Biased allosteric modulation at the CaSR engendered by structurally diverse calcimimetics

Running title: *Biasing CaSR signalling with calcimimetics*

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Summary

Background and purpose

The clinical use of cinacalcet in hyperparathyroidism is complicated by its tendency to induce hypocalcaemia, arising, at least in part, via activation of CaSRs in the thyroid and stimulation of calcitonin release. CaSR allosteric modulators that selectively bias signalling of the receptor towards pathways that mediate desired effects (e.g. PTH suppression) at the exclusion of those that mediate undesirable effects (e.g. elevated serum calcitonin), may offer superior therapies.

Experimental approach

We characterised the ligand-biased profile of novel calcimimetics in HEK293 cells stably expressing the human CaSR and monitoring effects on Ca^{2+}_i mobilisation, IP_1 accumulation, pERK1/2 and receptor expression.

Key results

Phenylalkylamine calcimimetics were biased towards allosteric modulation of Ca^{2+}_i mobilisation and IP_1 accumulation. *S,R*-calcimimetic B was biased only towards IP_1 accumulation. *R,R*-calcimimetic B and AC-265347 were biased towards IP_1 accumulation and pERK1/2. Nor-calcimimetic B was unbiased. In contrast to phenylalkylamines and calcimimetic B analogues, AC-265347 did not promote trafficking of a loss-of-expression naturally occurring CaSR mutation ($G^{670}E$).

Conclusions and implications

The ability of *R,R*-calcimimetic B and AC-265347 to bias signalling towards pERK1/2 and IP_1 accumulation may explain their ability to suppress PTH levels *in vivo* at concentrations that have no effect on serum calcitonin levels. The demonstration that AC-265347 promotes CaSR signalling but not trafficking reveals a

novel profile of ligand-biased modulation at the CaSR. The identification of allosteric modulators that bias CaSR signalling towards distinct intracellular pathways provides an opportunity to develop desirable biased signalling profiles *in vivo* for mediating selective physiological responses.

Abbreviations:

Ca^{2+}_o, extracellular calcium

Ca^{2+}_i, intracellular calcium

CaSR, calcium sensing receptor

FHH, familial hypocalciuric hypercalcaemia

Mg^{2+}_o, extracellular magnesium

NSHPT, neonatal severe hyperparathyroidism

pERK1/2, ERK1/2 phosphorylation

PTH, parathyroid hormone

Introduction

The human calcium sensing receptor (CaSR) is a family C G protein-coupled receptor (GPCR) primarily responsible for the regulation of extracellular calcium (Ca^{2+}_o) concentrations in the body. When Ca^{2+}_o rises, activation of the CaSR expressed in the parathyroid gland suppresses the secretion of parathyroid hormone (PTH). The drop in circulating PTH levels results in reduced renal Ca^{2+}_o reabsorption and reduced bone resorption (reviewed in Brown, 2013). Additionally, CaSR activation in the kidney by elevated serum Ca^{2+}_o inhibits Ca^{2+}_o reabsorption, leading to enhanced renal Ca^{2+}_o excretion independently of changes in PTH (Kantham *et al.*, 2009; Loupy *et al.*, 2012). Elevated serum Ca^{2+}_o also decreases bone resorption via CaSRs expressed on osteoblasts and osteoclasts (see Marie, 2010 for a review) and by stimulation of calcitonin secretion via CaSRs expressed on thyroid C cells (Freichel *et al.*, 1996).

The CaSR also has non-calciostatic roles. Thus, it mediates the modulation of blood pressure (see Smajilovic *et al.*, 2011 for a review) and protection against vascular calcification (Alam *et al.*, 2009), stimulation of gastrointestinal hormone secretion (Feng *et al.*, 2010; Mace *et al.*, 2012), modulation of electrolyte and water transport in the colon and kidney (reviewed in Macleod, 2013) and modulation of the proliferation and differentiation of numerous cell types, including colonic epithelial cells, keratinocytes, adipocytes and neurones.

Given its ubiquitous expression throughout the body and functionally diverse roles, drugs that target the CaSR may have therapeutic application in various clinical contexts. However, these drugs may also produce adverse effects arising from actions

in multiple tissues expressing the CaSR. Indeed, patients treated with the calcimimetic, cinacalcet ((α*R*)-(−)-α-methyl-*N*-[3-[3-[trifluoromethylphenyl]propyl]-1-napthalenemethanamine hydrochloride), a positive allosteric CaSR modulator indicated for the treatment of secondary and some forms of primary hyperparathyroidism, have a tendency to develop adverse effects that restrict its use to only severely affected patients. The most problematic adverse effect is hypocalcaemia (Chonchol *et al.*, 2009), likely resulting from both suppressed renal calcium reabsorption induced by CaSR activation in the kidney, and calcitonin-mediated inhibition of bone resorption via CaSR activation in the thyroid C-cells (Arenas *et al.*, 2013). Thus, novel calcimimetics that selectively stimulate CaSR-mediated signalling in the parathyroid gland without affecting CaSRs in other tissues may have an improved side effect profile and enable treatment of less severe grades of hyperparathyroidism.

One approach to directing desired physiological outcomes of GPCR activation is to selectively target those intracellular signalling pathways that couple to the anticipated effect, while avoiding those that couple to unwanted consequences. Such selectivity can be achieved with a drug that binds to and favours a receptor conformation that preferentially couples to a subset of desired intracellular signalling pathways (Kenakin, 2011). This concept is referred to as ligand-biased signalling, ligand-directed trafficking of receptor stimulus, functional selectivity or biased agonism (Kenakin & Christopoulos, 2013).

The CaSR is subject to ligand-biased signalling on a number of levels (Leach *et al.*, 2014). First, it binds multiple endogenous ligands, including Ca^{2+}_{o}, Mg^{2+}_{o}, L-amino

acids, polyamines and the glutamyl peptide, γ-glutathione. Ca^{2+}_o, spermine and L-phenylalanine have been demonstrated to preferentially activate distinct signalling pathways (Rey *et al.*, 2010; Thomsen *et al.*, 2012a), suggesting that each ligand has the propensity to stabilise a subset of preferred receptor states and subsequently stimulate the repertoire of intracellular signalling proteins that couple to these states. Second, positive allosteric modulators of the CaSR, such as cinacalcet, and negative CaSR modulators (calcilytics), such as NPS-2143 (2-chloro-6-[(2*R*)-3-[[1,1-dimethyl-2-(2-naphthalenyl)ethyl]amino-2-hydroxypropoxy]benzonitrile hydrochloride), engender biased allosteric modulation at the CaSR, such that they exhibit greater modulation of some pathways over others (Davey *et al.*, 2012; Leach *et al.*, 2013). Third, the "natural bias" of the CaSR can be altered in pathophysiological states. This has been demonstrated by naturally occurring mutations in the CaSR protein that alter its usual signalling bias (Leach *et al.*, 2012), a switch in CaSR signalling from $G_{i/o}$ to G_s in human breast cancer cells (Mamillapalli *et al.*, 2008), and an autoantibody directed against the CaSR in a patient with acquired hypocalciuric hypercalcemia, which potentiated inositol phosphate (IP) accumulation, yet inhibited ERK1/2 phosphorylation (pERK1/2) (Makita *et al.*, 2007). Finally, the complement of intracellular signalling proteins to which the CaSR couples differs between cell types, thus, the capacity of the CaSR to couple to different signalling pathways depends upon its tissue-specific expression.

Proof-of-concept that tissue-specific effects can be achieved by targeting the CaSR with drugs was evident from early experiments with the prototypical calcimimetic, NPS-R568. During the development of the phenylalkylamine calcimimetics (e.g. NPS-R568 and cinacalcet), it was recognised that the natural hypocalcaemic effects of

these drugs may be complicated by stimulation of calcitonin release via activation of CaSRs in the thyroid. Thus, the need to suppress PTH secretion with minimal effects on calcitonin secretion was acknowledged (Fox *et al.*, 1999a; Fox *et al.*, 1999b), but remains sub optimally addressed.

Third generation agents appear to have enhanced tissue-selective effects. This is evident from studies with the novel dibenzylamine calcimimetic, *R,R*-calcimimetic B (*R*-1-(6-methoxy-4'-(trifluoromethyl)-3-biphenylyl)-*N*-(*R*)-1-phenylethyl)ethanamine) and the structurally distinct calcimimetic, AC-265347 (1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol). Both calcimimetics inhibit PTH secretion at concentrations that do not induce calcitonin release in rats (Henley *et al.*, 2011; Ma *et al.*, 2011), demonstrating a means for normalising serum PTH and calcium levels without causing uncontrolled hypocalcaemia. How these compounds achieve this tissue specificity is unknown, but we hypothesise that it may be a result of ligand-biased allosteric modulation at the CaSR. This is based on the fact that distinct intracellular signalling pathways activated by the CaSR are responsible for its physiological effects, thus drugs may selectively promote suppression of PTH release by preferentially activating the pathways that couple to that response. For instance, CaSR suppression of PTH release is driven by phospholipase C (PLC)-mediated IP_3 production (Brown *et al.*, 1987; Kifor *et al.*, 1997) and pERK1/2 (Corbetta *et al.*, 2002) but there is some evidence that CaSR-mediated $Ca^{2+}{}_i$ release is not required for inhibition of PTH from bovine parathyroid cells (Russell *et al.*, 1999). Stimulation of both PLC and $Ca^{2+}{}_i$ mobilisation have been linked to the release of calcitonin (Liu *et al.*, 2003; McGehee *et al.*, 1997; Thomsen *et al.*, 2012b) but in rat 6-23 medullary thyroid carcinoma cells, inhibition of pERK1/2 has no effect on $Ca^{2+}{}_o$-mediated

stimulation of calcitonin release (Thomsen *et al.*, 2012b). Thus, drugs that bias CaSR signalling towards pERK1/2 may achieve tissue-selective suppression of PTH secretion in the absence of calcitonin release.

To probe the ligand-biased signalling profile(s) required to achieve drug tissue selectivity, pathways that mediate distinct physiological receptor functions should ideally be dissected in systems such as primary or immortalised cells that maintain their physiological function. However, for the CaSR, this has been hampered by a lack of relevant cell lines and methods to study, for instance, parathyroid cell function. We have developed techniques to measure signalling in, and PTH release from, primary human parathyroid cells (Avlani *et al.*, 2013; Broadhead *et al.*, 2011; Mun *et al.*, 2009) but performing high throughput experiments in these cells is at present not possible. Thus, most studies of this nature must rely on recombinant cell systems to investigate CaSR signalling in response to agonists and drugs. Nonetheless, recombinant systems can still be used to identify bias and validate whether compounds with desirable *in vivo* properties have unique pharmacology *in vitro*, and vice versa.

The current study thus primarily aimed to use a recombinant cell system to determine the potential for structurally distinct calcimimetics to engender ligand-biased signalling and subsequently promote coupling of the CaSR to three key signalling pathways that could mediate different physiological effects; IP_1 accumulation (a stable metabolite of IP_3), Ca^{2+}_i mobilisation and pERK1/2. Furthermore, we have previously shown that CaSR modulators can be biased in their ability to modulate signalling versus trafficking at the CaSR (Leach *et al.*, 2013). Therefore, in addition

to acute signalling at the CaSR, we determined the ability of the calcimimetics to act as pharmacochaperones of a naturally occurring mutant CaSR, $G^{670}E$. Differential effects on trafficking versus signalling may have important implications for the treatment of calcium handling disorders caused by mutations in the CaSR gene that result in a diverse range of molecular phenotypes.

Materials and Methods

Synthesis of calcimimetics

Synthesis of *R,R*-calcimimetic B (compound **3b** – appendix S1), its diastereoisomer *S,R*-calcimimetic B (compound **3a** – appendix S1) and nor-calcimimetic B (compound **3c** – appendix S1) was achieved using a two-step procedure derived from described literature (Harrington *et al.*, 2010). Full synthetic details and compound characterisation are given in Appendix S1. NPS-R568 and cinacalcet were prepared as described previously (Davey *et al.*, 2012). Calindol was purchased from Tocris Biosciences, whereas AC-265347 was from Sigma-Aldrich.

Cell culture

Generation of FlpIn HEK293 TRex cells (Invitrogen) stably expressing the human CaSR under the control of tetracycline has been described previously (Davey *et al.*, 2012; Leach *et al.*, 2012). Cells were maintained in DMEM with 10% FBS, 200 μg ml-1 hygromycin B and 5 μg ml-1 blasticidin.

Optimisation of assay conditions

The effect of ambient buffer Ca^{2+}_o on allosteric modulation at the CaSR has previously been published by us (Davey *et al.*, 2012). Because Ca^{2+}_o is both present in the buffer and added as the agonist, assay buffer Ca^{2+}_o was optimised to achieve the best possible assay signal while avoiding complications that arise from the presence of physiological Ca^{2+}_o concentrations (e.g. signalling desensitisation, potentiation of ambient Ca^{2+}_o signalling). In this same cell system, Mg^{2+}_o is nearly 3 fold less potent than Ca^{2+}_o as a CaSR agonist (data not shown). Thus, the presence of 1.18mM ambient Mg^{2+}_o has minimal effect on CaSR signalling. Therefore, all assays were performed under low Ca^{2+}_o but physiologically relevant Mg^{2+}_o conditions. For concentration-response curves to Ca^{2+}_o, data are plotted and analysed without the ambient Ca^{2+}_o concentration (i.e. only the added Ca^{2+}_o is considered).

Ca^{2+}_i mobilisation assays

Cells were seeded in a clear 96-well plate coated with poly-D-lysine (50 μg ml-1) at 80,000 cells per well and incubated overnight in the presence of 100 ng ml-1 tetracycline. The following day, cells were washed with 200 μl assay buffer (150 mM NaCl, 2.6 mM KCl, 1.18 mM $MgCl_2$, 10 mM D-Glucose, 10 mM HEPES, 0.1 mM Ca^{2+}_o, 0.5 % BSA and 4 mM probenecid at pH 7.4) and loaded with 100 μl Fluo-4 AM (1 μM) for 1 h at 37 ^oC.

Cells were washed again with 200 μl assay buffer prior to the addition of fresh assay buffer. In functional interaction studies between Ca^{2+}_o and the calcimimetics, the modulators were coadded with Ca^{2+}_o (in all assays measuring agonist-stimulated receptor signalling events, each well was treated with a single agonist and/or modulator concentration). The release of Ca^{2+}_i was measured at 37°C using a

Flexstation® 1 or 3 (Molecular Devices; Sunnyvale, California). Fluorescence was detected for 60 s at 485 nm excitation and 525 nm emission but the peak Ca^{2+}_i mobilisation response (approximately 12 seconds after agonist addition) was used for the subsequent determination of the agonist response. We have previously shown that when allosterism at the CaSR is quantified in Ca^{2+}_i mobilisation assays using the potency of Ca^{2+}_o obtained by plotting the area under the 60 second Ca^{2+}_i mobilisation trace, no significant difference in signalling or biased modulation is observed in comparison to parameters derived using the peak Ca^{2+}_i mobilisation response (Leach et al., 2013). Relative peak fluorescence units were normalised to the fluorescence stimulated by ionomycin to account for differences in cell number and loading efficiency, and further normalised to the maximum response observed for the WT CaSR in the absence of modulator.

Extracellular regulated kinase 1/2 (ERK1/2) phosphorylation assays

Cells were seeded at 80,000 cells per well into a poly-D-lysine coated (50 μg ml-1) transparent 96-well plate and grown overnight with 100 ng ml-1 tetracycline. The following day, cells were washed twice with PBS and serum-free DMEM containing 16 mM HEPES and 0.1 mM Ca^{2+}_o was added to wells. Vehicle or agonist (Ca^{2+}_o) with or without modulator were coadded to wells and incubated for 2.5 minutes (the time determined in prior assays for pERK1/2 to peak) at 37°C. All data were normalised to the response stimulated by 10% FBS and then further normalised to the maximum response stimulated by Ca^{2+}_o in the absence of modulator. pERK1/2 was determined using the SureFire pERK1/2 assay kit (kindly donated by Dr Michael Crouch, TGR biosciences, Adelaide) employing AlphaScreen technology

(PerkinElmer). All other details are as described previously (Leach *et al.*, 2013; Leach *et al.*, 2012).

IPone accumulation assays

Following overnight induction of receptor expression with 100 ng ml-1 tetracycline in a T175cm^2 flask (where appropriate), cells were harvested and resuspended in assay buffer (150 mM NaCl, 2.6 mM KCl, 1.18 mM $MgCl_2$, 10 mM D-Glucose, 10 mM HEPES, 0.1 mM Ca^{2+}_o, 50 mM LiCl, pH 7.4) at 1.43 x 10^6 cells ml-1. 7 μl agonist with or without modulator were added to wells of a 384 well white proxiplate (PerkinElmer) and 7 μl cells (1x10^4 cells) were added to these wells, centrifuged for 1 minute at 350 x g and incubated at 37°C for 45 minutes. The IP-One Tb™ assay kit (CisBio, France) was used to detect myo-inositol 1 phosphate (IP_1), based on fluorescence resonance energy transfer (FRET) between d2-conjugated IP_1 and Lumi4™-Tb cryptate conjugated anti-IP_1 antibody. These reagents were diluted 1:30 with lysis buffer and 3 μl of each was added to wells following agonist stimulation. Lysates were incubated for 1 hour and FRET was detected using an Envision plate reader (PerkinElmer) where emission of Lumi4™-Tb cryptate was detected at 620 nm and emission of d2-conjugated IP_1 at 665 nm. Results were calculated from the 665 nm / 620 nm ratio. Data were normalised to the maximum response stimulated by Ca^{2+}_o in the absence of modulator.

Flow cytometry analysis for receptor expression

FlpIn HEK293 TRex cells stably expressing the human wild-type (WT) or $G^{670}E$ mutant CaSR were seeded in a 96-well plate at a density of 80,000 cells per well in DMEM containing 100 ng ml-1 tetracycline and 0.3 μM or 3 μM allosteric modulator

and incubated overnight at 37°C. The next day, cells were harvested with PBS supplemented with 0.1 % BSA, 2 mM EDTA and 0.05% NaN_3 (washing buffer) and transferred to wells of a 96 well v-bottom plate, centrifuged for 3 min at 350 x *g*, 4°C and resuspended in 100 µl blocking buffer (PBS, 5% BSA, 2 mM EDTA and 0.05% NaN_3). Cells were incubated for 30 min in blocking buffer and subsequently incubated for 1 h with an AF647-conjugated 9E10 antibody (made in-house as described below), diluted in blocking buffer at 1 µg ml-1. Cells were subsequently washed with washing buffer and resuspended in washing buffer with Sytox blue stain (Molecular probes). The fluorescence signal was quantified using a FACS Canto (Becton Dickinson).

Production of anti-cMyc:AF647 (9E10:AF647)

Supernatant from the 9E10 hybridoma (ATCC Number: CRL-1729) was harvested and antibody purified over a HiTrap protein G sepharose column (GE Lifesciences). The purified antibody was coupled to AF647 Succinimidyl Ester (Life technologies) using standard protocols. Unincorporated fluor was removed using a 10kDa MWCO centrifugal concentrator (Merck Millipore). Degree of labeling was determined to be 3.6. The antibody conjugate was validated by titration in flow cytometry. A full description of antibody production, conjugation and validation can be found in the supplementary methods and results.

Data analysis

All nonlinear regression analysis was performed using GraphPad Prism® 6 (GraphPad Software, San Diego, CA). Parametric measures of potency, affinity and cooperativity were estimated as logarithms (Christopoulos, 1998). Data of the

functional CaSR concentration response curves obtained were fitted as logarithms to the following four-parameter concentration response curve equation (Equation 1)

$$Y=\text{Bottom}+\frac{(\text{Top-Bottom})\acute{}\ (A^{nH})}{A^{nH}+EC_{50}{}^{nH}} \quad (1)$$

where Y is the response, Bottom and Top represent the bottom and top asymptotes of the curve, respectively, A denotes the agonist concentration (excluding ambient $Ca^{2+}{}_o$ in the buffer), nH (Hill slope) describes the steepness of the curve, EC_{50} is the concentration of agonist that gives the mid-point response between Bottom and Top.

For functional interaction experiments between $Ca^{2+}{}_o$ and the allosteric modulators, pEC_{50} values obtained for each curve in the absence and presence of modulator were fitted to an allosteric ternary complex model (Equation 2)

$$pEC_{50}=\text{Log}\left[10^{\text{Log}\alpha\beta}\times[B]+10^{-pK_B}\right]-\text{Log}d \quad (2)$$

where pEC_{50} is the negative logarithm of the agonist EC_{50} in the presence of allosteric modulator, pK_B is the negative logarithm of the "functional" dissociation constant of the allosteric modulator determined in signalling assays, $\alpha\beta$ is the overall cooperativity between the allosteric modulator and orthosteric agonist, and d is the estimate of the EC50 in the absence of modulator. An extra sum of squares F test was used to determine whether data obtained in IP_1 accumulation, $Ca^{2+}{}_i$ mobilisation and pERK1/2 assays were fitted best when the allosteric modulator functional pK_B values were shared across the three different pathways. In a second analysis that constrained the functional pK_B across datasets (Supplemental Table 1, Supplemental Figure 9), an extra sum of squares F test was used to determine whether the cooperativities between the three pathways differed.

For the "cooperativity bias plot", the pEC_{50} of Ca^{2+}_o in the absence and presence of modulator in IP_1 accumulation, Ca^{2+}_i mobilisation and pERK1/2 assays was first fitted to equation 2 and 150 XY coordinates of points that defined the curve that best fit equation 2 were determined. Next, the XY coordinates for the different pathways were plotted against one another, with IP_1 accumulation or Ca^{2+}_i mobilisation data on the y-axis against pERK1/2 data on the x-axis. XY coordinates corresponding to the effects of 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 μM modulator are respresented by symbols on the plots. If the allosteric modulator shows equal cooperativity in the assays, the data points will be coincident and the cooperativity bias plots will overlap with the line of identity. If, however, the modulator exerts greater cooperativity in one of the pathways, the points will fall either side of this line towards the preferred pathway.

For agonist concentration response curves in the absence of Ca^{2+}_o and Mg^{2+}_o, data were fitted as logarithms to an operational model of agonism (Equation 3)

$$E=\frac{E_m \cdot t_B \cdot [B^n]}{K_B+[B^n] \cdot (t_B+1)} \quad (3)$$

where E is the effect (response) stimulated by the allosteric agonist, E_m is the maximum response of the system stimulated by the full agonist (Ca^{2+}_o), τ_B is an operational measure of allosteric agonist efficacy, defined as the inverse of the fraction of receptors that must be occupied by agonist to obtain the half-maximal response, [B] is the allosteric agonist concentration and n is the transducer slope.

Results

Rationale for choice of ligands and signalling pathways

The structures of the calcimimetics used in this study are shown in Figure 1. The prototypical phenylalkylamine calcimimetics, cinacalcet and NPS-R568 (3-(2-chlorophenyl)-*N*-((1*R*)-1-(3-methoxyphenyl)ethyl)-1-propanamine) have been well characterised *in vitro* and *in vivo* (Nemeth *et al.*, 2004; Nemeth *et al.*, 1998). Calindol ((*R*)-2-[*N*-(1-(1-naphthyl)ethyl)aminomethyl]indole) was the most potent calcimimetic identified at the Institut de Chimie des Substances Naturelles (ICSN, France) from a series of diamines based around the structure of NPS-R568 (Kessler *et al.*, 2004). *R,R*-calcimimetic B was the most potent CaSR ligand identified by Amgen in a dibenzylamine series and exhibited ideal *in vivo* pharmacodynamics. In an IP accumulation assay, *R,R*-calcimimetic B was estimated to have greater affinity than NPS-R568 (Harrington *et al.*, 2010; Henley *et al.*, 2011). The published synthesis of *R,R*-calcimimetic B employed a route yielding a diastereomeric ratio (d.r.) of 14:1 of *R,R*-calcimimetic B and the corresponding *S,R*-diastereoisomer (*S*-1-(6-methoxy-4'-(trifluoromethyl)-3-biphenylyl)-*N*-(*R*)-1-phenylethyl)ethanamine) respectively, which were then separated via HPLC (Harrington *et al.*, 2010). *S,R*-calcimimetic B was 100-fold less potent than *R,R*-calcimimetic B (Harrington *et al.*, 2010), comparable to the stereoselectivity of the *R*- and *S*-isomers of NPS- 568 and cinacalcet (Hammerland *et al.*, 1998; Nemeth *et al.*, 2004). Given the remarkable difference in potency of the individual diastereoisomers, we sought to isolate and further characterise each one independently. Adapting the synthesis of Harrington *et al*, we were able to generate a mixture of diastereoisomers with a d.r. of 4:1. These were successfully isolated by either chiral HPLC or preparative layer chromatography (PLC) (see Appendix S1 for full synthetic methods). Structurally, the contrasting pharmacological behaviour of each diastereoisomer can be attributed to the spatial orientation of the methyl group adjacent to the biphenyl and amino moieties. With this in mind, it was of interest to

evaluate the pharmacological activity of the 'nor' calcimimetic B derivative (*R*-*N*-((6-methoxy-4'-(trifluoromethyl)-3-biphenylyl)methyl)-1-phenylethanamine), with a methylene group replacing the methyl of interest. This was synthesised in a similar fashion to the *R,R*- and *S,R*-calcimimetic B derivatives. AC-265347 was identified in a screen by ACADIA Pharmaceuticals as a potent calcimimetic. It is structurally distinct from the phenylalkylamine calcimimetics and calcimimetic B, and was found to have improved potency over cinacalcet in an IP accumulation assay (Ma *et al.*, 2011).

We investigated the effects of the calcimimetics in Ca^{2+}_i mobilisation, IP accumulation and pERK1/2 assays because each of these pathways has been undeniably linked to CaSR regulation of PTH release from parathyroid chief cells and/or calcitonin release from thyroid C cells, as outlined in the introduction. Although additional pathways are also involved in the regulation of PTH and calcitonin release, we selected those for which assays can be reliably performed in a high throughput manner to enable robust quantification of allosteric modulation and biased signalling.

Calcimimetics are biased modulators of CaSR signalling

To evaluate the extent to which calcimimetics engender ligand-biased modulation at the CaSR, we first characterised their ability to potentiate the endogenous agonist, Ca^{2+}_o, in IP_1 accumulation, Ca^{2+}_i mobilisation and pERK1/2 assays. These experiments generated Ca^{2+}_o concentration-response curves in the absence and presence of the allosteric modulators.

As expected, cinacalcet, NPS-R568, calindol, AC-265347, *R,R*-calcimimetic B, *S,R*-calcimimetic B and nor-calcimimetic B, potentiated agonist-mediated activation of the CaSR in each assay, demonstrated by a leftward shift in the Ca^{2+}_o concentration-response curve, and a consequent increase in Ca^{2+}_o potency. In some instances, the calcimimetics elicited a concomitant increase in the baseline response due to potentiation of Ca^{2+}_o and Mg^{2+}_o in the buffer (Davey *et al.*, 2012) and/or agonist activity. No changes in the maximum response elicited by Ca^{2+}_o were observed in the presence of the calcimimetics. Experimental data from IP_1 accumulation assays for a representative calcimimetic from each class of compound are shown in Figure 2. Data for all calcimimetics across each pathway are shown in Appendix S3, Supplemental Figures 2-8.

We have previously demonstrated that both calcimimetics and calcilytics can exhibit biased allosteric modulation via two (albeit related) mechanisms. The first arises from the ability of modulators to bind with distinct affinities to CaSR conformations that mediate different signalling pathways (Davey *et al.*, 2012). Divergent affinities indicate that the modulators stabilise distinct receptor states, a requirement of ligand-biased signalling. The second arises from cooperativities between a modulator and the orthosteric agonist that differ at a given receptor state (Davey *et al.*, 2012; Leach *et al.*, 2013). Thus, an allosteric ternary complex model (equation 2) was used to quantify the parameters that governed the activity of the calcimimetics in each assay to estimate the functional affinity (functional pK_B) of the modulators and their overall cooperativity ($\alpha\beta$) with Ca^{2+}_o (Table 1). An F-test was used to determine whether the functional affinity and/or cooperativity of each calcimimetic differed across signalling assays. However, because functional affinity and cooperativity parameters

are correlated in the nonlinear regression algorithgm, it is sometimes difficult to separate out the two effects. Thus, results of nonlinear regression analyses that assumed the binding affinity to be the same or not the same across pathways are presented in Appendices 2 and 3 of the Supplemental data.

These analyses established a number of key findings. First, the phenylalkylamine calcimimetics, NPS-R568 and calindol, exhibited ligand-biased modulation that favoured activation of Ca^{2+}_i mobilisation and IP_1 accumulation. This was manifested as a lower functional affinity for the receptor state that coupled to pERK1/2 (Table 2, Figure 3A). Cinacalcet also demonstrated a tendency to modulate pERK1/2 less favourably than the other two pathways (Table 2, Figure 3A and B), but significance for this effect was only reached if its functional affinity was assumed to be the same across pathways (Supplemental Table 1, Supplemental Figure 9) and was thus indicative of weaker cooperativity in pERK1/2 assays. Second, *S,R*-calcimimetic B was biased towards modulation of IP_1 accumulation, but showed no preference between Ca^{2+}_i mobilisation or pERK1/2. Similar to cinacalcet, significance was only reached when its functional affinity was assumed to be the same across pathways (Supplemental Table 1, Supplemental Figure 9). Third, nor-calcimimetic B was relatively unbiased in its ability to modulate the three pathways, and its estimated functional affinities and cooperativities were comparable in all three assays. Finally, *R,R*-calcimimetic B and AC-265347 were biased towards modulation of pERK1/2 and IP_1 accumulation, either in terms of functional affinity (Figure 3A) or cooperativity (Figure 3B and Supplemental Figure 9). The bias arising from AC-265347 can be visualised in Figures 4A-C where the different effects of 0.1μM AC-265347 on Ca^{2+}_o signalling in the three different assays are apparent. The bias engendered by multiple

concentrations of AC-265347 can be visualised in the modulator "cooperativity bias plot" as shown in Figure 4D. This illustrates the impact of equivalent AC-265347 concentrations on Ca^{2+}_o potency in Ca^{2+}_i mobilisation or IP_1 assays on the y-axis, and pERK1/2 assays on the x-axis. If AC-265347 modulated both pathways equally, the data would converge on the line of identity. However, because it modulates one pathway to a greater degree than the other, the data points are distributed away from the line of identity towards the preferred pathway (i.e. towards IP_1 over pERK1/2 and towards pERK1/2 over Ca^{2+}_i).

Third generation calcimimetics are agonists at the CaSR

In IP_1 accumulation and Ca^{2+}_i mobilisation assays, the calcimimetics stimulated receptor activity in the presence of vehicle (buffer) alone. AC-265347, *R,R*-calcimimetic B and nor-calcimimetic B also did so in pERK1/2 assays. We previously simulated the effects of cinacalcet on signalling in the presence of an ambient concentration of agonist to reconstruct the experimental conditions under which our Ca^{2+}_i mobilisation and pERK1/2 assays are undertaken (Davey *et al.*, 2012). These simulations suggested that positive modulation of ambient agonists in the buffer (Ca^{2+}_o and Mg^{2+}_o) was expected. Accordingly, when we omitted ambient Ca^{2+}_o and Mg^{2+}_o from the assay buffer, Ca^{2+}_i mobilisation and IP_1 accumulation stimulated by cinacalcet, NPS-R568 and calindol on their own was largely inhibited (Figure 5), indicating that the observed "baseline effect" was primarily due to potentiation of ambient agonist activity. In contrast, AC-265347 and the calcimimetic B analogues retained activity in the absence of ambient Ca^{2+}_o and Mg^{2+}_o (Figure 5). The effects of omitting only Ca^{2+}_o from the buffer can be observed in Supplemental Figure 10.

We fitted the agonist activity of the calcimimetics (in the absence of Ca^{2+}_o and Mg^{2+}_o) to the standard operational model of agonism (Equation 3) (Black & Leff, 1983) to gain a second estimate of the functional affinity of the modulators at the CaSR. These estimates were similar to the affinities estimated for the modulators using the allosteric ternary complex model (Table 2). Of note is the comparable affinity of AC-265347 between Ca^{2+}_i mobilisation and IP_1 assays. This is in contrast to its affinity in "potentiation assays", which were strongly suggestive of a higher affinity for the receptor state that coupled to IP_1 accumulation (Table 1, Figure 3A, Supplemental Figure 8). Thus, the receptor state that mediates direct calcimimetic activation of the CaSR may be distinct from the state that modulates Ca^{2+}_o activity at the receptor.

Our analysis additionally derived an operational measure of agonism, defined as τ_B, which reflects both the degree to which the agonist can activate the receptor, and the stimulus-response coupling between the receptor and the intracellular signalling pathway. Interestingly, although Ca^{2+}_o is more potent in Ca^{2+}_i mobilisation than IP_1 assays, there was no significant difference in the activity of the modulators in the two assays ($p > 0.1$ unpaired t-test), indicating that they do not follow the same natural biased profile as the endogenous agonist.

Calcimimetics differentially modulate trafficking of a naturally occurring loss-of-expression mutant

We have previously shown that both calcimimetics and calcilytics are also biased in their abilities to modulate CaSR trafficking (Leach *et al.*, 2013). This may have important implications for patients with loss-of-expression CaSR mutations that cause

disorders of calcium metabolism such as familial hypocalciuric hypercalcaemia (FHH) and neonatal severe hyperparathyroidism (NSHPT). Thus, to determine the ability of each of the CaSR modulators to correct trafficking and signalling of defective CaSR mutants, we investigated the consequences of the modulators at the naturally occurring loss-of-expression mutant, $G^{670}E$ (Kobayashi *et al.*, 1997). Expression of this mutant receptor at the cell surface is greatly reduced but its affinity for cinacalcet is unaltered (Leach *et al.*, 2013; Leach *et al.*, 2012). This mutant also signals efficiently in Ca^{2+}_i mobilisation and pERK1/2 assays (Leach *et al.*, 2013; Leach *et al.*, 2012).

The affinities and cooperativities of AC-265347, cinacalcet, NPS-R568 and calindol were unaltered at the $G^{670}E$ mutation compared to the wildtype, as assessed in Ca^{2+}_i mobilisation assays (Table 3). The affinity of the calcimimetic B analogues, however, was reduced approximately 100-fold, although *R,R*-calcimimetic B and nor-calcimimetic B were still able to bind to the receptor and potentiate Ca^{2+}_o-mediated signalling.

Overnight treatment of HEK293 cells with cinacalcet, NPS-R568, calindol, *R,R*-calcimimetic B and nor-calcimimetic B restored cell surface expression of the $G^{670}E$ mutant (Table 3; Figure 6). *S,R*-calcimimetic B and AC-265347, however, had no effect on expression. In the case of *S,R*-calcimimetic B, this was likely due to lower receptor occupancy in comparison to the other calcimimetics due to its reduced functional affinity. The inability of AC-265347 to rescue $G^{670}E$ expression, however, was not due to reduced affinity or to reduced cooperativity, which were comparable to the other calcimimetics. The inability of AC-265347 to restore trafficking may be

related to its lower lipophilicity relative to the other compounds. This parameter can be represented by calculated partition coefficient (CLog P, see Figure 1), which for AC-265347 was found to be considerably lower than for the other allosteric modulators tested. Thus, AC-265347 may have a reduced propensity to cross cell membranes to pharmacochaperone misfolded receptors trapped in the ER and Golgi compartments.

Discussion

The present study evaluated the pharmacological activity of structurally related and diverse calcimimetics across multiple measures of receptor activity, identifying distinct ligand-biased profiles for each compound. Importantly, whereas phenylalkylamine modulators are biased towards Ca^{2+}_i mobilisation and IP_1 accumulation, *S,R*-calcimimetic B is biased only towards modulation of IP_1 accumulation, and nor-calcimimetic B is unbiased. *R,R*-calcimimetic B and AC-265347 on the other hand are biased *towards* pERK1/2 and IP_1 accumulation. Of note, although Ca^{2+}_i mobilisation via Gq-coupled receptors typically stems from the PLC-IP pathway, the divergence in bias between Ca^{2+}_i and IP1 assays observed herein suggests that CaSR-mediated Ca^{2+}_i mobilisation is also facilitated via alternative mechanisms. This is supported by a number of previous findings. In rat medullary thyroid carcinoma cells, Ca^{2+}_o activation of the CaSR resulted in Ca^{2+}_i influx via ion-gated calcium channels in addition to IP3-mediated calcium mobilisation (Thomsen *et al.*, 2012b). Sr^{2+}_o, on the other hand, stimulated CaSR-mediated PLC/IP3/Ca^{2+}_i mobilisation, but did not trigger opening of calcium channels in these cells (Thomsen *et al.*, 2012b). Similarly, although both Ca^{2+}_o and L-phenylalanine stimulated Ca^{2+}_i mobilisation in CaSR-transfected HEK293 cells, only Ca^{2+}_o promoted IP

accumulation and diacylglycerol production (Rey et al., 2005). Finally, we recently showed that truncation of the CaSR after R^{866} resulted in a complete inability of the receptor to stimulate Ca^{2+}_i mobilisation, whereas IP accumulation was reduced, but maintained (Goolam *et al.*, 2014). In the same study, mutations in intracellular loops 2 and 3 greatly impaired IP accumulation but had a weaker affect on Ca^{2+}_i mobilisation. These findings suggest Ca^{2+}_i mobilisation stimulated from the CaSR is in part driven via an IP-independent mechanism.

Intriguingly, although AC-265347 is a positive modulator of CaSR signalling, it is a neutral modulator of receptor trafficking. These findings build on our earlier studies of prototypical CaSR positive and negative allosteric modulators that initially identified bias in modulation by these compounds (Davey *et al.*, 2012; Leach *et al.*, 2013).

Ligand-biased signalling by CaSR modulators may be driven by ligand-specific stabilisation of distinct receptor states that couple preferentially to particular intracellular signalling pathways. This is suggested by the different functional affinities or cooperativities with the endogenous agonist estimated at each pathway. We introduced this concept several years ago (Leach *et al.*, 2007) and have subsequently observed biased allosteric modulation at the M_4 muscarinic (Leach *et al.*, 2010), A_1 adenosine (Aurelio *et al.*, 2009) and glucagon-like peptide 1 (GLP-1) (Koole *et al.*, 2011) receptors, indicating that pathway selectivity may be achieved with allosteric modulators acting at a number of GPCRs.

AC-265347 exhibited high cooperativity in pERK1/2 assays, maximally enhancing the potency of Ca^{2+}_o nearly 10-fold, in comparison to the 3-fold enhancement in potency observed with cinacalcet. This is consistent with previous findings indicating that AC-265347 is more potent than cinacalcet with respect to IP_1 accumulation assays but has comparable potency with respect to cellular proliferation (Ma *et al.*, 2011). This suggests that AC-265347 exhibits ligand-biased modulation of distinct CaSR signalling pathways. pERK1/2 plays a significant role in the suppression of PTH release (Corbetta *et al.*, 2002; Thomsen *et al.*, 2012b) but may be less important for CaSR-mediated stimulation of calcitonin release (Thomsen *et al.*, 2012b). Thus, compounds that favour pERK1/2 over Ca^{2+}_i mobilisation may have reduced propensity to induce calcitonin-dependent hypocalcaemia when compared to cinacalcet. Accordingly, there is pronounced separation (300-fold) in the concentration of *S*-AC-265347 required to suppress serum PTH levels versus the concentration that reduces serum Ca^{2+}_o levels in healthy rats (Ma *et al.*, 2011). Similarly, concentrations of calcimimetic B that maximally inhibit PTH secretion in nephrectomised rats have little effect on calcitonin release or serum Ca^{2+}_o levels (Henley *et al.*, 2011). In contrast, cinacalcet concentrations required to maximally suppress PTH secretion also stimulate calcitonin release and reduce serum Ca^{2+}_o levels in rats (Nemeth *et al.*, 2004), suggesting less selectivity of cinacalcet for suppression of PTH release. AC-265347 and *R,R*-calcimimetic B are thus potentially important lead compounds of value in elucidating the roles of pERK1/2 in CaSR-mediated regulation of PTH and calcitonin release.

The fact that third generation but not phenylalkylamine calcimimetics are agonists in their own right may also contribute to their parathyroid selectivity. When stimulus-

response coupling is strong, for instance in tissues such as the parathyroid glands where CaSR expression is high, partial agonist effects will become more pronounced.

The CaSR is promiscuous in its coupling to intracellular signalling pathways, and the influence of individual pathways to physiological outcomes such as regulation of hormone release from chief cells of the parathyroid and parafollicular C cells of the thyroid, and control of ion transport in the kidney, is still being elucidated. Although we have selected to investigate the modulatory effects of calcimimetics on three key signalling pathways that regulate some of the physiological actions of the CaSR, these pathways are not exhaustive. For instance, G12/13-mediated cytoskeletal rearrangements are important for CaSR-mediated suppression of PTH release (Quinn *et al.*, 2007) but experiments that measure G12/13-mediated membrane ruffling, for instance, are not amenable to high throughput screening techniques and have subsequently not been included in the present study. Our ongoing work aims to extend these studies to examine activity across multiple pathways in primary cell lines, to establish the link between signalling bias and *in vivo* pharmacological and physiological calcimimetic effects.

It must also be noted that allosterism may be influenced by the kinetics of ligand binding relative to the different time points underlying response generation in each experiment. Thus, an alternative explanation for the observed bias is that each ligand stabilises the same state with different kinetics. However, the same direction of bias towards Ca^{2+}_i mobilisation over pERK1/2 is also observed following preincubation of the CaSR with cinacalcet and NPS-R568 for 30 minutes prior to measurement of agonist-mediated receptor signalling (Davey et al., 2013). Thus, differences in

modulator bias in the different assays likely reflect true biased signalling and not an equilibrium artefact. For the detection of agonism, the transient nature of agonist-mediated Ca^{2+}_i mobilisation, pERK1/2 and indeed many other GPCR signalling responses means signalling will often subside before equilibrium binding can be reached. Thus, the receptor may no longer elicit a response once true equilibrium is obtained. Therefore, it is assumed that one of the most relevant responses for the purpose of detecting receptor signalling and indeed biased signalling is the response elicited upon first exposure of a cell to the activating agonist.

In addition to differences in agonist effects and biased modulation of different signalling pathways, we found that AC-265347, unlike the other calcimimetics tested, was unable to restore expression of the $G^{670}E$ loss-of-expression mutant. Importantly, this and our previous study have identified unique ligand-biased profiles whereby a drug can positively modulate CaSR signalling and trafficking (cinacalcet, NPS-R568, calindol, *R,R*-calcimimetic B and nor-calcimimetic B), negatively modulate CaSR signalling and positively modulate trafficking (NPS-2143) (Leach *et al.*, 2013) or positively modulate signalling without affecting trafficking (AC-265347). The inability of AC-265347 to rescue expression may be due to its lower lipophilicity, which makes it less likely to cross the cell membrane. Thus, compartmentalisation of receptors away from the cell surface restricts its access to only a subset of the available receptor pool. This represents an alternative means by which a drug can bias the activity of a receptor; one that is governed by its interaction with receptors that signal (cell surface receptors) versus those that can traffic to the cell surface (intracellular receptors).

660 The diverse pharmacological profile exhibited by each of the allosteric modulators
661 offers exciting possibilities for their use beyond treatments for secondary
662 hyperparathyroidism. For instance, future identification of pure "trafficking
663 modulators" may be beneficial in disease states where reduced CaSR expression has
664 been identified, such as colon cancer (Hizaki *et al.*, 2011; Singh *et al.*, 2012), and
665 primary and secondary hyperparathyroidism (Cetani *et al.*, 2000; Kifor *et al.*, 1996;
666 Yano *et al.*, 2000). Furthermore, drugs may be fine-tuned to the needs of distinct
667 patients carrying naturally occurring CaSR mutations, depending on the impact of
668 their mutation on receptor signalling and/or trafficking. The ability to tailor drug
669 therapies to patients harbouring naturally occurring mutations may become an
670 important consideration not just for the CaSR, but also for other GPCRs. Indeed,
671 naturally occurring mutations in the glucagon-like peptide 1 receptor, for instance,
672 engender signalling bias, with some mutations altering receptor coupling to only a
673 subset of intracellular signalling pathways (Koole *et al.*, 2011). Thus, a
674 pharmacogenomics approach may be essential for the future treatment of certain
675 patient subtypes.
676
677 In conclusion, the current study has characterised structurally diverse calcimimetics
678 and identified distinct ligand-biased signalling engendered by different classes of
679 compounds. Although at present it is unclear which biased profile will be desirable in
680 different disease states, the identification of biased ligands provides novel tools to
681 probe the *in vivo* consequences of differentially promoting CaSR signalling and
682 trafficking.
683
684

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AUTHOR CONTRIBUTIONS:

AC, ADC, AEC and KL planned and coordinated the study, AEC, KJG and KL performed experimental assays, SNM synthesised calcimimetic B analogues, SGBF prepared and evaluated the AF647-conjugated 9E10 antibody, AEC, SNM, KJG, PMS, PJS, ADC, AC and KL wrote the manuscript.

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Figure 1 Structure of the CaSR allosteric modulators examined in this study. Calculated partition coefficient (CLog P) obtained from PerkinElmer ChemBioDraw software are shown.

Figure 2 Structurally distinct calcimimetics potentiate Ca^{2+}_o-mediated receptor activation with different potencies. Ca^{2+}_o-mediated IP_1 accumulation in the presence of 0 (●), 0.003 μM (□), 0.01 μM (○), 0.03 μM (○), 0.1 μM (△), 0.3 μM (◇), 1 μM (▽), 3 μM (⬭) and 10 μM (⊗) cinacalcet (A), *R,R*-calcimimetic B (B) and AC-265347 (C). Data are mean + s.e.m from at least 4 independent experiments performed in duplicate.

Figure 3 Calcimimetics display distinct functional affinities and/or cooperativities for CaSR conformations that couple to different signalling pathways. Modulator functional affinities (functional pK_B) and cooperativities (αβ) were determined as described in the Methods, by fitting the Ca^{2+}_o pEC_{50} in the absence and presence of modulator determined in Ca^{2+}_i mobilisation (white bars), pERK1/2 (grey bars) and IP_1 accumulation (black bars) assays to an allosteric ternary complex model (equation 2). The affinity of the modulator was unconstrained in each pathway. Statistical differences shown by asterisks are demonstrated where an F-test determined that the data were fitted best when the modulator affinities and cooperativies were different between the three pathways. Data are mean + s.e.m from at least 4 independent experiments performed in duplicate.

Figure 4 AC-265347 preferentially modulates pERK1/2 and IP_1 accumulation over Ca^{2+}_i mobilisation. Ca^{2+}_o-mediated Ca^{2+}_i-mobilisation (A), pERK1/2 (B) and IP_1 accumulation (C) in the absence (●) and presence of 0.1μM (Δ) AC-265347. A "bias plot" (D) depicts AC-265347's preferential modulation of pERK1/2 and IP_1 accumulation versus Ca^{2+}_i-mobilisation. Ca^{2+}_o pEC_{50} in the absence and presence of modulator was determined in IP_1 accumulation, Ca^{2+}_i mobilisation and pERK1/2 assays and fitted to an allosteric ternary complex model (equation 2) to determine 150 XY coordinates of points that defined the curve that best described the model. The XY coordinates for the different pathways are plotted against one another, with IP_1 accumulation or Ca^{2+}_i mobilisation data on the y-axis against pERK1/2 data on the x-axis. Grey and black dashed lines join IP_1 accumulation and Ca^{2+}_o mobilisation XY coordinates, respectively, corresponding to the effects of 0 (●), 0.003 μM (□), 0.01 μM (○), 0.03 μM (⭘), 0.1 μM (Δ), 0.3 μM (◇), 1 μM (∇), 3 μM (⬭) and 10 μM (⊗) AC-265347. The dotted line represents the line of identity, which is a theoretical representation of how the data would look if the pathways were modulated equally by AC-265347.

Figure 5 Calcimimetics are agonists at the CaSR. Activity of calcimimetics in the absence of ambient Ca^{2+}_o and Mg^{2+}_o measured in Ca^{2+}_i-mobilisation (closed circles) and IP_1 accumulation assays (open circles). Data are mean + s.e.m from 3 independent experiments performed in triplicate.

Figure 6 CaSR modulators differentially rescue the $G^{670}E$ loss-of-expression mutant. Whereas overnight treatment with the calcimimetics has minimal effect on the expression of the WT CaSR in HEK cells, cinacalcet, NPS-R568, calindol, *R*,*R*-

calcimimetic B and nor-calcimimetic B rescue the expression of the $G^{670}E$ mutant. AC-265347 and *S,R*-calcimimetic B, however, do not rescue cell surface expression. Data are mean + s.e.m from at least 4 independent experiments.

DISCLOSURE STATEMENT:

AEC, SNM, KJG, SGBF, PJS, PMS, ADC and KL have nothing to declare. AC has previously published work on the CaSR in collaboration with researchers from Amgen.

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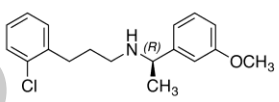
COMPETEING INTERESTS:

None



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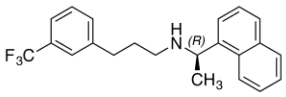

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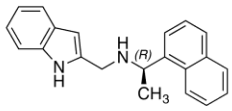

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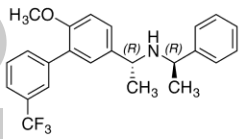
CLogP: 4.14



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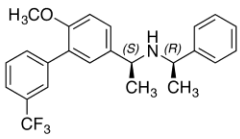

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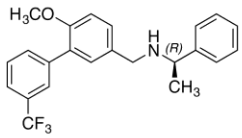

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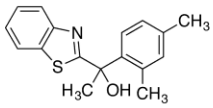
CLogP: 5.16



AC-265347
CLogP: 3.74

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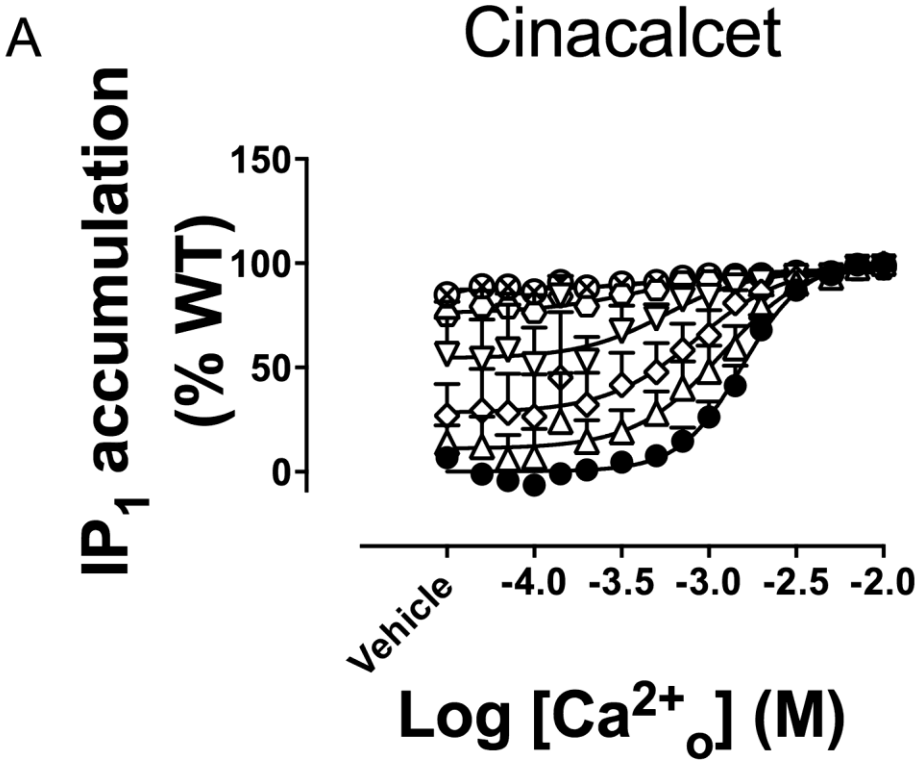

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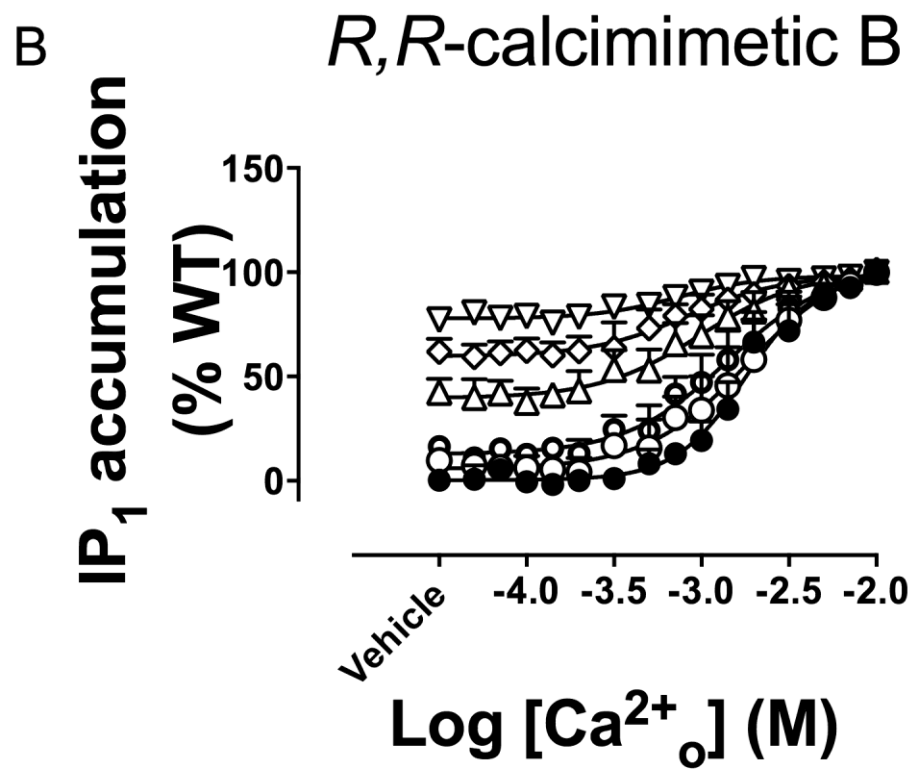

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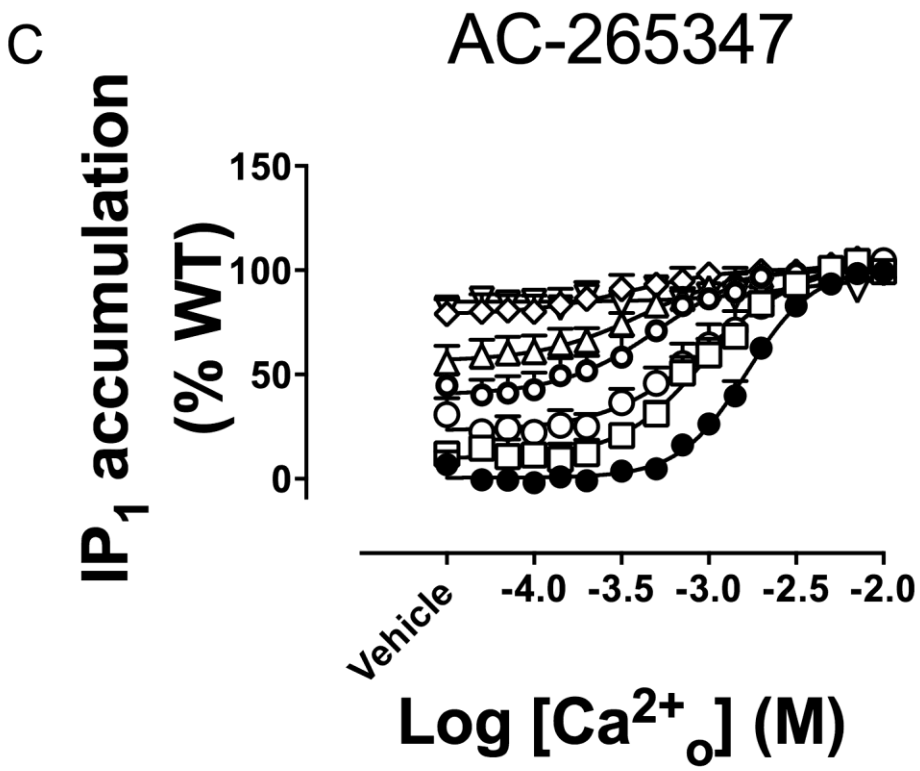

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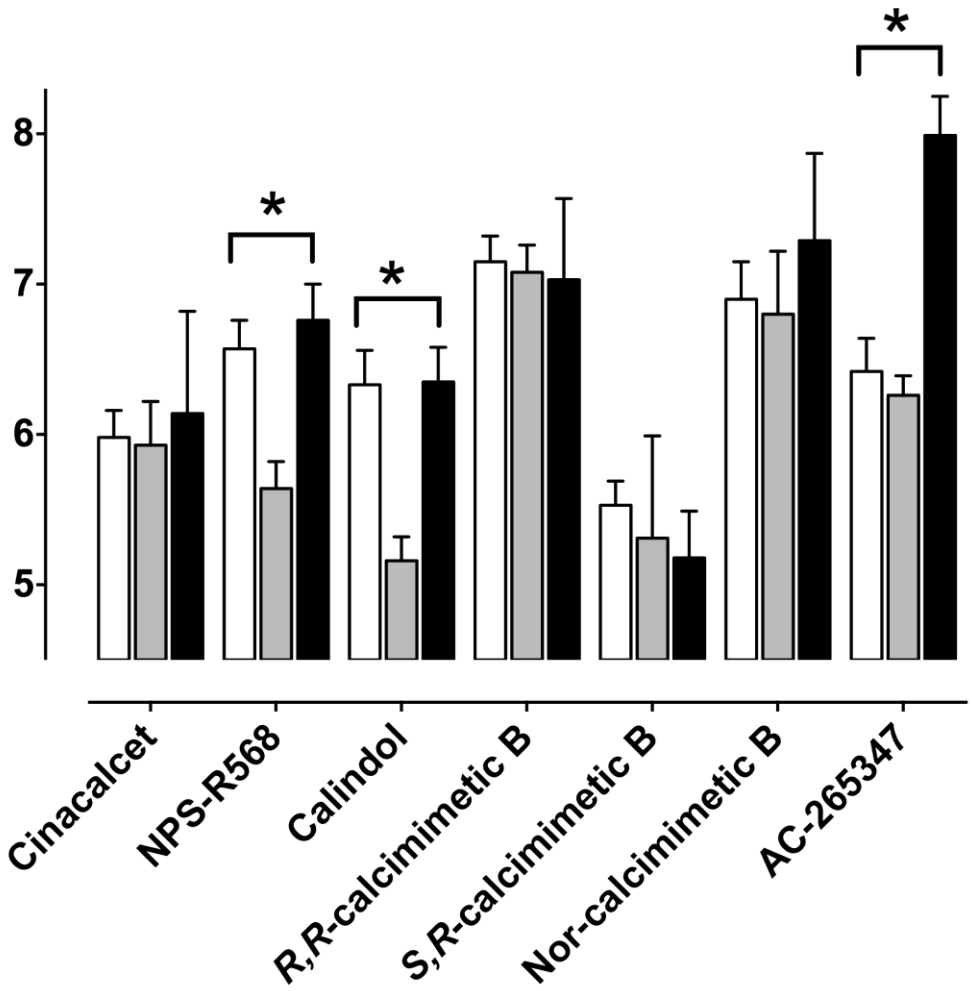

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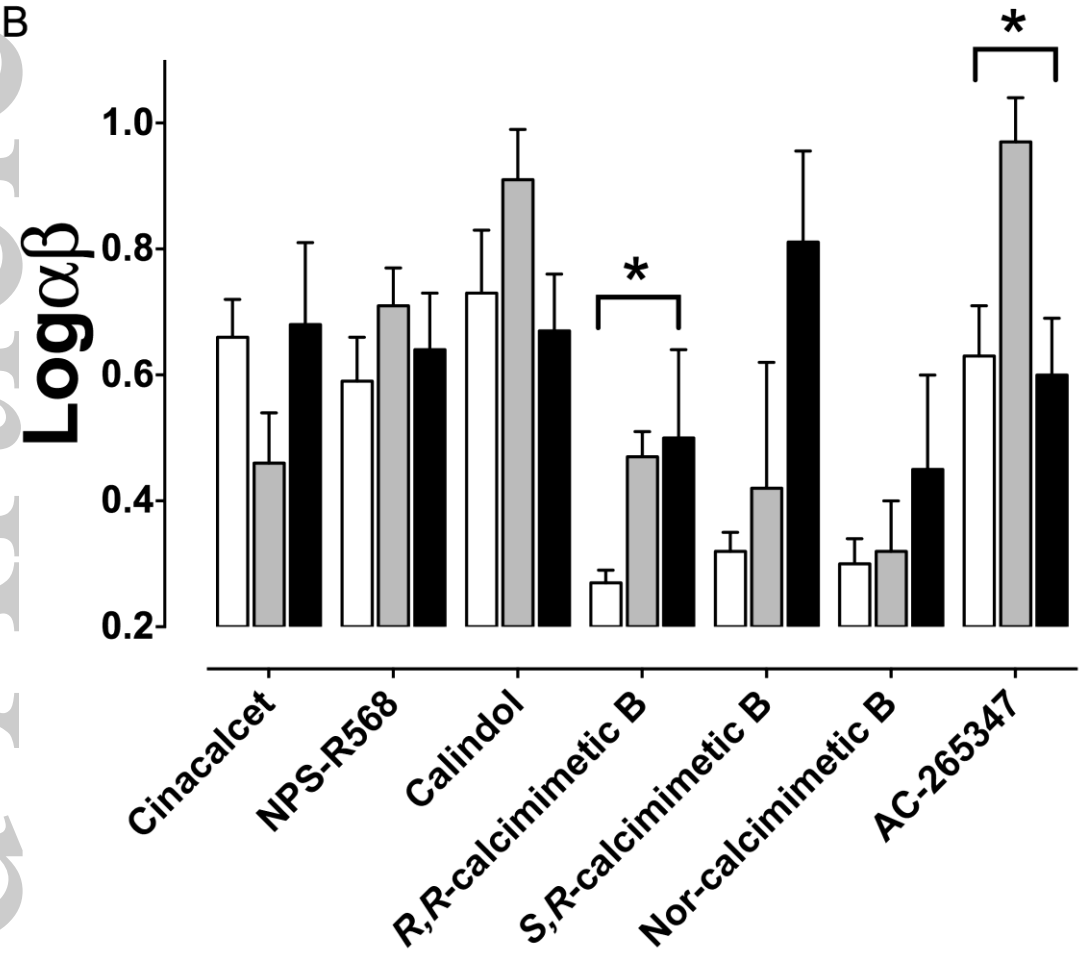

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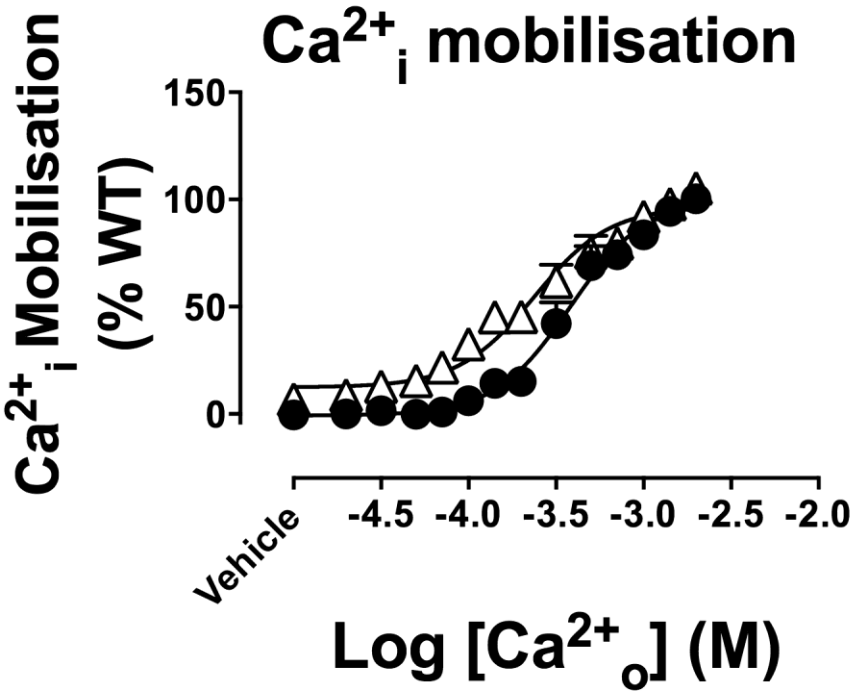

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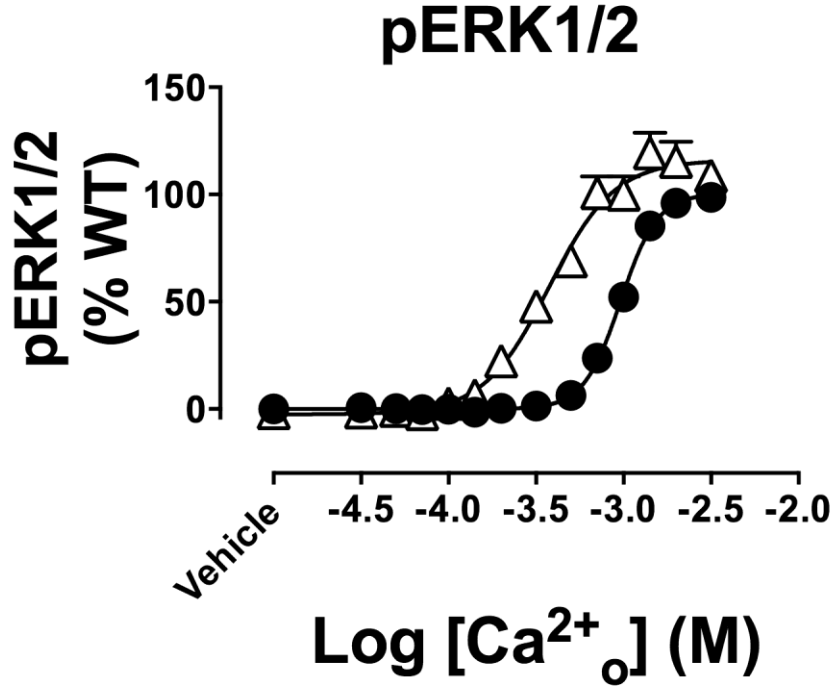

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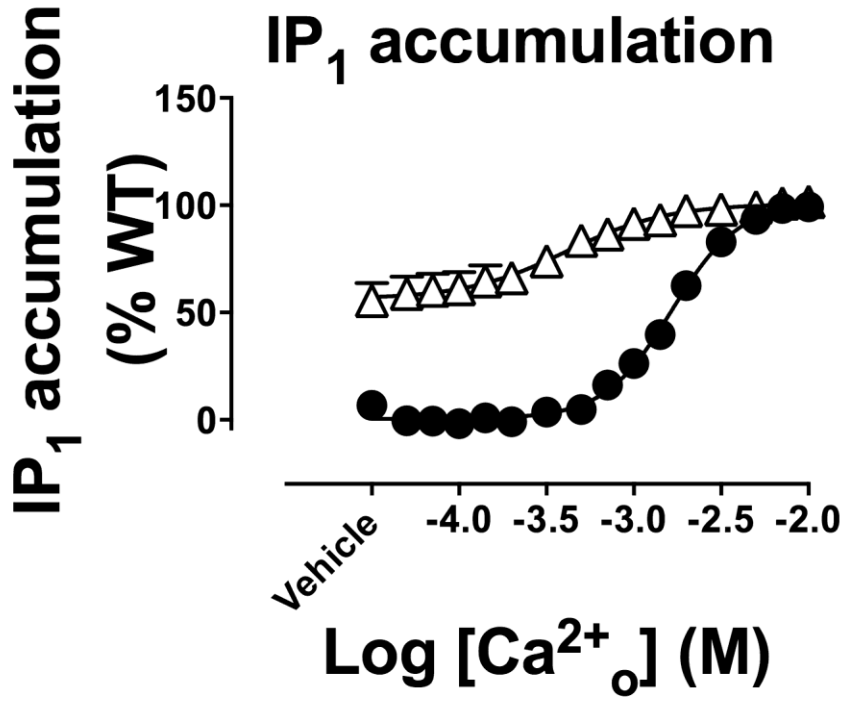

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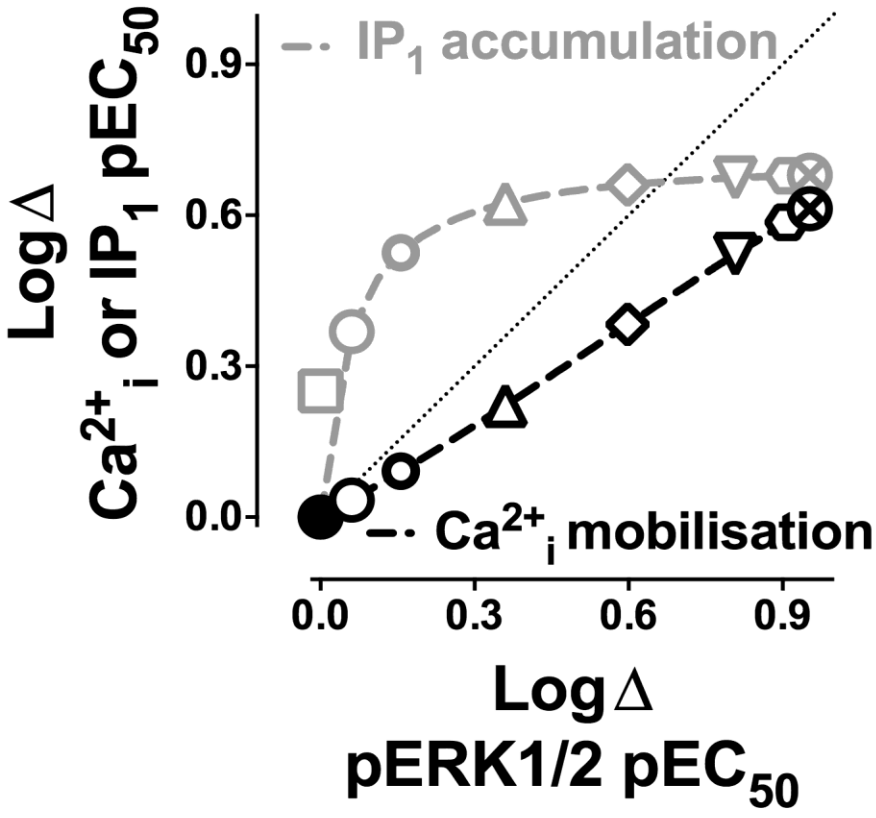

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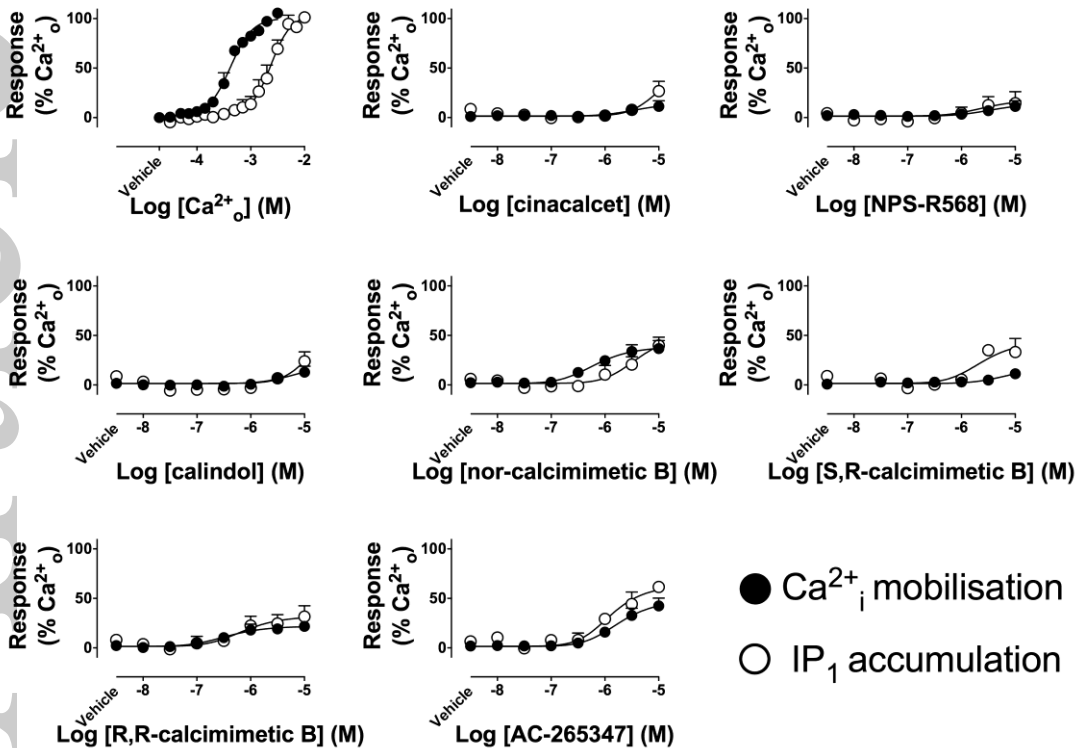

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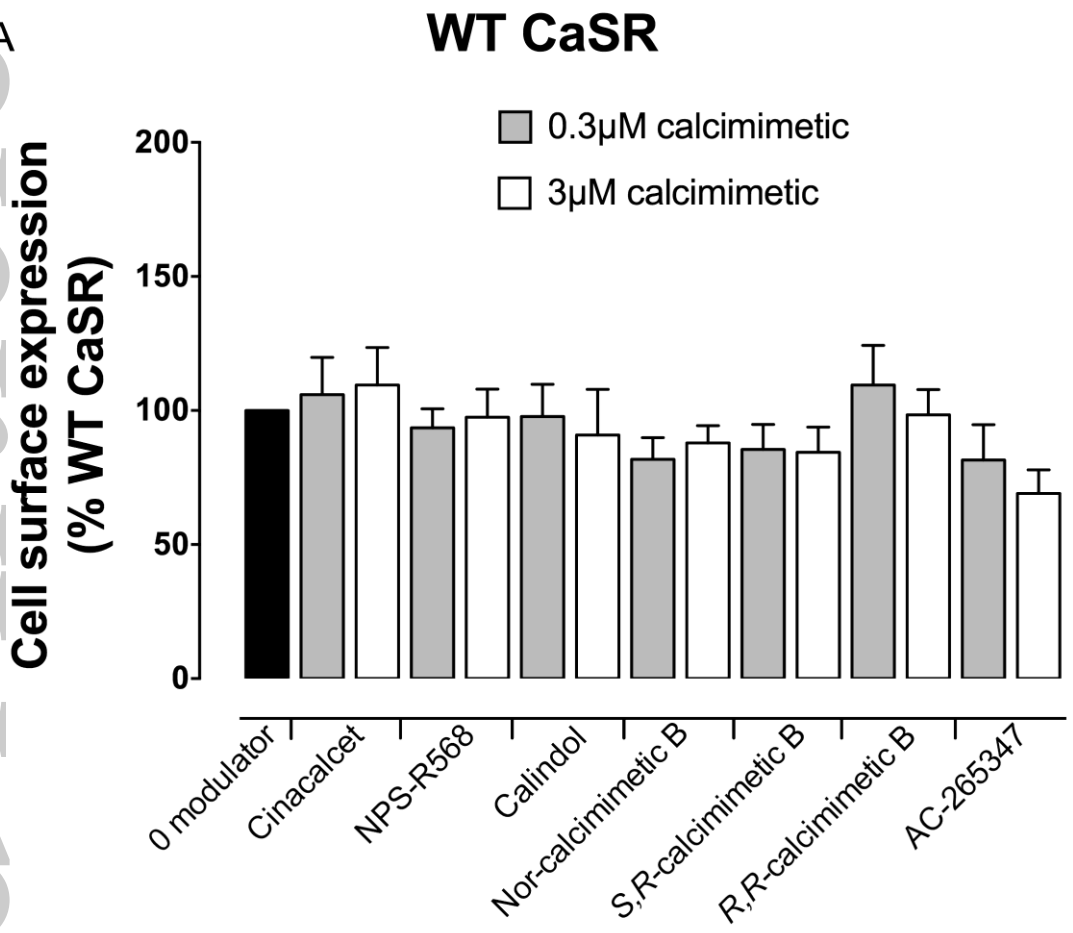

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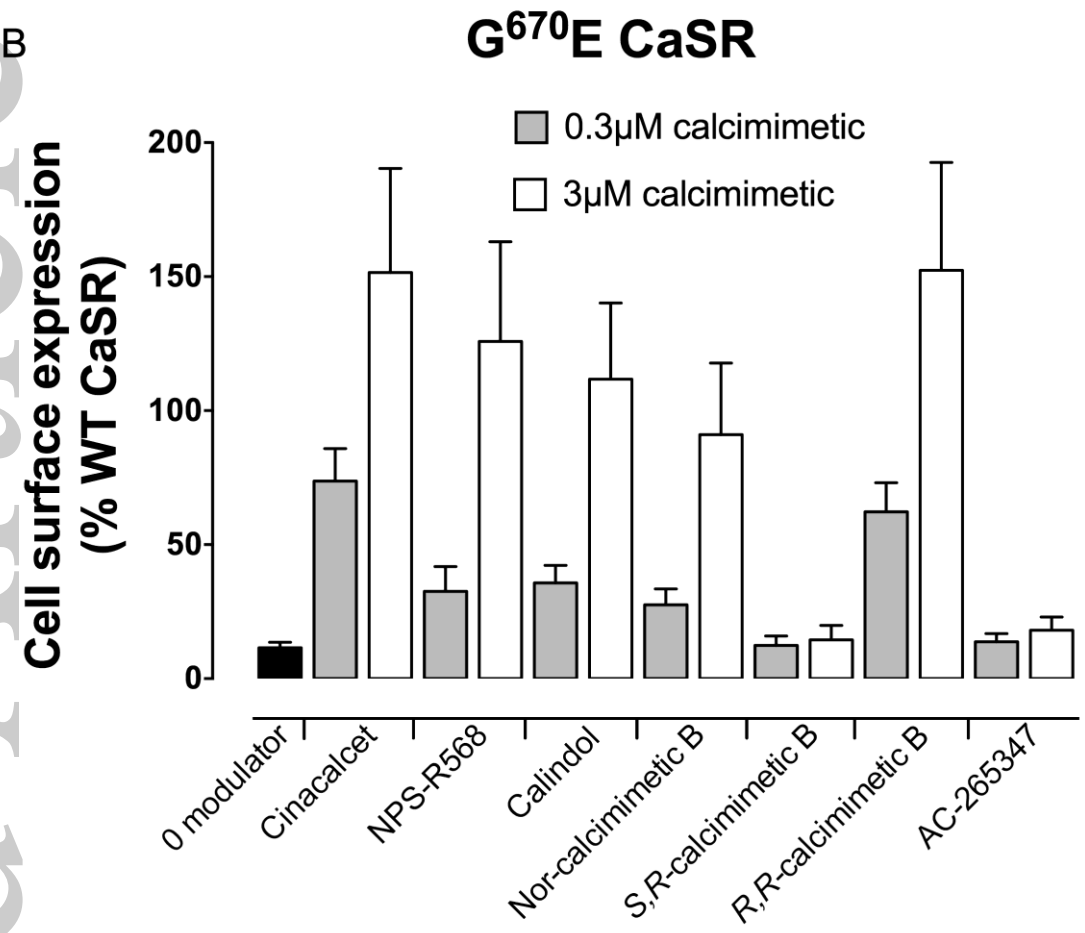

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Table 1. Pharmacological parameters that govern the allosteric activity of CaSR modulators in Ca^{2+}_i mobilisation, pERK1/2 and IP_1 accumulation assays. The potency of Ca^{2+}_o in the presence of increasing concentrations of modulator was fitted to an allosteric ternary complex model (Equation 2) to quantify the equilibrium dissociation constant (pK_B) and cooperativity ($\alpha\beta$) of the modulators at the human CaSR, using a model in which the binding affinity was not constrained across pathways.

Grouped data analysis

	Ca^{2+}_i mobilisation		**pERK1/2**		**IP_1 accumulation**	
	pK_B ± s.e.m. (n)	***Log$\alpha\beta$ ± s.e.m. ($\alpha\beta$)***	***pK_B ± s.e.m. (n)***	***Log$\alpha\beta$ ± s.e.m. ($\alpha\beta$)***	***pK_B ± s.e.m. (n)***	***Log$\alpha\beta$ ± s.e.m. ($\alpha\beta$)***
Cinacalcet	5.98 ± 0.18 (18)[a]	0.66 ± 0.06 (4.6)[a]	5.93 ± 0.29 (13)[a]	0.46 ± 0.08 (2.9)[a]	6.14 ± 0.33 (4)	0.68 ± 0.13 (4.8)
NPS-R568*	6.57 ± 0.19 (15)	0.59 ± 0.07 (3.9)	5.64 ± 0.18 (4)	0.71 ± 0.06 (5.1)	6.76 ± 0.24 (4)	0.64 ± 0.09 (4.3)
Calindol*	6.33 ± 0.23 (4)	0.73 ± 0.10 (5.4)	5.16 ± 0.16 (4)	0.91 ± 0.08 (8.1)	6.35 ± 0.23 (4)	0.67 ± 0.09 (4.7)
***S,R*-Calcimimetic B**	5.53 ± 0.16 (4)	0.32 ± 0.03 (2.1)	5.31 ± 0.68 (3)	0.42 ± 0.20 (2.6)	5.18 ± 0.31 (3)	0.81 ± 0.14 (6.5)
R,R*-Calcimimetic B	7.15 ± 0.17 (4)	0.27 ± 0.02 (1.9)	7.08 ± 0.18 (4)	0.47 ± 0.04 (3.0)	7.03 ± 0.54 (4)	0.50 ± 0.14 (3.2)
nor-calcimimetic B	6.90 ± 0.25 (7)	0.30 ± 0.04 (2.0)	6.80 ± 0.42 (5)	0.32 ± 0.08 (2.1)	7.29 ± 0.58 (4)	0.45 ± 0.15 (3.0)

AC-265347*	6.42 ± 0.22 (5)	0.63 ± 0.08 (4.3)	6.26 ± 0.13 (4)	0.97 ± 0.07 (9.3)	7.99 ± 0.26 (4)	0.60 ± 0.09 (4.0)

[a]Data sets taken from those used in (Leach *et al.*, 2013)

* Significant difference in pK_B and/or $Log\alpha\beta$ between pathways ($p<0.05$, F test)

Table 2. Pharmacological parameters that govern calcimimetic agonism at the CaSR. Agonist concentration-response curves were fitted to an operational model of agonism (Equation 3) (Black & Leff, 1983) to quantify the equilibrium dissociation constant (pK_B) of the calcimimetics and their operational measure of agonism (τ_B).

	Ca^{2+}_i mobilisation		**IP_1 accumulation**	
	pK_B ± s.e.m. (n)	***$Log\tau_B$ ± s.e.m. (τ_B)***	***pK_B ± s.e.m. (n)***	***$Log\tau_B$ ± s.e.m. (τ_B)***
***R,R*-Calcimimetic B**	6.77 ± 0.23 (3)	-0.27 ± 0.04 (0.54)	6.48 ± 0.28 (3)	-0.16 ± 0.06 (0.69)
***S,R*-Calcimimetic B**	5.44 ± 0.29 (3)	-0.10 ± 0.10 (0.79)	5.89 ± 0.26 (3)	-0.06 ± 0.07 (0.87)
nor-calcimimetic B	6.44 ± 0.14 (3)	-0.10 ± 0.03 (0.79)	5.61 ± 0.29 (3)	-0.008 ± 0.09 (0.98)
AC-265347	5.94 ± 0.14 (3)	-0.02 ± 0.14 (0.95)	6.04 ± 0.18 (3)	0.08 ± 0.05 (1.1)

Table 3. Pharmacological properties of CaSR modulators at the naturally occurring $G^{670}E$ mutant. Cell surface expression of the mutant following overnight treatment with modulator was determined by FACS analysis. The potency of Ca^{2+}_o in Ca^{2+}_i mobilisation assays in the presence of increasing concentrations of modulator was fitted to an allosteric ternary complex model (Equation 2) to quantify the equilibrium dissociation constant (pK_B) and cooperativity ($\alpha\beta$) of the modulators at the $G^{670}E$ mutant.

	Cell surface expression (% WT)			**Ca^{2+}_i mobilisation**	
	0 modulator	***0.3 μM***	***3 μM***	***pK_B ± s.e.m. (n)***	***Log$\alpha\beta$ ± s.e.m. ($\alpha\beta$)***
Cinacalcet	12 ± 2	74 ± 12	152 ± 39	6.00 ± 0.19 (7)[a]	0.59 ± 0.06 (3.9)[a]
NPS-R568		33 ± 9	126 ± 37	6.61 ± 0.14 (4)	0.74 ± 0.14 (5.5)
Calindol		36 ± 7	112 ± 28	6.33 ± 0.31 (3)	0.53 ± 0.10 (3.4)
***R,R*-calcimimetic B**		62 ± 11	152 ± 40	5.27 ± 0.37 (4)	0.51 ± 0.12 (3.2)
***S,R*-calcimimetic B**		12 ± 3	14 ± 5	Not performed	Not performed
nor-calcimimetic B		28 ± 6	91 ± 27	6.21 ± 0.23 (3)	0.42 ± 0.06 (2.6)
AC-265347		14 ± 3	18 ± 5	6.62 ± 0.23 (3)	0.72 ± 0.10 (5.2)

[a]Data sets taken from those used in (Leach *et al.*, 2013)