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Characterization of Highly Efficacious Allosteric Agonists of the Human Calcium-Sensing Receptor

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Running Title

Novel calcium-sensing receptor agonists

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CaSR: calcium-sensing receptor

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Abstract

We discovered structurally novel human calcium-sensing receptor (CaSR) allosteric agonists, and compared their pharmacology to phenylalkylamine calcimimetics. AC-265347 activated CaSR signaling in cellular proliferation and phosphatidyl inositol (PI) hydrolysis assays with potencies of 30 and 10 nM, respectively. (S)-AC-265347, the (S) enantiomer of AC-265347, was approximately 10 to 20-fold more potent than (R)-AC-265347. The phenylalkylamines cinacalcet and calindol had similar activity to AC-265347 in cellular proliferation assays, but less activity in PI assays. All compounds had reduced activity when extracellular Ca^{2+} was removed indicating they cooperate with Ca^{2+} to activate CaSRs, and all activated CaSR isoforms with the N-terminal extracellular domain deleted, indicating they interact with the transmembrane (TM) domains. In both cases AC-265347 and therefore (S)-AC-265347 were significantly more efficacious than the phenylalkylamines. Mutations E837A$^{7.39}$ and I841A$^{7.43}$ strongly reduced phenylalkylamine-induced signaling, but not AC-265347 or (S)-AC-265347 induced signaling, suggesting different modes of binding. AC-265347 and (S)-AC-265347 stimulated significantly greater responses than cinacalcet or calindol at each of four loss-of-function human polymorphic CaSR variants. AC-265347 did not inhibit the CYP2D6 cytochrome p450 isozyme, unlike cinacalcet which is a potent CYP2D6 inhibitor. In rats, AC-265347, (S)-AC-265347 and (R)-AC-265347 each reduced serum parathyroid hormone (PTH) with a rank order potency correlated to their in vitro potencies. AC-265347 and (S)-AC-265347 also reduced plasma ionizable calcium ($[Ca^{2+}]_o$). AC-265347 was orally active, and its plasma concentrations correlated well with its effects on

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serum PTH. Thus these highly efficacious CaSR allosteric agonists represent leads for developing therapeutic agents with potential advantages over existing therapies.

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Introduction

Extracellular calcium ($[Ca^{2+}]_o$) is able to function as a 'first' messenger, affecting a wide array of cellular processes, and therefore blood levels of $[Ca^{2+}]_o$ are subject to extremely tight control (Brown, 1991). This regulation is mediated primarily by a calcium-sensing receptor (CaSR), first cloned from bovine parathyroid cells (Brown et al, 1993). The CaSR is a member of the G-protein coupled receptor (GPCR) superfamily and belongs to the Group C family of GPCRs which also includes the GABA (B) receptor and the metabotropic glutamate receptors. These receptors contain a large N-terminal extracellular domain that binds ligand, a seven transmembrane spanning region, and a C-terminal domain that transduces intracellular signals (Brown and MacLeod, 2001). Besides extracellular calcium, CaSR activity is modulated by other divalent and trivalent cations like Mg^{2+}, Cd^{2+}, Ba^{2+}, La^{3+}, and Gd^{3+}, amino acids (especially aromatic amino acids), spermine, amyloid beta-peptides, and ionic strength (Chang and Shoback, 2004).

The CaSR primarily functions to maintain systemic $[Ca^{2+}]_o$ homeostasis mainly by suppressing parathyroid hormone (PTH) secretion by the parathyroid glands, and by influencing rates of renal tubular calcium reabsorption and secretion of calcitonin by C-cells of the thyroid (Hauache, 2001). Increases in $[Ca^{2+}]_o$ also affect secretion of many other hormones including adrenocorticotropic hormone (ACTH), gastrin, insulin, growth hormone, and PTH-related peptide (PTHrP), though in some cases these changes may occur through Ca^{2+} ion channels rather than the CaSR itself (Brown and MacLeod, 2001).

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Consistent with its main physiological function, the CaSR receptor is highly expressed in tissues involved in mineral ion homeostasis including the parathyroid, thyroidal C cells, kidney, bone (including osteoclasts, osteoblasts and osteocytes), chondrocytes (cartilage forming cells), intestine (including duodenum and ileum), and placenta. However the CaSR is also found in organs not involved in maintaining $[Ca^{2+}]_o$ homeostasis such as brain with highest levels in subfornical organ (hypothalamic thirst center); neurons, astrocytes, and microglia; in pituitary gland; bone marrow and peripheral blood (including platelets and monocytes); keratinocytes; the gastrointestinal system including the esophagus, stomach, small intestine and colon; and in the pancreas where it may affect insulin and glucagon secretion (Brown and MacLeod, 2001). Thus, besides its well documented role in maintaining $[Ca^{2+}]_o$ homeostasis, the CaSR may also function as a nutrient sensor, an osmolarity regulator, and a regulator of hormone secretion, cellular chemotaxis, proliferation, differentiation, apoptosis and gene expression.

The physiological roles of the CaSR have been further validated through correlating human CaSR polymorphisms with diseases of $[Ca^{2+}]_o$ homeostasis. Inactivating mutations in the human CaSR lead to familial hypocalciuric hypercalcemia (FHH or familial benign hypercalcemia (FBH)), and neonatal severe hyperparathyroidism (NSHPT) while activating mutations in the human CaSR cause autosomal dominant hypocalcemia with hypercalciuria (ADHH) and Bartter syndrome (Thakker, 2004). Auto-immune antibodies to the CaSR cause autoimmune hypocalciuric hypercalcaemia (AHH) and acquired hypoparathyroidism (AH) (Thakker, 2004).

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Small molecules that modulate the sensitivity of CaSR to Ca^{2+} have been described including calcimimetics which allosterically increase the sensitivity and responsiveness of CaSR to $[Ca^{2+}]_o$ (Hammerland et al, 1998; Goodman, 2005; Nemeth et al, 1998; Dauban et al, 2000; Kessler et al, 2004), and calcilytics which allosterically decrease the sensitivity and responsiveness of CaSR to $[Ca^{2+}]_o$ (Nemeth et al, 2001; Kessler et al, 2006; Arey et al, 2005). One calcimimetic called cinacalcet (marketed as Sensipar® in the United States, Mimpara® in Europe) is approved for the clinical treatment of secondary hyperparathyroidism (SHPT) and for the treatment of parathyroid carcinoma (Dong, 2005). SHPT occurs in patients suffering from chronic kidney disease and end-stage renal disease, and is characterized by elevated serum levels of PTH and disturbances in calcium and phosphorus metabolism. By activating the CaSR, cinacalcet lowers serum PTH and normalizes calcium and phosphorus metabolism.

We have identified a structurally novel benzothiazole class of human CaSR allosteric agonists and compared their pharmacology to the phenylalkylamine calcimetics cinacalcet and calindol in a variety of *in vitro* and *in vivo* functional assays. The structural differences of these benzothiazoles from existing calcimimetics translated into greater activity at CaSR's carrying a variety of artificial and naturally occurring loss-of-function mutations. Thus these novel CaSR allosteric agonists represent important new leads for the development of drugs with potential advantages over existing therapies.

Methods

Ligands – $MgCl_2$ and $CaCl_2$ were from Sigma (Louisville, KY). Pharmacy grade Cincancalet hydrochloride (Sensipar®) tablets were dissolved into DMSO stock solutions immediately prior to use. Calindol hydrochloride was from Toronto Research Chemicals (North York, Ontario). AC-265347 (1-Benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol) and its resolved enantiomers (S)-AC-265347 ((S)- 1-Benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol) and (R)-AC-265347 ((R)- 1-Benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol) were synthesized at ACADIA Pharmaceuticals (see Gustafsson et al, 2010). Compound structure was verified by NMR. Purity was greater than 99% measured by HPLC and gas chromatography.

Cell culture – NIH-3T3 cells (ATCC (Manassas, VA) CRL 1658) were incubated at 37 °C in a humidified atmosphere (5% CO2) in Dulbecco's modified Eagles medium (DMEM) (Invitrogen-Gibco, Carlsbad, CA) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 U per ml penicillin G, 50 U per ml streptomycin (Invitrogen-Gibco, Carlsbad, CA) and 10% calf serum (Sigma, St Louis, MO) or 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD). HEK293T (ATCC (Manassas, VA) CRL-11268) cells were cultured similarly except 10% fetal calf serum was substituted for 10% calf serum.

Constructs. The human parathyroid cell calcium-sensing receptor (CaSR) receptor used in this study was cloned from a human kidney cDNA library by polymerase chain reaction using oligonucleotides derived from the GenBank accession entry U20759, and

subcloned into pSI and pCI (Promega Corp.) expression vectors. PCRs were performed using Pfu Turbo (Stratagene, La Jolla, CA). The N-terminal truncated CaSR was constructed by amplifying the C-terminal 479 amino acids of the wild-type CaSR with a consensus kozac sequence and an initiating methionine incorporated in frame, and subcloning the truncated receptor into the expression vectors. All point mutations described were generated using the Quickchange mutagenesis protocol (Stratagene). All clones were sequence verified.

Cellular proliferation assays - Receptor Selection and Amplification Technology (R-SAT®) assays were performed as described (Burstein et al, 2006; Gardell et al, 2008) with the following modifications. Briefly: Cells were plated one day before transfection using 7 x 10^3 cells in 0.1 ml of media per well of a 96-well plate (Falcon). Cells were transiently transfected with 10 ng of receptor DNA and 30 ng pSI-β-galactosidase (Promega, Madison, WI) per well of a 96-well plate using Polyfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. One day after transfection medium was changed and cells were combined with ligands in DMEM supplemented with 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD) instead of calf serum to a final volume of 200 µl per well. After five days in culture β-galactosidase levels were measured essentially as described (Burstein et al, 2006; Gardell et al, 2008). Cells were rinsed with phosphate buffered saline (PBS), pH = 7.4 before the addition of 200 µl PBS supplemented with 3.5 mM O-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma, St Louis, MO). After incubation (2-4 h) the plates were read at 420 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices).

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Phosphatidyl Inositol (PI) hydrolysis assays – PI hydrolysis assays were performed using diluted SPA beads (80 µl; GE Healthcare, Chalfont St. Giles, UK), followed by 30 µl of each cell lysate per well of PerkinElmer Life and Analytical Sciences Pico plates as described previously (Gardell et al., 2008). The 'high Ca^{2+}' buffer was identical to the buffer used for the cellular proliferation assays with the addition of 10 mM LiCl as described previously. The 'no Ca^{2+}' buffer was composed of Hank's Buffered Salt Solution (HBSS, HyClone, Logan, UT) plus 0.5 mM $MgCl_2$, 0.2% BSA and 10 mM LiCl, and did not contain antibiotics.

Data analysis – Concentration-response graphs for all functional assays were plotted and EC50 values were determined by nonlinear regression analysis using Prism software (GraphPad version 4.0, San Diego, CA, USA) according to the following equation:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*Hillslope))

where X is the logarithm of concentration. Y is the response; Y starts at Bottom and goes to Top with a sigmoid shape.

PTH releasing assays – Male Sprague-Dawley rats (~150 - 200g) were housed with free access to rat chow/water at 2 animals per cage for at least 2 days prior to use. Drugs were dissolved in 5% DMSO/5% Water/90% PEG400 and and dosed either sub-

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cutaneously (s.c.) or orally (p.o.). The controls were given the same volume of vehicle as in test groups. Blood samples were obtained at the indicated times, the plasma fraction was separated and stored at -80 degrees C until further use. PTH levels were analyzed using a rat intact PTH RIA kit according to the manufacturer's instructions (Immutopics, San Clemente, CA).

Analysis of plasma phosphate and Ca^{2+} concentrations – Plasma phosphate levels were analyzed using a phosphate assay kit with an improved Malachite Green dye from BioAssay Systems (cat# DIPI-500, Hayward, CA) according to the manufacturers' instructions. Plasma levels of ionizable Ca^{2+} were determined using an ion-selective electrode blood-gas analyzer (IDEXX, West Sacremento, CA).

Analysis of AC-265347 concentrations – Plasma levels of AC-265347 were measured in rats dosed orally with 10 or 30 mg/kg. Plasma samples were collected at 0, 0.5, 1, 2, 4, 6 and 24 hr, and analyzed by LC/MS/MS. The LC/MS/MS analysis was performed using a 4000 QTRAP (Applied Bioscience, Foster City, CA) hybrid triple quadrupole linear ion-trap mass spectrometer equipped with electrospray ionization (ESI) and operated in multiple reaction monitoring (MRM) mode. AC-265347 ion pair was 284.2 / 266. The mass spectrometer was coupled to a HPLC system consisting of two Shimadzu (Columbia, MD) LC-20AD high performance pumps interfaced with a Shimadzu CBM-20A controller and a CTC HTC PAL (Carrboro, NC) autosampler. Separation was performed using a 50 x 2.1 -mm Hypersil Gold aQ (Bellefonte, PA) reversed-phase C18 column equipped with a guard column. LC solvent A was water and

B was acetonitrile, each containing 1% formic acid. Data collection and processing were performed using Analyst software v 1.4.2.

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Results

Using a cellular proliferation assay (R-SAT®, see Burstein et al, 2006) we screened the human parathyroid calcium-sensing receptor against a diverse chemical library containing over 250,000 compounds and identified a number of active chemical classes of compounds. Based on structurally interesting features compared with known calcimimetics, chemical optimization of a benzothiazole class of compounds was undertaken. We pharmacologically characterized one compound from this chemical series called AC-265347 (compound 13 in Gustafsson et al, 2010), and its enantiomers (S)-AC-265347 and (R)-AC-265347 in greater detail, and compared them to the phenylalkylamine calcimimetics cinacalcet and calindol (see **Figure 1)**.

AC-265347 activated CaSR signaling in cellular proliferation and phosphatidyl inositol (PI) hydrolysis assays with potencies of 30 and 10 nM, respectively (**Figure 2, Table 1, Table 2**). The enantiomers of AC-265347 were each active, with (S)-AC-265347 about 10 to 20-fold more potent than (R)-AC-265347. The phenylalkylamine calcimimetics cinacalcet (marketed as Sensipar®, Nemeth et al, 2004) and calindol had similar activity to AC-265347 in cellular proliferation assays, but less activity in PI hydrolysis assays. Under assay conditions of high ambient Ca^{2+}, all of these compounds stimulated 85-100% of the maximal functional response to $MgCl_2$ in cellular proliferation assays, and 75-85% of the maximal functional response to $CaCl_2$ in PI hydrolysis assays. It was not possible to use $CaCl_2$ as a reference standard in cellular proliferation assays due to its cytotoxicity at high concentrations. No responses to AC-265347, cinacalcet, or $MgCl_2$ were observed in cells transfected only with reporter, confirming the specificity of the

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assay, and AC-265347 did not activate human GABAb receptors, or human type 1 parathyroid hormone (PTH1) receptors, confirming its specificity for the CaSR (data not shown). The phenylalkylamine calcimimetics were derived from fendiline, a voltage-gated Ca^{2+} channel blocker (Nemeth et al, 1998). As expected given its structural differences, AC-265347 displayed negligible binding to L-type voltage gated Ca^{2+} channels, nor did it inhibit the CYP2D6 cytochrome p450 isoform. In contrast, cinacalcet is a potent CYP2D6 inhibitor (IC_{50} = 87 nM, see Nakashima et al, 2007) and both cinacalcet and calidnol have been shown to block L-type Ca2+ channels (Thakore & Vanessa, 2010).

All of the calcimimetics described to date including cinacalcet allosterically increase the sensitivity and responsiveness of CaSR to $[Ca^{2+}]_o$ (Jensen and Brauner-Osborne, 2007). Therefore we retested all of these compounds in PI hydrolysis assays in the absence of extracellular Ca^{2+} (see **Figure 2E and 2F, Table 2**). The maximum responses and potencies of all of the compounds were greatly reduced in the absence of extracellular Ca^{2+} indicating they cooperate with Ca^{2+} to activate CaSRs. Under these conditions, significant differences in the efficacy of these compounds became apparent, with AC-265347 and (S)-AC-265347 stimulating significantly greater responses than cinacalcet, calindol or (R)-AC-265347. Besides these two buffer systems, we tested these ligands in PI assays in buffers lacking penicillin and streptomycin but containing a range of Ca^{2+} concentrations (0.5 mM to 2 mM) and observed that removal of these antibiotics did not alter the rank order activity of these compounds (data not shown).

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The natural ligands for Family C GPCRs bind to the large, extracellular N-terminal domain characteristic of this receptor family whereas drugs and other artificial compounds targeting Family C GPCRs interact primarily with the transmembrane helical domains of these receptors (Bräuner-Osborne et al, 2007). Therefore we tested all of the compounds on cells expressing a CaSR isoform with the N-terminal domain deleted (**Figure 3**, **Table 2**). All the compounds activated CaSR isoforms with the N-terminal extracellular domain deleted (deltaN), however AC-265347 and (S)-AC-265347 stimulated significantly greater responses than Cincalcet, calindol or (R)-AC-265347. As expected, deletion of the N-terminus of the CaSR abolished activation by $CaCl_2$. We retested all the compounds at deltaN CaSRs in the absence of extracellular Ca^{2+} and observed that they still retained agonist activity. Under these conditions, maximum responses to all compounds were further reduced; however the potencies were not significantly different to the potencies at deltaN CaSRs in the presence of extracellular Ca^{2+}. These results are in agreement with previous studies suggesting CaSRs may contain more than one Ca^{2+}-binding site (Ray et al, 2005). These results indicate both classes of compounds interact with the transmembrane (TM) domains of CaSRs, both have intrinsic agonist activity, but AC-265347 and (S)-AC-265347 have greater intrinsic activity than the phenylalkylamine calcimimetics.

A large number of mutations have been introduced into the human CaSR in order to define and differentiate the interactions of calcimimetics and calcilytics with CaSRs (Hu and Spiegel, 2007; Jensen and Brauner-Osborne, 2007). In particular, residues $E837^{7.39}$, and to a lesser extent $I841^{7.43}$ are thought to play crucial roles in mediating binding and

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activation of CaSRs by positive and negative modulators (Hu et al, 2002; 2005; 2006; Petrel et al, 2004). In agreement with previous studies, the activity of cinacalcet was strongly reduced at E837A$^{7.39}$, and its efficacy was significantly reduced at I841A$^{7.43}$ (**Figure 4**, **Table 1, Table 2**). Calindol activity was similarly affected. In contrast, the activities of AC-265437 and (S)-AC-265347 were affected to a much smaller degree at the E837A$^{7.39}$ mutant, and essentially unaffected at I841A$^{7.43}$. Very similar results were obtained in cellular proliferation and PI hydrolysis assays.

A large number of both activating and inactivating naturally occurring polymorphisms in the human CaSR have been described (Hu and Spiegel, 2007). We constructed four of these naturally occurring CaSR mutants (R66C, T138M, R185Q, and R795W) that have previously been shown to be functionally impaired (see Bai et al. 1996), and compared the abilities of the benzothiazols and phenylalkylamines to activate these receptors in cellular proliferation and PI hydrolysis assays (**Figure 5**, **Table 1, Table 2)**. In agreement with previous results, all four of these CaSR polymorphic variants displayed impaired responses to $MgCl_2$ or $CaCl_2$ compared to wild-type CaSR, with reduced potency, reduced maximum response, or both. AC-265347 and (S)-AC-265347 consistently stimulated significantly greater responses than cinacalcet or calindol at each of these four polymorphic variants.

CaSRs play a crucial role in regulating blood concentrations of PTH, $[Ca2+]_o$, phosphorus and calcitonin (Hauache, 2001; Brown and McCleod, 2001), and treatment with calcimimetics lowers serum levels of parathyroid hormone (PTH) and $[Ca^{2+}]_o$

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(Nemeth et al, 2004). Treatment of normal male Sprague-Dawley rats with AC-265347, (S)-AC-265347 and (R)-AC-265347 each reduced serum PTH (**Figure 6**). The approximate ED_{50} values for PTH suppression were 0.01 mg/kg for AC-265347 and (S)-AC-265347, and 0.1 mg/kg for (R)-AC-265347, a rank order potency correlated to their in vitro potencies. cinacalcet also suppressed serum PTH, with a similar maximal effect, and an ED_{50} of approximately 0.1 mg/kg. (S)-AC-265347 also suppressed serum ionizable calcium $[Ca^{2+}]_o$ in a dose-dependent manner, though it required much higher doses than it needed to suppress serum PTH (**Figure 7A**). A similar difference between the potency required to suppress serum PTH and that required to suppress $[Ca^{2+}]_o$ has been observed for phenylalkylamine calcimimetics such as cinacalcet (Fox et al, 1999; Nemeth et al, 2004). AC-265347 also produced a significant suppression of serum $[Ca^{2+}]_o$ (data not shown), however although rats treated with (R)-AC-265347 did have lowered serum $[Ca^{2+}]_o$, the trend was not statistically significant (**Figure 7B**).

Oral administration of AC-265347 rapidly suppressed serum PTH levels in rats, with the maximal suppression of plasma PTH occurring 30 minutes after drug administration (**Figure 8A**). Lower levels of plasma PTH were maintained for 6 hours at 30 mg/kg AC-265347. Plasma concentrations of AC-265347 after oral administration were determined over a 24 hour time course (**Figure 8B**). Peak plasma concentrations of AC-265347 were 67 and 311 ng/ml for the 10 mg/kg and 30 mg/kg groups, respectively, and occurred at 1 hour post-dose. These levels compare favorably with those reported previously for cinacalcet (73 and 124 ng/ml at 10 and 36 mg/kg, p.o., respectively, see Nemeth et al.,

2004). The plasma concentration-time curves of AC-265347 correlated very well with its observed effects on serum PTH levels.

Discussion

We have discovered a structurally novel class of benzothiazol CaSR allosteric agonists, and pharmacologically compared them with the phenylalkylamine class of calcimemetics cinacalcet and calindol. Compared to the phenylalkylamine calcimimetics, the benzothiazols showed greater potency and efficacy at wild-type CaSRs, and at a variety of artificial and naturally occurring mutant forms of CaSRs.

The novel CaSR allosteric agonists described herein displayed stereoselectivity in their actions on CaSRs, with the (S)-enantiomer approximately 10 to 20-fold more active than the (R)-enantiomer in both in vitro and in vivo functional assays. These novel compounds demonstrated potent activity lowering serum PTH and serum $[Ca^{2+}]_o$ *in vivo*, actions expected for calcimimetics. The in vivo actions of these compounds were well correlated with their plasma concentrations and with their in vitro potencies.

The benzothiazol CaSR allosteric agonists appear to interact with CaSRs differently than the phenylalkylamines calcimimetics. Although studies using CaSRs lacking the N-terminal extracellular domain indicated both classes of compounds interact with the transmembrane domain spanning regions of the CaSR (see **Figure 3**), studies using point mutations within transmembrane domain 7 clearly indicate they utilize different amino acid residues to bind to CaSRs. We observed that mutations E837A$^{7.39}$ and I841$^{7.43}$ strongly reduced phenylalkylamine-induced signaling, in agreement with previous studies on cinacalcet and other structurally related calcimimetics and calcilytics (Petrel et al,

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2004). In contrast, these mutations had little effect on AC-265347 or (S)-AC-265347 induced signaling (see **Figure 4**).

The different interactions of AC-265347 and its analogs may translate into certain clinical advantages over cinacalcet and other phenylalkylamine derivatives. The CaSR is highly polymorphic, and a large number of loss-of-function polymorphic variants have been described (see www.casrdb.mcgill.ca). We tested four loss-of-function CaSR polymorphic variants and found consistently that AC-265347 and especially (S)-AC-265347 were able to activate these receptors better than either cinacalcet or calindol (see **Figure 5** above). The specific polymorphic variants we tested are thought to be quite rare in normal healthy people, however each has been found in people with disorders in calcium management, specifically FHH and NSHPT, and are significantly associated with these diseases (Pollak et al, 1993; Chou et al, 1995). The improved activation of these loss-of-function polymorphic variants by the benzothiazol CaSR allosteric agonists could stem from their different modes of binding CaSRs, or could simply be due to the fact that they have higher intrinsic activity than the phenylalkylamines. These results suggest that AC-265347, or its analogs might provide greater efficacy than cinacalcet in patients harboring loss-of-function CaSR polymorphisms.

Recently several studies reported that the calcimimetic NPS R-568 improves the signal transduction characteristics of loss-of-function polymorphic variants of the human CaSR associated with human disease (Rus et al., 2008; White et al., 2009; Lu et al., 2009). NPS R-568 is structurally very similar to cinacalcet, and therefore one would expect

cinacalcet to have similar effects on those polymorphic receptors, and *vice versa*, one would expect NPS R-568 to have similar effects as cinacalcet and calindol on the polymorphic receptors studied here. The polymorphisms studied in those previous reports were different from the ones report in this study, and thus direct comparisons of this study to those studies are not possible. In addition, there are a large number of other polymorphic variants of the CaSR known to exist that were not tested in this study or any of the studies cited above (Pidasheva et al., 2004). It seems reasonable to speculate that benzothiazol CaSR allosteric agonists like AC-265347 may stimulate greater responses than cinacalcet at these other variant receptors too.

Cinacalcet is a potent inhibitor of the CYP2D6 cytochrome p450 isoform with a Ki of 87 nM (Nakanisha et al, 2007). A wide variety of drugs are metabolized by CYP2D6, and the potential for cinacalcet to cause significant drug-drug interactions has been documented (Harris et al, 2007). In contrast, we observed no significant interaction of AC-265347 with CYP2D6 at concentrations up to 100 uM. Thus drug-drug interactions should be less of a concern with AC-265347 or its analogs.

In conclusion, the data presented herein suggest AC-265347 and structural analogs of AC-265347 have the potential to be developed into effective calcimimetics, and may provide therapeutic advantages over cinacalcet in certain patient populations.

Authorship Contributions

Participated in research design: Burstein, Ma, Olsson, and Tabatabaei

Conducted experiments: Ma, Schmeltzer, Owens

Contributed new reagents or analytic tools: Gustafsson, Jensen

Performed data analysis: Burstein, Ma, and Owens

Wrote or contributed to the writing of the manuscript: Burstein

References

Arey BJ, Seethala R, Ma Z, Fura A, Morin J, Swartz J, Vyas V, Yang W, Dickson JK Jr, Feyen JH (2005) A novel calcium-sensing receptor antagonist transiently stimulates parathyroid hormone secretion in vivo. *Endocrinology*. **146**:2015-2022.

Bai M, Quinn S, Trivedi S, Kifor O, Pearce SH, Pollak MR, Krapcho K, Hebert SC, Brown EM (1996) Expression and characterization of inactivating and activating mutations in the human Ca2+o-sensing receptor. *J Biol Chem*. **271**:19537-19545.

Baubet V, Le Mouellic H, Campbell AK, Lucas-Meunier E, Fossier P, Brulet P (2000) Chimeric green fluorescent protein-aequorin as bioluminescent Ca2+ reporters at the single-cell level. *Proc Natl Acad Sci U S A*. **97**:7260-7265.

Bräuner-Osborne H, Wellendorph P, Jensen AA (2007) Structure, pharmacology and therapeutic prospects of family C G-protein coupled receptors. *Curr Drug Targets*. **8**:169-184.

Brown EM (1991) Extracellular Ca2+ sensing, regulation of parathyroid cell function, and role of Ca2+ and other ions as extracellular (first) messengers. *Physiol Rev*. **71**:371-411.

Brown EM, MacLeod RJ (2001) Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev*. **81**:239-297.

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

Burstein ES, Piu F, Ma JN, Weissman JT, Currier EA, Nash NR, Weiner DM, Spalding TA, Schiffer HH, Del Tredici AL, Brann MR (2006) Integrative functional assays, chemical genomics and high throughput screening: harnessing signal transduction pathways to a common HTS readout. Curr Pharm Des. 12:1717-1729.

Chang W, Shoback D (2004) Extracellular Ca2+-sensing receptors-an overview. *Cell Calcium*. **35**:183-196.

Chou YH, Pollak MR, Brandi ML, Toss G, Arnqvist H, Atkinson AB, Papapoulos SE, Marx S, Brown EM, Seidman JG, Seidman CE (1995) Mutations in the human Ca(2+)-sensing-receptor gene that cause familial hypocalciuric hypercalcemia. *Am J Hum Genet*. **56**:1075-1079.

Dauban P, Ferry S, Faure H, Ruat M, Dodd RH (2000) N1-Arylsulfonyl-N2-(1-aryl)ethyl-3-phenylpropane-1,2-diamines as novel calcimimetics acting on the calcium sensing receptor. *Bioorg Med Chem Lett*. **10**:2001-2004.

Dong BJ (2005) Cinacalcet: An oral calcimimetic agent for the management of hyperparathyroidism. *Clin Ther*. **27**:1725-1751.

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

Gardell LR, Ma JN, Seitzberg JG, Knapp AE, Schiffer HH, Tabatabaei A, Davis CN, Owens M, Clemons B, Wong KK, Lund B, Nash NR, Gao Y, Lameh J, Schmelzer K, Olsson R, Burstein ES (2008) Identification and characterization of novel small-molecule protease-activated receptor 2 agonists. J Pharmacol Exp Ther. 327:799-808.

Garrett JE, Capuano IV, Hammerland LG, Hung BC, Brown EM, Hebert SC, Nemeth EF, Fuller F (1995) Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *J Biol Chem*. **270**:12919-12925.

Goodman WG (2005) Calcimimetics: a remedy for all problems of excess parathyroid hormone activity in chronic kidney disease? *Curr Opin Nephrol Hypertens*. **14**:355-360.

Gustafsson M, Jensen J, Bertozzi SM, Currier EA, Ma J, Burstein ES, Olsson R (2010) Discovery of 1-(benzothiazol-2-yl)-1-phenylethanols, a Novel Class of

Calcium Sensing Receptor Positive Allosteric Modulators. Bioorg Med Chem Lett. **20**:5918-5921.

Hammerland LG, Garrett JE, Hung BC, Levinthal C, Nemeth EF (1998) Allosteric activation of the Ca2+ receptor expressed in Xenopus laevis oocytes by NPS 467 or NPS 568. *Mol Pharmacol*. **53**:1083-1088.

Hauache OM (2001) Extracellular calcium-sensing receptor: structural and functional features and association with diseases. *Braz J Med Biol Res*. **34**:577-584.

Harris RZ, Salfi M, Posvar E, Hoelscher D, Padhi D (2007) Pharmacokinetics of desipramine HCl when administered with cinacalcet HCl. Eur J Clin Pharmacol. **63**:159-163.

Hu J, Reyes-Cruz G, Chen W, Jacobson KA, Spiegel AM (2002) Identification of acidic residues in the extracellular loops of the seven-transmembrane domain of the human Ca2+ receptor critical for response to Ca2+ and a positive allosteric modulator. *J Biol Chem.* **277**:46622-46631.

Hu J, McLarnon SJ, Mora S, Jiang J, Thomas C, Jacobson KA, Spiegel AM (2005) A region in the seven-transmembrane domain of the human Ca2+ receptor critical for response to Ca2+. *J Biol Chem*. **280**:5113-5120.

Hu J, Jiang J, Costanzi S, Thomas C, Yang W, Feyen JH, Jacobson KA, Spiegel AM (2006) A missense mutation in the seven-transmembrane domain of the human Ca2+ receptor converts a negative allosteric modulator into a positive allosteric modulator. *J Biol Chem*. **281**:21558-21565.

Hu J, Spiegel AM (2007) Structure and function of the human calcium-sensing receptor: insights from natural and engineered mutations and allosteric modulators. *J Cell Mol Med*. **11**:908-922.

Jensen AA and Brauner-Osborne H (2007) Allosteric modulation of calcium-sensing receptor. *Curr. Neuropharmacol*. **5**:180-186.

Kessler A, Faure H, Petrel C, Ruat M, Dauban P, Dodd RH (2004) N2-benzyl-N1-(1-(1-naphthyl)ethyl)-3-phenylpropane-1,2-diamines and conformationally restrained indole analogues: development of calindol as a new calcimimetic acting at the calcium sensing receptor. *Bioorg Med Chem Lett*. **14**:3345-3349.

Kessler A, Faure H, Petrel C, Rognan D, Césario M, Ruat M, Dauban P, Dodd RH (2006) N1-Benzoyl-N2-[1-(1-naphthyl)ethyl]-trans-1,2-diaminocyclohexanes: Development of 4-chlorophenylCaSRboxamide (calhex 231) as a new calcium sensing receptor ligand demonstrating potent calcilytic activity. *J Med Chem*. **49**:5119-5128.

Lu JY, Yang Y, Gnacadja G, Christopoulos A, Reagan JD (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.

Nakashima D, Takama H, Ogasawara Y, Kawakami T, Nishitoba T, Hoshi S, Uchida E, Tanaka H (2007) Effect of cinacalcet hydrochloride, a new calcimimetic agent, on the pharmacokinetics of dextromethorphan: in vitro and clinical studies. J Clin Pharmacol. **47**:1311-1319.

Nemeth EF, Steffey ME, Hammerland LG, Hung BC, Van Wagenen BC, DelMar EG, Balandrin MF (1998) Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proc Natl Acad Sci U S A.* **95**:4040-4045.

Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, Conklin RL, Gowen M, Gleason JG, Bhatnagar PK, Fox J (2001) Calcilytic compounds: potent and selective Ca2+ receptor antagonists that stimulate secretion of parathyroid hormone. *J Pharmacol Exp Ther*. **299**:323-331.

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, Harris R, 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, Dauban 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.

Pidasheva S, D'Souza-Li L, Canaff L, Cole DE, Hendy GN (2004) CASRdb: calcium-sensing receptor locus-specific database for mutations causing familial (benign) hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. *Hum Mutat*. **24**:107-111.

Pollak MR, Brown EM, Chou YH, Hebert SC, Marx SJ, Steinmann B, Levi T, Seidman CE, Seidman JG (1993) Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell*. **75**:1297-1303.

Ray K, Tisdale J, Dodd RH, Dauban P, Ruat M, Northup JK (2005) Calindol, a positive allosteric modulator of the human Ca(2+) receptor, activates an extracellular ligand-binding domain-deleted rhodopsin-like seven-transmembrane structure in the absence of Ca(2+). J Biol Chem. 280:37013-37020.

Rus R, Haag C, Bumke-Vogt C, Bähr V, Mayr B, Möhlig M, Schulze E, Frank-Raue K, Raue F, Schöfl C (2008) Novel inactivating mutations of the calcium-sensing

receptor: the calcimimetic NPS R-568 improves signal transduction of mutant receptors. J Clin Endocrinol Metab. 93:4797-4803.

Thakker RV (2004) Diseases associated with the extracellular calcium-sensing receptor. *Cell Calcium*. **35**:275-282.

Thakore P, Vanessa Ho WS (2010) Vascular actions of calcimimetics: role of Ca(2+) -sensing receptors versus Ca(2+) influx through L-type Ca(2+) channels. Br J Pharmacol. Oct. 20 epub.

White E, McKenna J, Cavanaugh A, Breitwieser GE (2009) Pharmacochaperone-mediated rescue of calcium-sensing receptor loss-of-function mutants. Mol Endocrinol. 23:1115-1123.

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Figure legends

Figure 1. Structures of calcimimetics.

Figure 2. AC-265347 activates CaS receptors. The indicated compounds were tested for proliferative responses using transiently transfected NIH3T3 cells in cellular proliferation assays (the R-SAT® functional assay; **A** and **B**) or using transiently transfected HEK 293T cells in phosphatidyl inositol (PI) hydrolysis assays (**C**, **D**, **E** and **F**) as described in the methods. Assays were conducted in a buffer system containing 1.6 mM $CaCl_2$, 0.8 mM $MgCl_2$ ('High Ca^{2+} buffer' – see methods, **A**, **B**, **C**, and **D**) or no $CaCl_2$, 0.5 mM $MgCl_2$ ('No Ca^{2+} buffer', **E** and **F**). Proliferative responses were quantified using a beta-galactosidase reporter system and normalized to the responses to $MgCl_2$. It was not possible to use $CaCl_2$ as a reference standard in cellular proliferation assays due to its cytotoxicity at high concentrations. Responses in PI hydrolysis assays were normalized to the responses to $CaCl_2$. 100% represents 5 to 10-fold responses over baseline in typical experiments in both assays. Data points represent the means of duplicate determinations. Graphs are representative of at least three independent experiments. Legends: AC-265347, filled circles; (S)-AC-265347, filled squares; (R)-AC-265347, open squares; cinacalcet, open circles; calindol, open inverted triangles; $MgCl_2$ or $CaCl_2$ where indicated, filled diamonds.

Figure 3. AC-265347 activates CaSRs through the transmembrane domains. The indicated compounds were tested for responses in PI hydrolysis assays as described above using HEK 293T cells transiently transfected with CaSRs with the N-terminal

extracellular domain deleted. Assays were conducted in a buffer system containing 1.6 mM $CaCl_2$, 0.8 mM $MgCl_2$ (**A**) or no $CaCl_2$, 0.5 mM $MgCl_2$ (**B**). Data points represent the means of duplicate determinations. Graphs are representative of at least three independent experiments. Responses were normalized to the responses to $CaCl_2$ by wild-type CaSRs. Legends: (S)-AC-265347, filled squares; cinacalcet, open circles; calindol, open inverted triangles; $CaCl_2$, filled diamonds.

Figure 4. AC-265347 activates CaSRs through a novel binding site. The indicated compounds were tested for functional responses in cellular proliferation (**A** and **B**) or PI hydrolysis (**C** and **D**) assays as described above; using cells transiently transfected with CaSRs mutated as follows: E837A$^{7.39}$ (**A** and **C**); I841$^{7.43}$ (**B** and **D**). Data points represent the means of duplicate determinations. Graphs are representative of at least three independent experiments. Responses were normalized to the responses to $MgCl_2$ (**A** and **B**) or $CaCl_2$ (**C** and **D**) by wild-type CaSRs. Legends: (S)-AC-265347, filled squares; cinacalcet, open circles; calindol, open inverted triangles; $MgCl_2$ (**A** and **B**) or $CaCl_2$ (**C** and **D**), filled diamonds.

Figure 5. CaSR allosteric agonists differentially activate 'loss-of-function' polymorphic variants. The indicated compounds were tested for functional responses in cellular proliferation (**A, B, C,** and **D**) or PI hydrolysis (**E, F, G,** and **H**) assays as described above; using cells transiently transfected with CaSRs mutated as follows: T138M (**A** and **E**); R185Q (**B** and **F**); R795W (**C** and **G**); R66C (**D** and **F**). Data points represent the means of duplicate determinations. Graphs are representative of at least

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three independent experiments. Responses were normalized to the responses to $MgCl_2$ (**A, B, C,** and **D**) or $CaCl_2$ (**E, F, G,** and **H**) by wild-type CaSRs. Legends: (S)-AC-265347, filled squares; cinacalcet, open circles; $MgCl_2$ (**A, B, C,** and **D**) or $CaCl_2$ (**E, F, G,** and **H**), filled diamonds.

Figure 6. AC-265347 lowers plasma PTH in rats. The indicated doses (mg/kg, sub-cutaneous) of (S)-AC-265347, (R)-AC-265347, AC-265347 or cinacalcet (**A**, **B**, **C** or **D**, respectively) were administered to male Sprague-Dawley rats (N=6 per drug group; N=12 vehicle), blood samples taken at 1 hour post-dose, and plasma levels of parathyroid hormone (PTH, reported as ng/ml) were measured as described in the Methods. *$p<0.05$; **$p<0.01$ compared to vehicle analyzed by ANOVA with Dunnett post-test analysis.

Figure 7. AC-265347 lowers plasma Ca^{2+} in rats. The indicated doses (mg/kg, sub-cutaneous) of (S)-AC-265347 or (R)-AC-265347 (**A** or **B**, respectively) were administered to male Sprague-Dawley rats (N=6 per drug group; N=12 vehicle), blood samples taken at 1 hour post-dose, and plasma levels of ionizable calcium ($[Ca^{2+}]_o$, reported as mM) were measured as described in the Methods. *$p<0.05$; **$p<0.01$ compared to vehicle analyzed by ANOVA with Dunnett post-test analysis.

Figure 8. AC-265347 is orally active. **A**) The indicated doses of AC-265347 were orally administered to catheterized male Sprague-Dawley rats (N=5 per drug group, N=8 vehicle), blood samples taken at the indicated time points, and plasma PTH levels measured as described in the Methods. The PTH levels in the 10 mg/kg group were

significantly ($p<0.01$) lower than vehicle at 0.5 and 1 hour post-dose, and significantly ($p<0.05$) lower than vehicle at 2 hours post-dose. The PTH levels in the 30 mg/kg group were significantly ($p<0.01$) lower than vehicle at 0.5, 1 and 2 hours post-dose. Significance was assessed by ANOVA with Dunnett post-test analysis. Legends: vehicle, filled inverted triangles; 10 mg/kg, open squares; 30 mg/kg, filled squares. **B**) Blood samples were taken at the indicated time intervals, and analyzed by LC/MS for concentrations of AC-265347 as described in the Methods. Legends: 10 mg/kg, open squares; 30 mg/kg, filled squares.

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Receptors	Ligands	pEC50	Eff(%) vs WT	Hill #
WT	AC-265347	7.6 ± 0.2	97 ± 8	1.10 ± 0.06
	(S)-265347	8.0 ± 0.2	89 ± 9	1.10 ± 0.10
	(R)-265347	7.0 ± 0.2	89 ± 12	1.13 ± 0.20
	Calindol	7.4 ± 0.2	103 ± 9	1.01 ± 0.09
	Cinacalcet	7.6 ± 0.1	86 ± 6	1.09 ± 0.05
	$MgCl_2$	2.4 ± 0.1	100 ± 4	2.90 ± 0.38
R66C	AC-265347	5.7 ± 0.3	16 ± 13	nc
	(S)-265347	nc	10 ± 3	nc
	(R)-265347	nc	3 ± 2	nc
	Calindol	nc	4 ± 1	nc
	Cinacalcet	nc	11 ± 6	nc
	$MgCl_2$	1.3 ± 0.5	28 ± 16	nc
T138M	AC-265347	6.3 ± 0.2	99 ± 12	1.39 ± 0.14
	(S)-265347	6.4 ± 0.2	100 ± 13	1.16 ± 0.21
	(R)-265347	5.5 ± 0.1	58 ± 17	0.94 ± 0.12
	Calindol	6.6 ± 0.1	57 ± 1	1.63 ± 0.05
	Cinacalcet	6.6 ± 0.1	53 ± 7	1.05 ± 0.15
	$MgCl_2$	1.2 ± 0.2	107 ± 13	3.29 ± 0.34
R185Q	AC-265347	6.1 ± 0.2	77 ± 11	1.83 ± 0.18
	(S)-265347	6.4 ± 0.1	76 ± 9	1.77 ± 0.10
	(R)-265347	<5.0	30 ± 4	1.69 ± 0.12
	Calindol	5.6 ± 0.3	31 ± 3	1.28 ± 0.32
	Cinacalcet	6.0 ± 0.3	25 ± 5	0.97 ± 0.15
	$MgCl_2$	1.7 ± 0.2	13 ± 5	2.48 ± 0.53
R795W	AC-265347	<5.0	44 ± 10	nc
	(S)-265347	5.4 ± 0.2	62 ± 7	1.31 ± 0.24
	(R)-265347	nc	9 ± 6	nc
	Calindol	nc	15 ± 3	nc
	Cinacalcet	nc	6 ± 1	nc
	$MgCl_2$	0.8 ± 0.2	53 ± 8	2.01 ± 0.41
E837A	AC-265347	6.4 ± 0.2	66 ± 14	1.24 ± 0.13
	(S)-265347	6.5 ± 0.3	72 ± 18	1.17 ± 0.31
	(R)-265347	5.9 ± 0.4	48 ± 20	nc
	Calindol	<5.0	18 ± 4	nc

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	Cinacalcet	**<5.0**	**29 ± 8**	**nc**
	$MgCl_2$	**2.5 ± 0.2**	**71 ± 9**	**3.07 ± 0.30**
I841A	**AC-265347**	**7.5 ± 0.3**	**103 ± 9**	**0.98 ± 0.19**
	(S)-265347	**7.8 ± 0.2**	**101 ± 10**	**1.34 ± 0.13**
	(R)-265347	**5.6 ± 0.6**	**69 ± 13**	**1.29 ± 0.13**
	Calindol	**6.9 ± 0.3**	**25 ± 5**	**0.98 ± 0.19**
	Cinacalcet	**7.8 ± 0.3**	**24 ± 7**	**0.74 ± 0.20**
	$MgCl_2$	**2.5 ± 0.1**	**104 ± 9**	**2.02 ± 0.16**

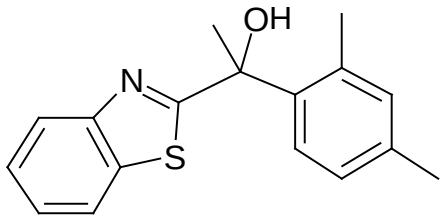
Table 1. **Agonist activation of WT and mutant CaSRs by CaSR ligands in cellular proliferation assays**. The indicated compounds were tested for proliferative responses using transiently transfected NIH3T3 cells in cellular proliferation assays (the R-SAT® functional assay, see Burstein et al., 2006) as described in the methods. Assays were conducted in a buffer system containing 1.6 mM $CaCl_2$, 0.8 mM $MgCl_2$. Proliferative responses were quantified using a beta-galactosidase reporter system and normalized to the responses to $MgCl_2$. It was not possible to use $CaCl_2$ as a reference standard in cellular proliferation assays due to its cytotoxicity at high concentrations. 100% represents 5 to 10-fold responses over baseline in typical experiments. Data represent the means ± SEM of three or more independent experiments. nc indicates not calculated.

		$Ca2^+$ medium			no $Ca2^+$ medium		
Receptor	Ligand	pEC50	EFF(%) vs WT	HILL #	pEC50	EFF(%) vs WT	HILL #
WT	AC-265347	8.1 ± 0.3	84 ± 24	0.72 ± 0.25	5.6 ± 0.1	28 ± 12	1.22 ± 0.24
	(S)-265347	8.3 ± 0.3	86 ± 27	0.64 ± 0.18	5.4 ± 0.5	45 ± 19	1.05 ± 0.35
	(R)-265347	7.0 ± 0.5	75 ± -	0.70 ± -	nc	9 ± 1	nc
	Calindol	6.9 ± 0.3	80 ± 13	0.74 ± 0.18	5.4 ± 0.3	14 ± 4	1.15 ± 0.36
	Cinacalcet	7.3 ± 0.3	75 ± 14	0.74 ± 0.26	5.3 ± 0.3	19 ± 6	1.10 ± 0.27
	$CaCl_2$	2.8 ± 0.1	99 ± 11	1.86 ± 0.51	2.6 ± 0.0	99 ± 4	3.29 ± 0.51
Delta-N	AC-265347	6.5 ± 0.3	44 ± 21	0.63 ± 0.14	6.5 ± 0.6	27 ± 8	0.80 ± 0.22
	(S)-265347	6.6 ± 0.4	52 ± 31	0.65 ± 0.07	6.4 ± 0.5	34 ± 7	0.79 ± 0.02
	(R)-265347	6.0 ± 0.1	27 ± 18	0.85 ± 0.47	nc	15 ± 1	nc
	Calindol	6.0 ± 0.2	35 ± 16	0.85 ± 0.13	5.8 ± 0.1	28 ± 8	0.89 ± 0.41
	Cinacalcet	6.2 ± 0.2	39 ± 18	0.61 ± 0.13	6.1 ± 0.3	30 ± 9	0.90 ± 0.46
	$CaCl_2$	nc	3 ± 3	nc	nc	4 ± -	nc
R66C	AC-265347	6.4 ± 0.1	21 ± 3	0.95 ± 0.08			
	(S)-265347	6.4 ± 0.0	24 ± 3	0.88 ± 0.01			
	Calindol	5.6 ± 0.2	15 ± 3	1.13 ± 0.05			
	Cinacalcet	5.5 ± 0.2	16 ± 5	0.92 ± 0.22			
	$CaCl_2$	2.4 ± 0.2	27 ± 2	2.53 ± 0.42			
T138M	AC-265347	6.2 ± 0.0	64 ± 11	0.95 ± 0.05			
	(S)-265347	6.3 ± 0.1	68 ± 4	0.84 ± 0.08			
	Calindol	5.7 ± 0.4	32 ± 5	1.02 ± 0.33			
	Cinacalcet	5.3 ± 0.5	32 ± 3	nc			

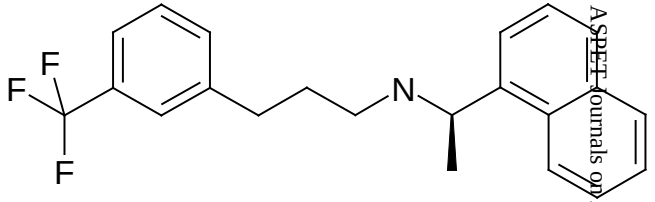
	$CaCl_2$	2.3	±	0.2	111	±	16	2.82	±	0.64
R185Q	AC-265347	6.1	±	0.1	55	±	12	1.29	±	0.09
	(S)-265347	6.2	±	0.1	64	±	14	0.98	±	0.04
	Calindol	5.6	±	0.2	16	±	6	1.13	±	0.30
	Cinacalcet	<5.0			21	±	3	nc		
	$CaCl_2$	1.8	±	0.2	18	±	3	2.23	±	0.67
R795W	AC-265347	5.7	±	0.1	15	±	5	1.42	±	0.28
	(S)-265347	5.8	±	0.1	18	±	4	1.17	±	0.16
	Calindol	nc			6	±	3	nc		
	Cinacalcet	nc			9	±	3	nc		
	$CaCl_2$	2.4	±	0.2	14	±	2	3.57	±	0.91
E837A	AC-265347	6.4	±	0.2	55	±	6	0.96	±	0.05
	(S)-265347	6.6	±	0.5	47	±	4	0.78	±	0.11
	Calindol	<5.0			27	±	1	nc		
	Cinacalcet	<5.0			30	±	4	nc		
	$CaCl_2$	2.7	±	0.1	65	±	10	1.84	±	0.37
I841A	AC-265347	7.6	±	0.4	44	±	8	0.59	±	0.06
	(S)-265347	8.0	±	0.7	43	±	7	0.56	±	0.13
	Calindol	nc			17	±	4	nc		
	Cinacalcet	nc			16	±	4	nc		
	$CaCl_2$	2.9	±	0.2	54	±	4	1.73	±	0.47

Table 2. **Agonist activation of WT and mutant CaSRs by CaSR ligands in phosphatidyl inositol (PI) hydrolysis assays**. The indicated compounds were tested in PI hydrolysis using transiently transfected HEK 293T cells as described in the methods. Assays were conducted in buffer systems containing either 1.6 mM $CaCl_2$, 0.8 mM $MgCl_2$ (Ca^{2+} medium) or 0.5 mM $MgCl_2$ (no Ca^{2+} medium). Data were normalized to the responses to $CaCl_2$. 100% represents 5 to 10-fold responses over baseline in typical experiments. Data represent the means $\pm$ SEM of three or more independent experiments. nc indicates not calculated.

Figure 1. Structures of calcimimetics



AC-265347



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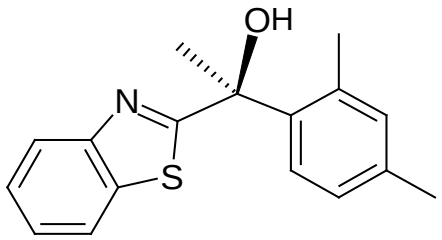

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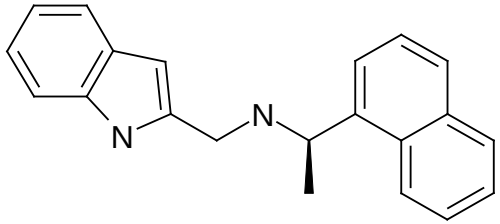


Calindol

Figure 2. AC-265347 activates CaS receptors

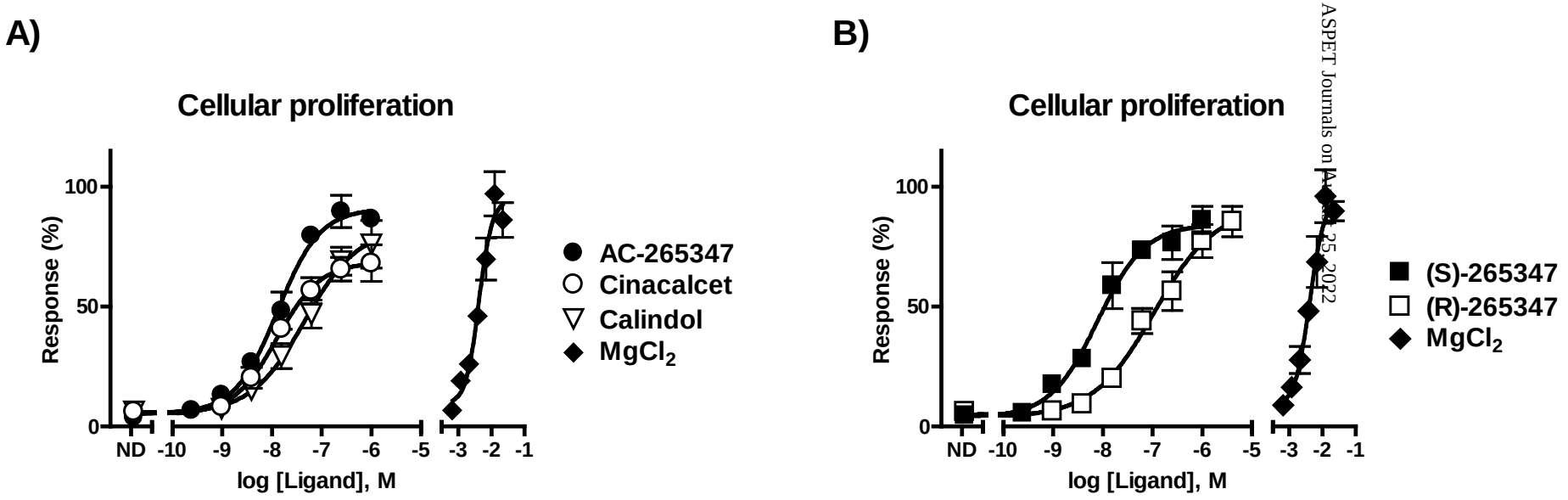


Figure 2. AC-265347 activates CaS receptors

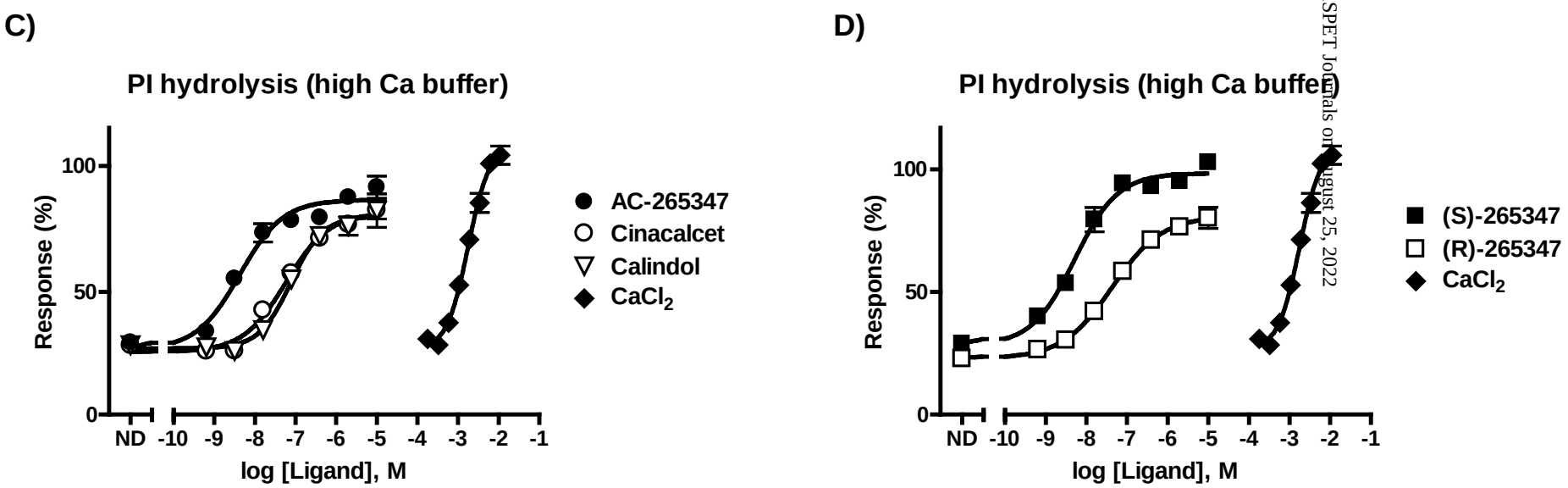
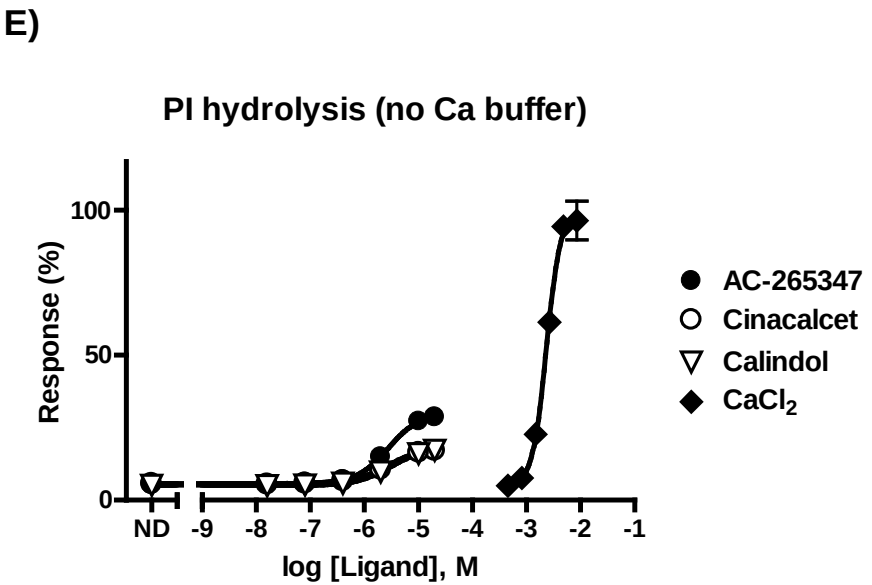


Figure 2. AC-265347 activates CaS receptors



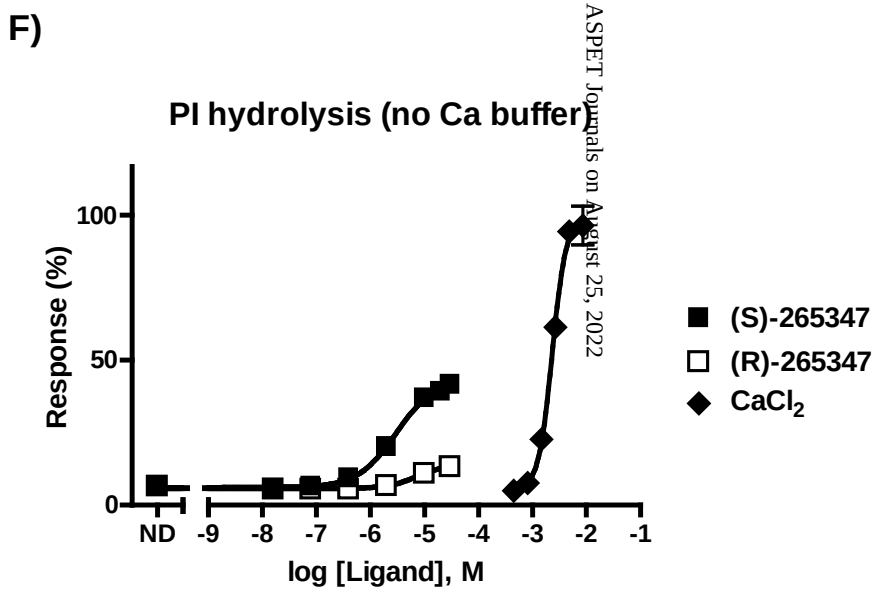


Figure 3. AC-265347 activates CaSRs through the transmembrane domains.

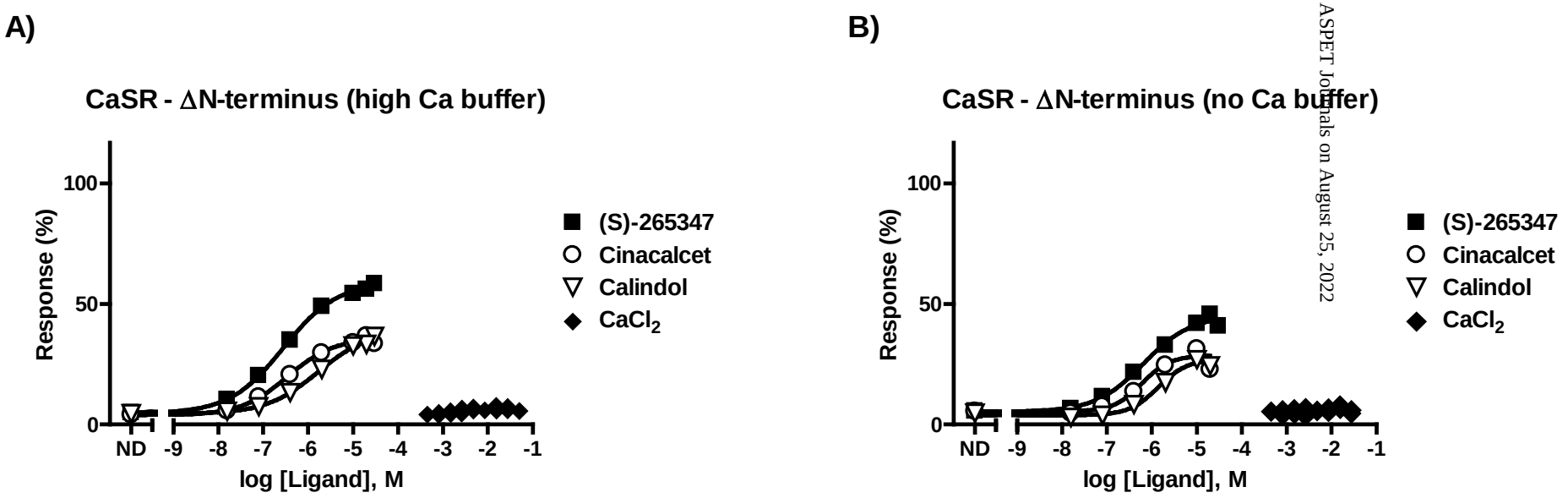


Figure 4. AC-265347 activates CaSRs through a novel binding site.

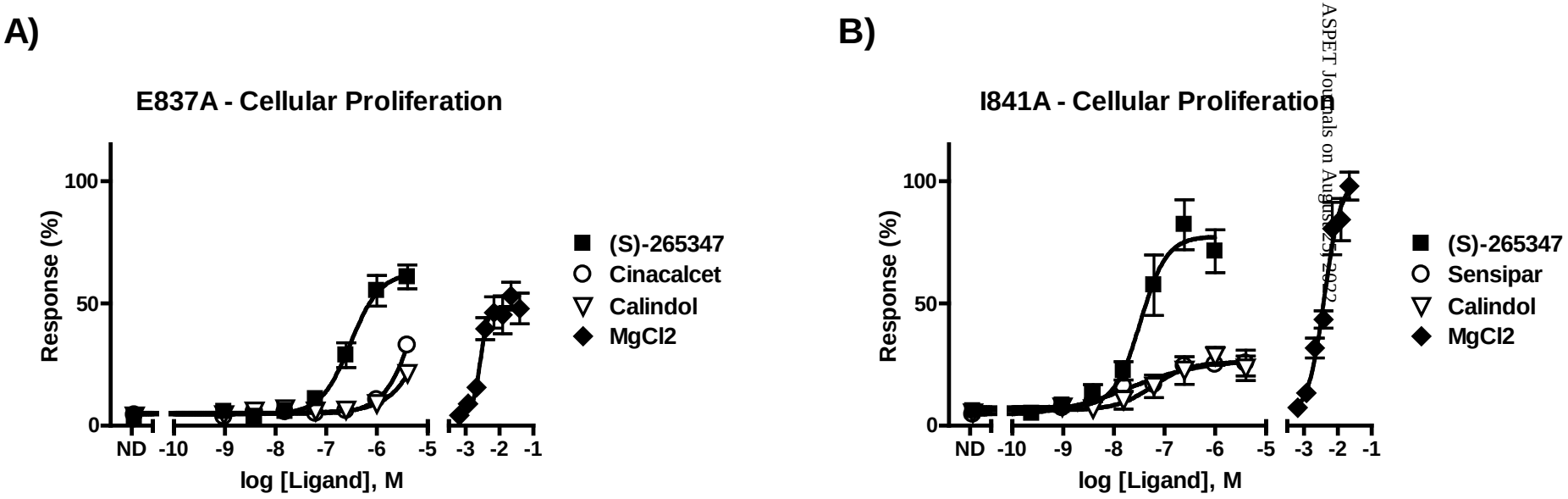


Figure 4. AC-265347 activates CaSRs through a novel binding site.

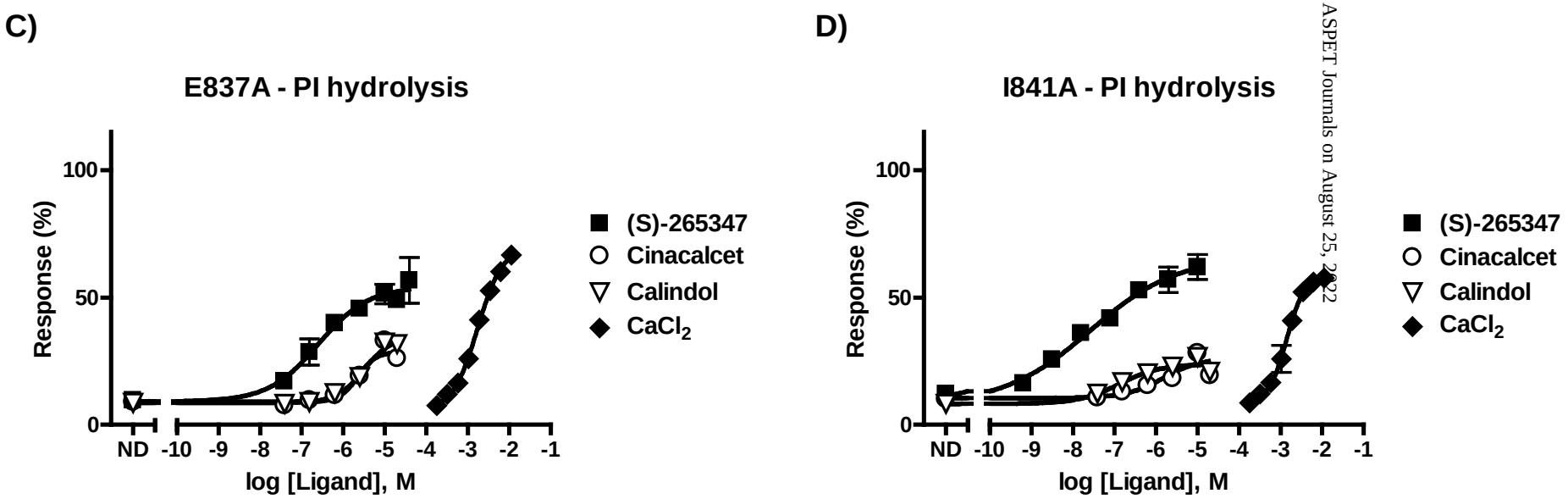


Figure 5. CaSR agonists differentially activate ‘loss-of-function’ polymorphic variants

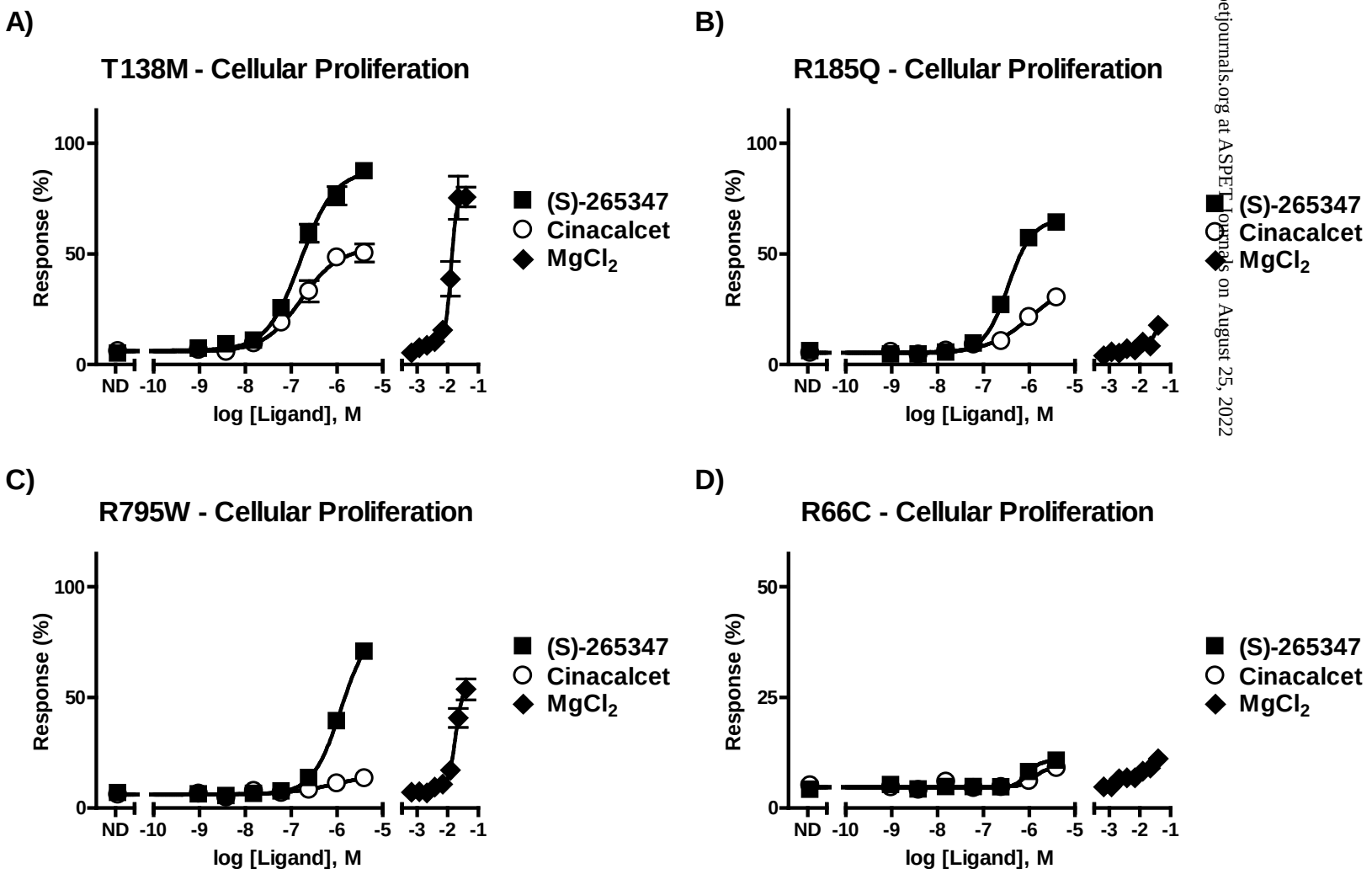


Figure 5. CaSR agonists differentially activate ‘loss-of-function’ polymorphic variants

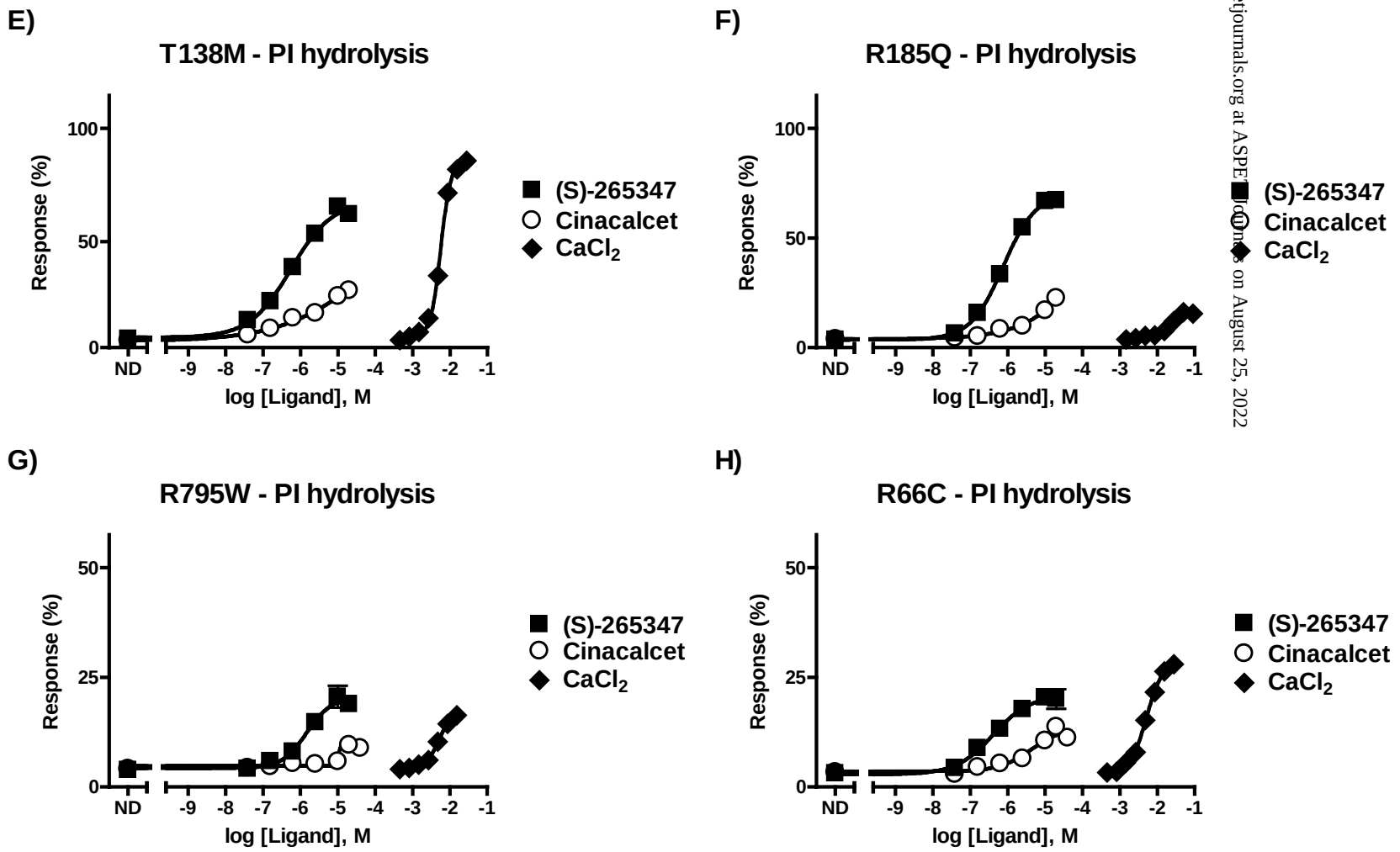


Figure 6. AC-265347 lowers plasma PTH in rats

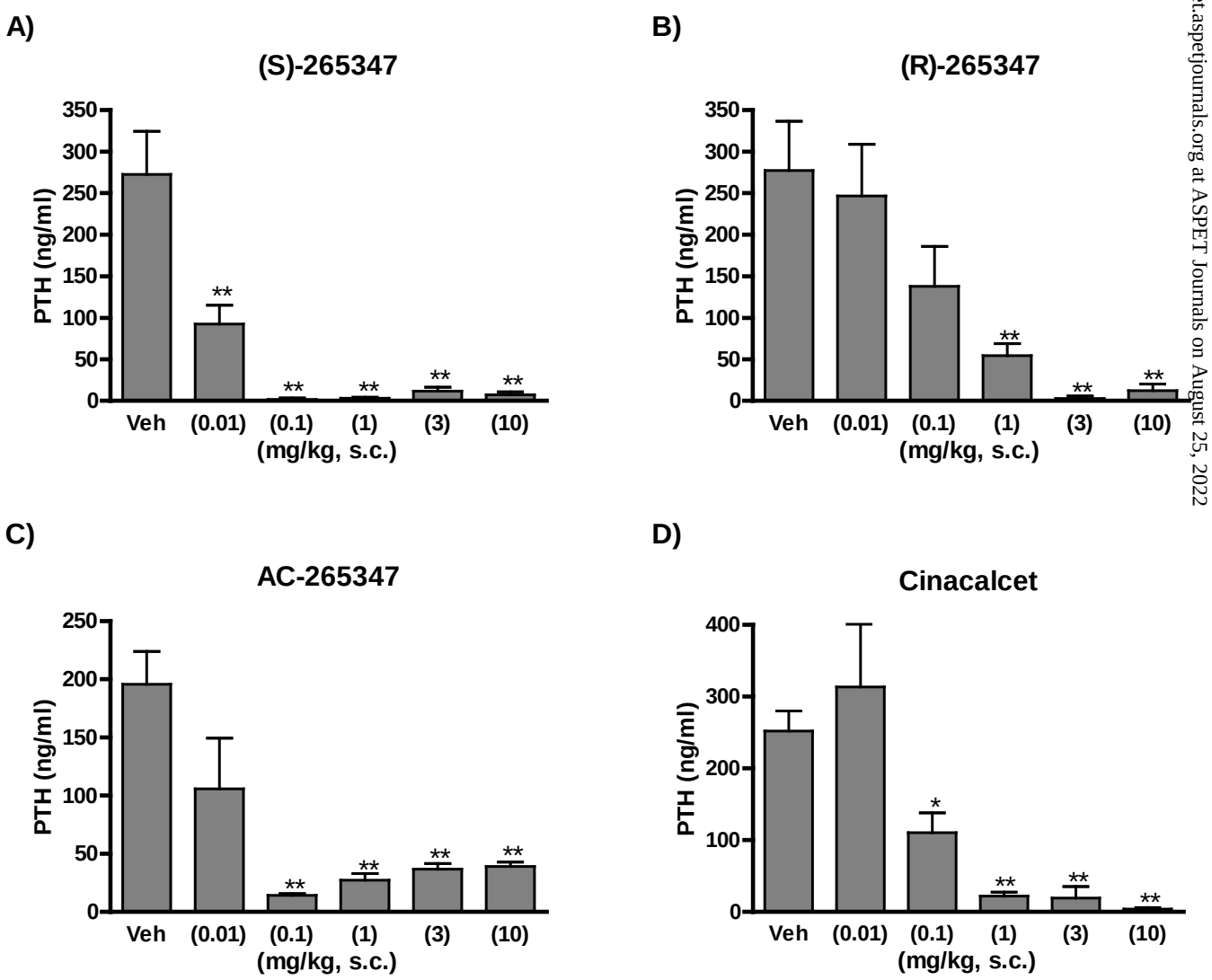


Figure 7. AC-265347 lowers plasma Ca^{2+} in rats

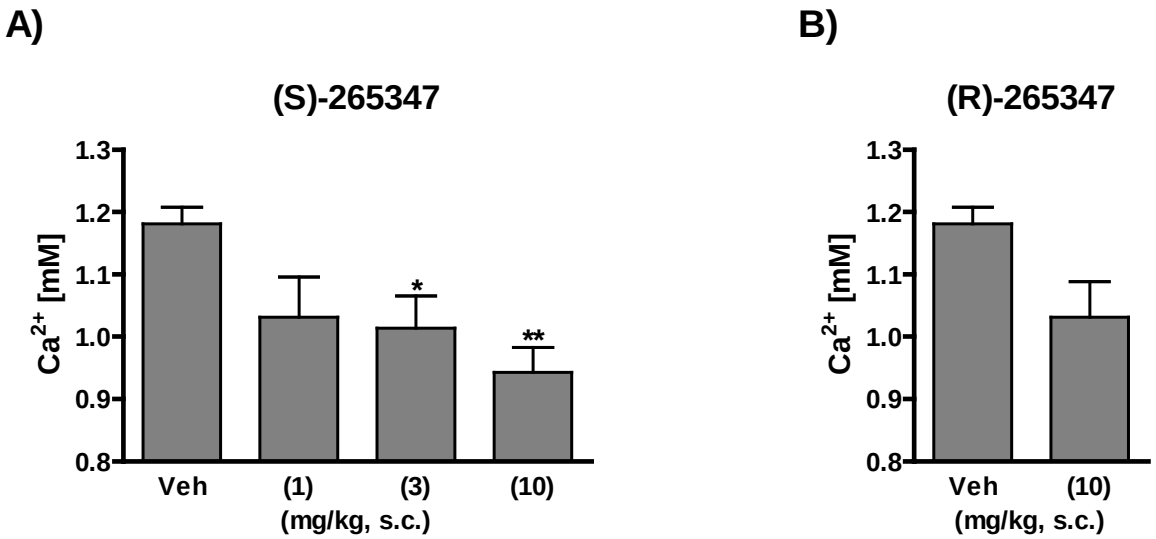


Figure 8A. AC-265347 is orally active.

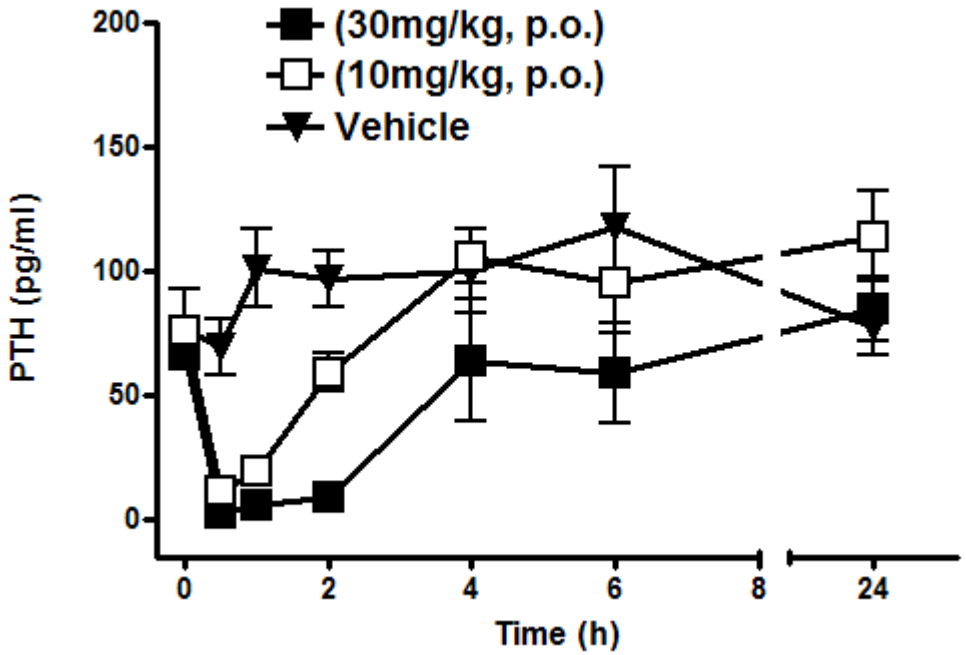


Figure 8B. AC-265347 is orally active.