004) Pharmacodynamics of the type II calcimimetic compound cinacalcet HCl. *J Pharmacol Exp Ther* 308: 627-635.

Petrel C, Kessler A, Duaban P, Dodd RH, Rognan D, Ruat M (2004) Positive and negative allosteric modulators of the Ca^{2+}-sensing receptor interact within overlapping but not identical binding sites in the transmembrane domain. *J Biol Chem* 279: 18990-18997.

Richman JG, Kanemitsu-Parks M, Gaidarov I, Cameron JS, Griffin P, Zheng H, Guerra NC, Cham L, Maciejewski-Lenoir D, Behan DP, Boatman D, Chen R, Skinner P, Ornelas P, Waters MG, Wright SD, Semple G, Connolly DT (2007) Nicotinic acid receptor agonists differentially activate downstream effectors *J Biol Chem* 282: 18028-18036.

Sørensen OH, Hellensen C, Hindberg I (1970) The effect of diet and age on the response to calcitonin in rats. *Acta Endocrinol* 65: 316-322.

Timmers HJ, Karperien M, Hamdy NA, and de Boer H, Hermus AR (2006) Normalization of serum calcium by cinacalcet in a patient with hypercalcaemia due to a *de novo* inactivating mutation of the calcium-sensing receptor. *J Intern Med* 260: 177-182.

Wilcox, JS. (1993) Fundamental principles of in situ hybridization. *J. Histochem. Cytochem.* 41: 1721-1723.

Footnotes

This research was wholly funded by Amgen, Inc.

Legends for Figures

Figure 1

Dose response of Calcimimetic B and R-568 on HEK293 cells stably expressing hCaSR in the presence of 1 mM $CaCl_2$ (A-C) or 0 mM $CaCl_2$ (D). Calcium flux was measured using FLIPR (A) or aequorin (B). Inositol phosphate accumulation was measured by preloading the cells with tritiated inositol (C,D) as described in the Methods. Figure 2D shows a typical dose response curve of $CaCl_2$ as compared to R-568 and Calcimimetic B in Ca^{2+} free buffer. Curves were fitted to the four parameter logistic equation. Results from a representative experiment performed in duplicate (inositol phosphate, FLIPR assays) or triplicate (aequorin assay) are shown. Error bars represent standard error of the mean. Similar results were obtained from at least four additional experiments.

Figure 2

hCaSR mediated ERK phosphorylation. Dose response curves of R-568 (A, B) and Calcimimetic B (C, D) on HEK293 cells stably expressing hCaSR in the presence or absence of 1 mM $CaCl_2$. Cells were treated with the indicated concentrations of compound and samples were analyzed for ERK phosphorylation by Western Blot as described in the Methods. Results from a representative experiment performed in duplicate are shown. Similar results were obtained from at least two additional experiments. Error bars represent standard error of the mean.

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 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, α, 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 β 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 while $\beta<1$ results in reduced responsiveness. τ_A and τ_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,C, D) Simulations of the effect of α, β and τ_B on the orthosteric agonist dose response curve. $\tau_A = 63$ (full agonist) in all three simulations.

Figure 4

Effect of Calcimimetic B and R-568 on the $CaCl_2$ dose response curve of 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 and data was globally fit to Equation 1 as described in Methods. Similar results were obtained from at least two additional experiments. Results from a representative experiment performed in duplicate are shown. Error bars represent standard error of the mean.

Figure 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 upon ionized Ca^{2+} lowering compared to 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 draw at the indicated times. Levels of Ca^{2+}, iPTH and calcitonin were measured as described in the Methods. Results are from a representative experiment repeated at least 3 times. Error bars represent standard error of the mean. (D) In situ hybridization (right panel) with rat CaSR antisense probe showing strong expression of CaSR mRNA in the parathyroid tissue relative to the surrounding thyroid tissue. H & E stain is show on the left panel (4X magnification).

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The membrane was probed with an hCaSR antibody and developed with a chemiluminescence reagent as previously described (Lu, et al. 2009) . Molecular weight of the protein markers is measured in kilodaltons. (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 buffer without Ca^{2+} and serum starved for 2 h. Cells were then treated with 1mM $CaCl_2$ or 1mM $CaCl_2$ plus increasing concentrations of calcimimetic for 12h. The concentration of calcitonin in the culture medium was determined by ELISA. Error bars represent standard error of the mean. Results are from a representative experiment repeated two additional times.

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1

	Log EC_{50}	
	R-568	Calcimimetic B
Ca^{2+} Flux (FLIPR)	-7.2 ± 0.14 (n = 9)	-8.1* ± 0.35 (n = 37)
Ca^{2+} Flux (Aequorin)	-6.4 ± 0.10 (n = 3)	-7.8* ± 0.14 (n = 9)
I.P. Accumulation	-6.4 ± 0.13 (n = 33)	-7.7* ± 0.15 (n = 32)

* significantly different compared to R-568 $P < 0.001$ (t-test)

Table Legend 1

Potency values ($logEC_{50}$) for R-568 and Calcimimetic B to the hCaSR in FLIPR, aequorin and inositol phosphate accumulation assays. Parameter values were obtained by nonlinear regression analysis using the variable slope Hill equation as described in Methods. Values represent the mean ± standard deviation.

JPET Fast Forward. Published on March 21, 2011 as DOI: 10.1124/jpet.110.178681
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2

Inositol Phosphate Accumulation Assay

Compound	Log EC_{50}	Log K_B	Log τ_B	Log $\alpha\beta$	$\alpha\beta$
R-568	-2.6 ± 0.047	-6.2 ± 0.12	-0.073 ± 0.062	0.58 ± 0.20	3.8 (2.4-5.9)
Calc. B	-2.7 ± 0.021	-6.8* ± 0.082	0.086* ± 0.039	0.82 ± 0.17	6.6 (4.5-9.8)

Results of 3 to 5 experiments
* significantly different compared to R-568, $P \leq 0.05$ (t-test)

Table Legend 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. Log K_B is the logarithm of the equilibrium dissociation of calcimimetic at the free receptor, log EC_{50} is the logarithm of the potency of extracellular Ca^{2+} in absence of calcimimetic, log τ_B is the logarithm of the efficacy of calcimimetic at the free receptor, log $\alpha\beta$ is the logarithm of the composite cooperativity factor and $\alpha\beta$ is the antilogarithm (mean) of the composite cooperativity factor with the range in parentheses based on estimated log $\alpha\beta$ values. Parameter values were obtained using equation 1 as described in Methods. Values represent the mean ± standard deviation of 3 to 4 experiments.

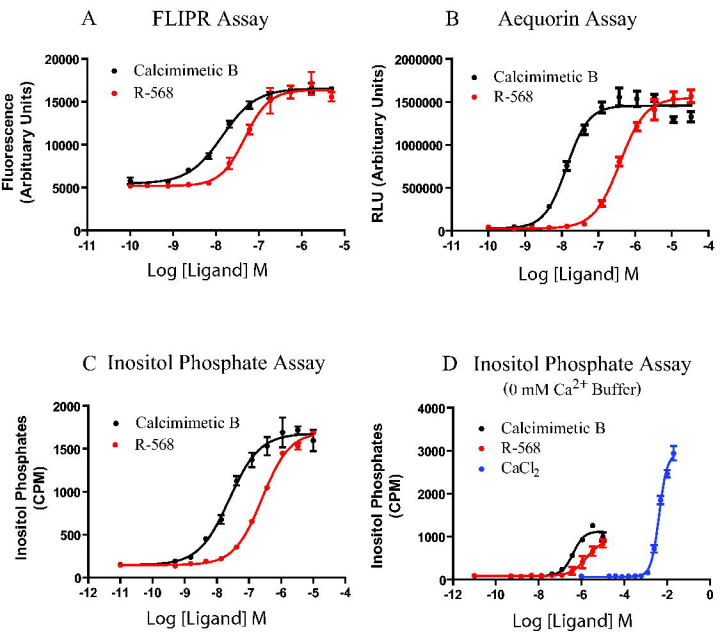

A FLIPR Assay
Calcimimetic B
R-568
Fluorescence (Arbituary Units)
20000
15000
10000
5000
0
-11 -10 -9 -8 -7 -6 -5
Log [Ligand] M
B Aequorin Assay
Calcimimetic B
R-568
RLU (Arbituary Units)
2000000
1500000
1000000
500000
0
-11 -10 -9 -8 -7 -6 -5 -4
Log [Ligand] M
C Inositol Phosphate Assay
Calcimimetic B
R-568
Inositol Phosphates (CPM)
2000
1500
1000
500
0
-12 -11 -10 -9 -8 -7 -6 -5 -4
Log [Ligand] M
D Inositol Phosphate Assay
(0 mM Ca2+ Buffer)
Calcimimetic B
R-568
CaCl2
Inositol Phosphates (CPM)
4000
3000
2000
1000
0
-12 -10 -8 -6 -4 -2 0
Log [Ligand] M

Figure 1

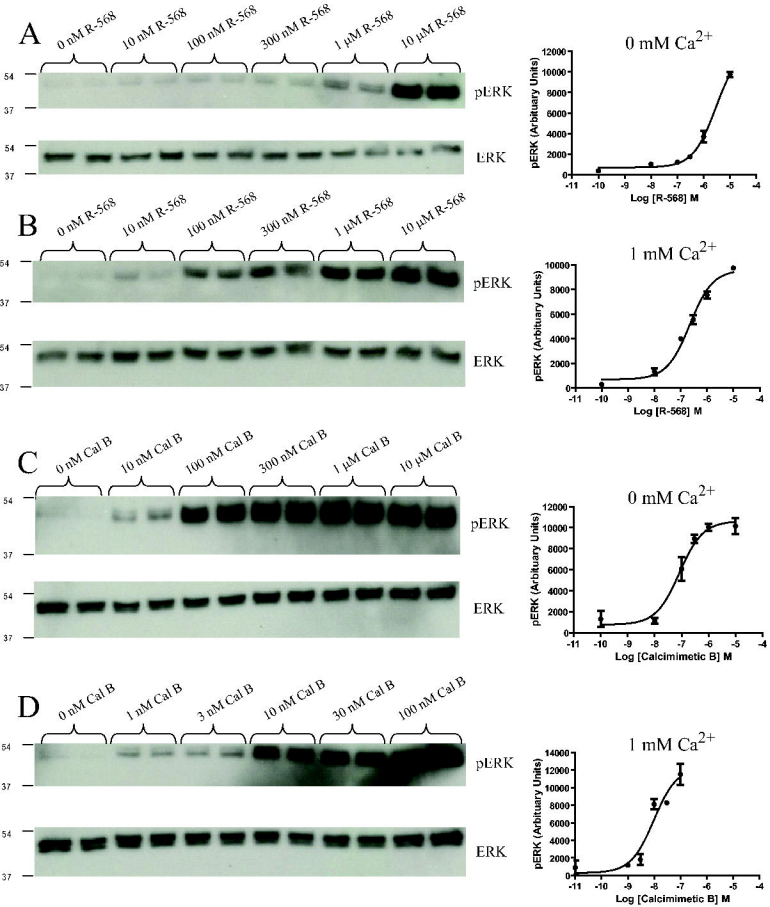
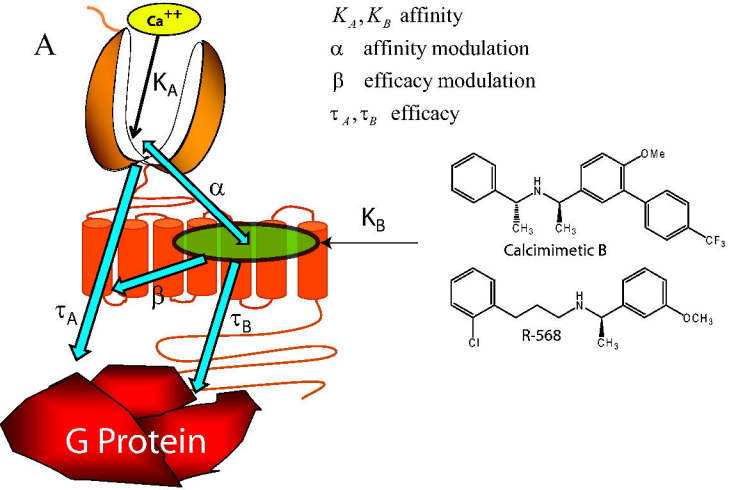
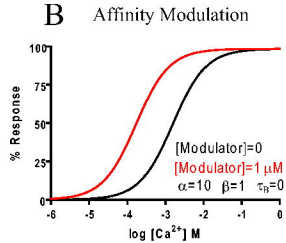
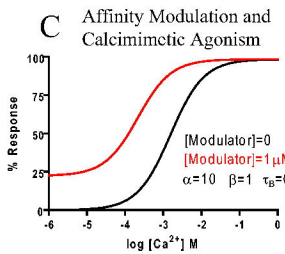

A
0 nM R-568
10 nM R-568
100 nM R-568
300 nM R-568
1 µM R-568
10 µM R-568
54
37
pERK
ERK
0 mM Ca2+
pERK (Arbitrary Units)
Log [R-568] M
B
1 mM Ca2+
C
0 nM Cal B
10 nM Cal B
100 nM Cal B
300 nM Cal B
1 µM Cal B
10 µM Cal B
Log [Calcimimetic B] M
D
1 nM Cal B
3 nM Cal B
30 nM Cal B
100 nM Cal B

Figure 2


A
Ca++
K_A
α
K_B
β
τ_A
τ_B
G Protein
K_A, K_B affinity
α affinity modulation
β efficacy modulation
τ_A, τ_B efficacy
OMe
CF3
Calcimimetic B
Cl
OCH3
R-568


B
Affinity Modulation
% Response
log [Ca2+] M
[Modulator]=0
[Modulator]=1 μM
α=10 β=1 τB=0


C
Affinity Modulation and
Calcimimetic Agonism
% Response
log [Ca2+] M
[Modulator]=0
[Modulator]=1 μM
α=10 β=1 τB=0.3

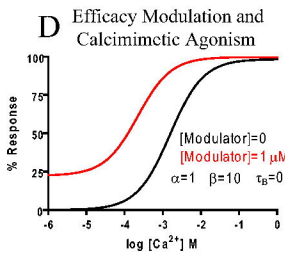
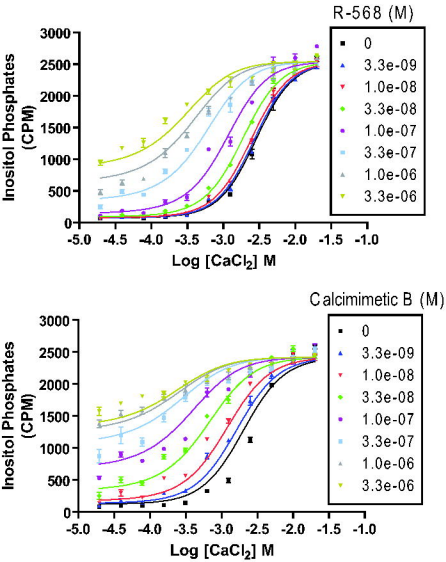

D
Efficacy Modulation and
Calcimimetic Agonism
% Response
log [Ca2+] M
[Modulator]=0
[Modulator]=1 μM
α=1 β=10 τB=0.3

Figure 3


R-568 (M)
0
3.3e-09
1.0e-08
3.3e-08
1.0e-07
3.3e-07
1.0e-06
3.3e-06
Inositol Phosphates (CPM)
3000
2500
2000
1500
1000
500
0
-5.0 -4.5 -4.0 -3.5 -3.0 -2.5 -2.0 -1.5 -1.0
Log [CaCl2] M

Calcimimetic B (M)
0
3.3e-09
1.0e-08
3.3e-08
1.0e-07
3.3e-07
1.0e-06
3.3e-06
Inositol Phosphates (CPM)
3000
2500
2000
1500
1000
500
0
-5.0 -4.5 -4.0 -3.5 -3.0 -2.5 -2.0 -1.5 -1.0
Log [CaCl2] M

Figure 4

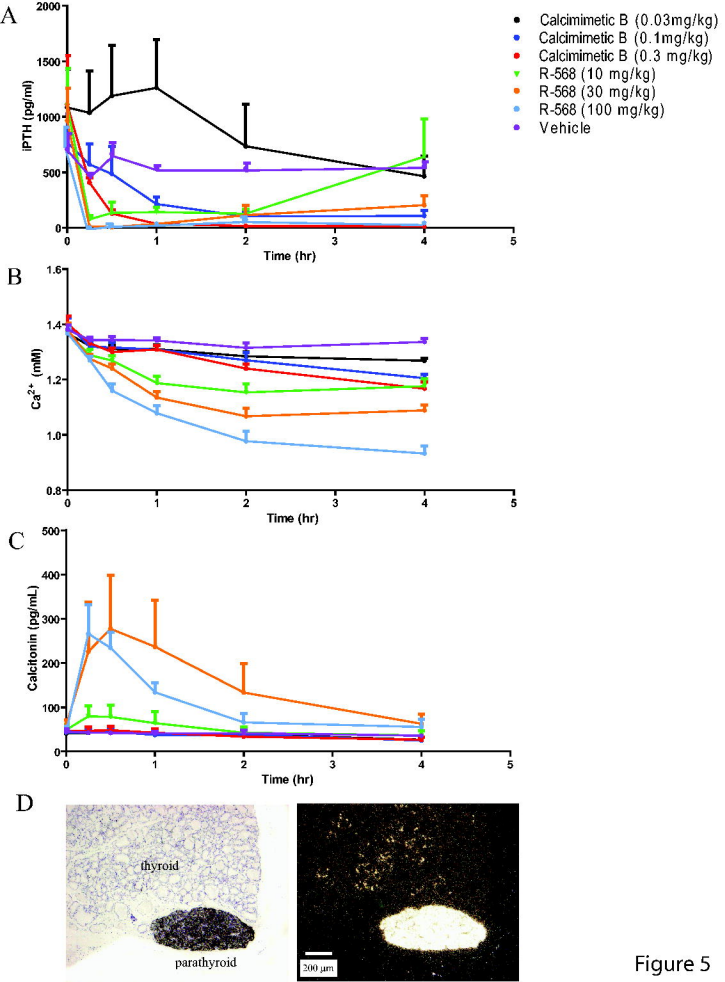
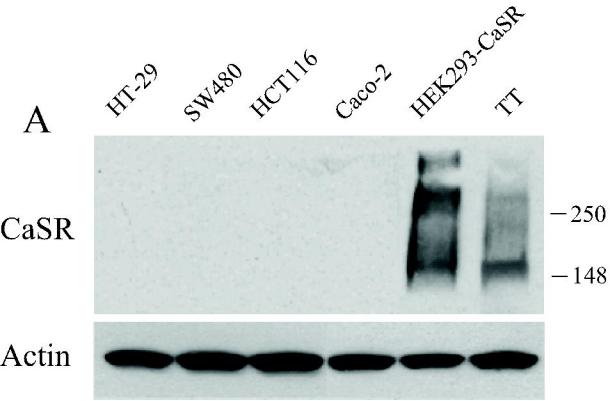

A
iPTH (pg/ml)
2000
1500
1000
500
0
0
1
2
3
4
5
Time (hr)
Calcimimetic B (0.03mg/kg)
Calcimimetic B (0.1mg/kg)
Calcimimetic B (0.3 mg/kg)
R-568 (10 mg/kg)
R-568 (30 mg/kg)
R-568 (100 mg/kg)
Vehicle
B
Ca2+ (mM)
1.6
1.4
1.2
1.0
0.8
0
1
2
3
4
5
Time (hr)
C
Calcitonin (pg/mL)
500
400
300
200
100
0
0
1
2
3
4
5
Time (hr)
D
thyroid
parathyroid
200 µm

Figure 5


A
HT-29
SW480
HCT116
Caco-2
HEK293-CaSR
TT
CaSR
250
148
Actin

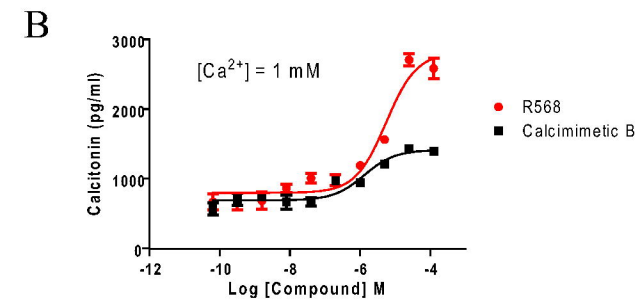

B
[Ca2+] = 1 mM
Calcitonin (pg/ml)
3000
2000
1000
0
-12
-10
-8
-6
-4
Log [Compound] M
R568
Calcimimetic B

Figure 6