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1 **Biased allosteric modulation at the CaSR engendered by structurally diverse**
2 **calcimimetics**

3 Running title: Biasing CaSR signalling with calcimimetics

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22

23 **Summary**

24 Background and purpose

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

31 Experimental approach

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

35 Key results

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

42 Conclusions and implications

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

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

51

52

53 **Abbreviations:**

54 Ca^{2+}_{o}, extracellular calcium

55 Ca^{2+}_{i}, intracellular calcium

56 CaSR, calcium sensing receptor

57 FHH, familial hypocalciuric hypercalcaemia

58 Mg^{2+}_{o}, extracellular magnesium

59 NSHPT, neonatal severe hyperparathyroidism

60 pERK1/2, ERK1/2 phosphorylation

61 PTH, parathyroid hormone

62

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65

66 Introduction

67 The human calcium sensing receptor (CaSR) is a family C G protein-coupled receptor
68 (GPCR) primarily responsible for the regulation of extracellular calcium (Ca^{2+}_o)
69 concentrations in the body. When Ca^{2+}_o rises, activation of the CaSR expressed in the
70 parathyroid gland suppresses the secretion of parathyroid hormone (PTH). The drop
71 in circulating PTH levels results in reduced renal Ca^{2+}_o reabsorption and reduced bone
72 resorption (reviewed in Brown, 2013). Additionally, CaSR activation in the kidney
73 by elevated serum Ca^{2+}_o inhibits Ca^{2+}_o reabsorption, leading to enhanced renal Ca^{2+}_o
74 excretion independently of changes in PTH (Kantham et al., 2009; Loupy et al.,
75 2012). Elevated serum Ca^{2+}_o also decreases bone resorption via CaSRs expressed on
76 osteoblasts and osteoclasts (see Marie, 2010 for a review) and by stimulation of
77 calcitonin secretion via CaSRs expressed on thyroid C cells (Freichel et al., 1996).
78
79 The CaSR also has non-calciostatic roles. Thus, it mediates the modulation of blood
80 pressure (see Smajilovic et al., 2011 for a review) and protection against vascular
81 calcification (Alam et al., 2009), stimulation of gastrointestinal hormone secretion
82 (Feng et al., 2010; Mace et al., 2012), modulation of electrolyte and water transport in
83 the colon and kidney (reviewed in Macleod, 2013) and modulation of the proliferation
84 and differentiation of numerous cell types, including colonic epithelial cells,
85 keratinocytes, adipocytes and neurones.
86
87 Given its ubiquitous expression throughout the body and functionally diverse roles,
88 drugs that target the CaSR may have therapeutic application in various clinical
89 contexts. However, these drugs may also produce adverse effects arising from actions

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90 in multiple tissues expressing the CaSR. Indeed, patients treated with the
91 calcimimetic, cinacalcet ((αR)-(−)-α-methyl-N-[3-[3-[trifluoromethylphenyl]propyl]-
92 1-napthalenemethanamine hydrochloride), a positive allosteric CaSR modulator
93 indicated for the treatment of secondary and some forms of primary
94 hyperparathyroidism, have a tendency to develop adverse effects that restrict its use to
95 only severely affected patients. The most problematic adverse effect is
96 hypocalcaemia (Chonchol et al., 2009), likely resulting from both suppressed renal
97 calcium reabsorption induced by CaSR activation in the kidney, and calcitonin-
98 mediated inhibition of bone resorption via CaSR activation in the thyroid C-cells
99 (Arenas et al., 2013). Thus, novel calcimimetics that selectively stimulate CaSR-
100 mediated signalling in the parathyroid gland without affecting CaSRs in other tissues
101 may have an improved side effect profile and enable treatment of less severe grades of
102 hyperparathyroidism.
103
104 One approach to directing desired physiological outcomes of GPCR activation is to
105 selectively target those intracellular signalling pathways that couple to the anticipated
106 effect, while avoiding those that couple to unwanted consequences. Such selectivity
107 can be achieved with a drug that binds to and favours a receptor conformation that
108 preferentially couples to a subset of desired intracellular signalling pathways
109 (Kenakin, 2011). This concept is referred to as ligand-biased signalling, ligand-
110 directed trafficking of receptor stimulus, functional selectivity or biased agonism
111 (Kenakin & Christopoulos, 2013).
112
113 The CaSR is subject to ligand-biased signalling on a number of levels (Leach et al.,
114 2014). First, it binds multiple endogenous ligands, including Ca^{2+}_o, Mg^{2+}_o, L-amino

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115 acids, polyamines and the glutamyl peptide, γ-glutathione. Ca^{2+}_o, spermine and L-
116 phenylalanine have been demonstrated to preferentially activate distinct signalling
117 pathways (Rey et al., 2010; Thomsen et al., 2012a), suggesting that each ligand has
118 the propensity to stabilise a subset of preferred receptor states and subsequently
119 stimulate the repertoire of intracellular signalling proteins that couple to these states.
120 Second, positive allosteric modulators of the CaSR, such as cinacalcet, and negative
121 CaSR modulators (calcilytics), such as NPS-2143 (2-chloro-6-[(2R)-3-[[1,1-dimethyl-
122 2-(2-naphthalenyl)ethyl]amino-2-hydroxypropoxy]benzonitrile hydrochloride),
123 engender biased allosteric modulation at the CaSR, such that they exhibit greater
124 modulation of some pathways over others (Davey et al., 2012; Leach et al., 2013).
125 Third, the "natural bias" of the CaSR can be altered in pathophysiological states. This
126 has been demonstrated by naturally occurring mutations in the CaSR protein that alter
127 its usual signalling bias (Leach et al., 2012), a switch in CaSR signalling from $G_{i/o}$ to
128 G_s in human breast cancer cells (Mamillapalli et al., 2008), and an autoantibody
129 directed against the CaSR in a patient with acquired hypocalciuric hypercalcemia,
130 which potentiated inositol phosphate (IP) accumulation, yet inhibited ERK1/2
131 phosphorylation (pERK1/2) (Makita et al., 2007). Finally, the complement of
132 intracellular signalling proteins to which the CaSR couples differs between cell types,
133 thus, the capacity of the CaSR to couple to different signalling pathways depends
134 upon its tissue-specific expression.
135
136 Proof-of-concept that tissue-specific effects can be achieved by targeting the CaSR
137 with drugs was evident from early experiments with the prototypical calcimimetic,
138 NPS-R568. During the development of the phenylalkylamine calcimimetics (e.g.
139 NPS-R568 and cinacalcet), it was recognised that the natural hypocalcaemic effects of

140 these drugs may be complicated by stimulation of calcitonin release via activation of
141 CaSRs in the thyroid. Thus, the need to suppress PTH secretion with minimal effects
142 on calcitonin secretion was acknowledged (Fox et al., 1999a; Fox et al., 1999b), but
143 remains sub optimally addressed.
144
145 Third generation agents appear to have enhanced tissue-selective effects. This is
146 evident from studies with the novel dibenzylamine calcimimetic, R,R-calcimimetic B
147 (R-1-(6-methoxy-4'-(trifluoromethyl)-3-biphenylyl)-N-(R)-1-phenylethyl)ethanamine)
148 and the structurally distinct calcimimetic, AC-265347 (1-benzothiazol-2-yl-1-(2,4-
149 dimethyl-phenyl)-ethanol). Both calcimimetics inhibit PTH secretion at
150 concentrations that do not induce calcitonin release in rats (Henley et al., 2011; Ma et
151 al., 2011), demonstrating a means for normalising serum PTH and calcium levels
152 without causing uncontrolled hypocalcaemia. How these compounds achieve this
153 tissue specificity is unknown, but we hypothesise that it may be a result of ligand-
154 biased allosteric modulation at the CaSR. This is based on the fact that distinct
155 intracellular signalling pathways activated by the CaSR are responsible for its
156 physiological effects, thus drugs may selectively promote suppression of PTH release
157 by preferentially activating the pathways that couple to that response. For instance,
158 CaSR suppression of PTH release is driven by phospholipase C (PLC)-mediated IP_3
159 production (Brown et al., 1987; Kifor et al., 1997) and pERK1/2 (Corbetta et al.,
160 2002) but there is some evidence that CaSR-mediated Ca^{2+}_i release is not required for
161 inhibition of PTH from bovine parathyroid cells (Russell et al., 1999). Stimulation of
162 both PLC and Ca^{2+}_i mobilisation have been linked to the release of calcitonin (Liu et
163 al., 2003; McGehee et al., 1997; Thomsen et al., 2012b) but in rat 6-23 medullary
164 thyroid carcinoma cells, inhibition of pERK1/2 has no effect on Ca^{2+}_o-mediated

165 stimulation of calcitonin release (Thomsen et al., 2012b). Thus, drugs that bias CaSR
166 signalling towards pERK1/2 may achieve tissue-selective suppression of PTH
167 secretion in the absence of calcitonin release.
168
169 To probe the ligand-biased signalling profile(s) required to achieve drug tissue
170 selectivity, pathways that mediate distinct physiological receptor functions should
171 ideally be dissected in systems such as primary or immortalised cells that maintain
172 their physiological function. However, for the CaSR, this has been hampered by a
173 lack of relevant cell lines and methods to study, for instance, parathyroid cell
174 function. We have developed techniques to measure signalling in, and PTH release
175 from, primary human parathyroid cells (Avlani et al., 2013; Broadhead et al., 2011;
176 Mun et al., 2009) but performing high throughput experiments in these cells is at
177 present not possible. Thus, most studies of this nature must rely on recombinant cell
178 systems to investigate CaSR signalling in response to agonists and drugs.
179 Nonetheless, recombinant systems can still be used to identify bias and validate
180 whether compounds with desirable in vivo properties have unique pharmacology in
181 vitro, and vice versa.
182
183 The current study thus primarily aimed to use a recombinant cell system to determine
184 the potential for structurally distinct calcimimetics to engender ligand-biased
185 signalling and subsequently promote coupling of the CaSR to three key signalling
186 pathways that could mediate different physiological effects; IP_1 accumulation (a stable
187 metabolite of IP_3), $Ca^{2+}{}_i$ mobilisation and pERK1/2. Furthermore, we have
188 previously shown that CaSR modulators can be biased in their ability to modulate
189 signalling versus trafficking at the CaSR (Leach et al., 2013). Therefore, in addition

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190 to acute signalling at the CaSR, we determined the ability of the calcimimetics to act
191 as pharmacochaperones of a naturally occurring mutant CaSR, $G^{670}E$. Differential
192 effects on trafficking versus signalling may have important implications for the
193 treatment of calcium handling disorders caused by mutations in the CaSR gene that
194 result in a diverse range of molecular phenotypes.
195

196 **Materials and Methods**

197

198 Synthesis of calcimimetics
199 Synthesis of R,R-calcimimetic B (compound **3b** – appendix S1), its diastereoisomer
200 S,R-calcimimetic B (compound **3a** – appendix S1) and nor-calcimimetic B
201 (compound **3c** – appendix S1) was achieved using a two-step procedure derived from
202 described literature (Harrington et al., 2010). Full synthetic details and compound
203 characterisation are given in Appendix S1. NPS-R568 and cinacalcet were prepared
204 as described previously (Davey et al., 2012). Calindol was purchased from Tocris
205 Biosciences, whereas AC-265347 was from Sigma-Aldrich.
206

207 Cell culture
208 Generation of FlpIn HEK293 TRex cells (Invitrogen) stably expressing the human
209 CaSR under the control of tetracycline has been described previously (Davey et al.,
210 2012; Leach et al., 2012). Cells were maintained in DMEM with 10% FBS, 200 µg
211 ml-1 hygromycin B and 5 µg ml-1 blasticidin.
212

213 Optimisation of assay conditions

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

225

226 $Ca^{2+}{}_{i}$ mobilisation assays

227 Cells were seeded in a clear 96-well plate coated with poly-D-lysine (50 µg ml-1) at
228 80,000 cells per well and incubated overnight in the presence of 100 ng ml-1
229 tetracycline. The following day, cells were washed with 200 µl assay buffer (150 mM
230 NaCl, 2.6 mM KCl, 1.18 mM $MgCl_2$, 10 mM D-Glucose, 10 mM HEPES, 0.1 mM
231 $Ca^{2+}{}_{o}$, 0.5 % BSA and 4 mM probenecid at pH 7.4) and loaded with 100 µl Fluo-4
232 AM (1 µM) for 1 h at 37 °C.

233

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

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239 Flexstation® 1 or 3 (Molecular Devices; Sunnyvale, California). Fluorescence was
240 detected for 60 s at 485 nm excitation and 525 nm emission but the peak Ca^{2+}_i
241 mobilisation response (approximately 12 seconds after agonist addition) was used for
242 the subsequent determination of the agonist response. We have previously shown that
243 when allosterism at the CaSR is quantified in Ca^{2+}_i mobilisation assays using the
244 potency of Ca^{2+}_o obtained by plotting the area under the 60 second Ca^{2+}_i mobilisation
245 trace, no significant difference in signalling or biased modulation is observed in
246 comparison to parameters derived using the peak Ca^{2+}_i mobilisation response (Leach
247 et al., 2013). Relative peak fluorescence units were normalised to the fluorescence
248 stimulated by ionomycin to account for differences in cell number and loading
249 efficiency, and further normalised to the maximum response observed for the WT
250 CaSR in the absence of modulator.
251
252 Extracellular regulated kinase 1/2 (ERK1/2) phosphorylation assays
253 Cells were seeded at 80,000 cells per well into a poly-D-lysine coated (50 μg ml-1)
254 transparent 96-well plate and grown overnight with 100 ng ml-1 tetracycline. The
255 following day, cells were washed twice with PBS and serum-free DMEM containing
256 16 mM HEPES and 0.1 mM Ca^{2+}_o was added to wells. Vehicle or agonist (Ca^{2+}_o)
257 with or without modulator were coadded to wells and incubated for 2.5 minutes (the
258 time determined in prior assays for pERK1/2 to peak) at 37°C. All data were
259 normalised to the response stimulated by 10% FBS and then further normalised to the
260 maximum response stimulated by Ca^{2+}_o in the absence of modulator. pERK1/2 was
261 determined using the SureFire pERK1/2 assay kit (kindly donated by Dr Michael
262 Crouch, TGR biosciences, Adelaide) employing AlphaScreen technology

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263 (PerkinElmer). All other details are as described previously (Leach et al., 2013; Leach
264 et al., 2012).
265
266 IPone accumulation assays
267 Following overnight induction of receptor expression with 100 ng ml-1 tetracycline in
268 a T175cm^2 flask (where appropriate), cells were harvested and resuspended in assay
269 buffer (150 mM NaCl, 2.6 mM KCl, 1.18 mM $MgCl_2$, 10 mM D-Glucose, 10 mM
270 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
271 with or without modulator were added to wells of a 384 well white proxiplate
272 (PerkinElmer) and 7 µl cells (1x10^4 cells) were added to these wells, centrifuged for 1
273 minute at 350 x g and incubated at 37°C for 45 minutes. The IP-One Tb™ assay kit
274 (CisBio, France) was used to detect myo-inositol 1 phosphate (IP_1), based on
275 fluorescence resonance energy transfer (FRET) between d2-conjugated IP_1 and
276 Lumi4™-Tb cryptate conjugated anti-IP_1 antibody. These reagents were diluted 1:30
277 with lysis buffer and 3 µl of each was added to wells following agonist stimulation.
278 Lysates were incubated for 1 hour and FRET was detected using an Envision plate
279 reader (PerkinElmer) where emission of Lumi4™-Tb cryptate was detected at 620 nm
280 and emission of d2-conjugated IP_1 at 665 nm. Results were calculated from the 665
281 nm / 620 nm ratio. Data were normalised to the maximum response stimulated by
282 $Ca^{2+}{}_o$ in the absence of modulator.
283
284 Flow cytometry analysis for receptor expression
285 FlpIn HEK293 TRex cells stably expressing the human wild-type (WT) or $G^{670}E$
286 mutant CaSR were seeded in a 96-well plate at a density of 80,000 cells per well in
287 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

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

315
$$\mathrm{Y{=}Bottom{+}}\frac{\mathrm{(Top\text{-}Bottom)}\quad (\mathrm{A}^{\mathrm{nH}})}{\mathrm{A}^{\mathrm{nH}}+\mathrm{EC}_{50}{}^{\mathrm{nH}}} \qquad (1)$$

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

320

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

324
$$\mathrm{pEC_{50}{=}Log}\left[10^{\mathrm{Log}} \quad \times[\mathrm{B}]+10^{\text{-}\mathrm{pK_B}}\right]\text{-}\,\mathrm{Logd} \qquad (2)$$

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

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337 For the “cooperativity bias plot”, the pEC_{50} of $Ca^{2+}{}_{o}$ in the absence and presence of
338 modulator in IP_1 accumulation, $Ca^{2+}{}_{i}$ mobilisation and pERK1/2 assays was first fitted
339 to equation 2 and 150 XY coordinates of points that defined the curve that best fit
340 equation 2 were determined. Next, the XY coordinates for the different pathways
341 were plotted against one another, with IP_1 accumulation or $Ca^{2+}{}_{i}$ mobilisation data on
342 the y-axis against pERK1/2 data on the x-axis. XY coordinates corresponding to the
343 effects of 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 μM modulator are respresented by
344 symbols on the plots. If the allosteric modulator shows equal cooperativity in the
345 assays, the data points will be coincident and the cooperativity bias plots will overlap
346 with the line of identity. If, however, the modulator exerts greater cooperativity in
347 one of the pathways, the points will fall either side of this line towards the preferred
348 pathway.
349

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

352 $$E=\frac{E_m \quad {}_{B} \quad [B^n]}{K_B+[B^n] \quad \left(\quad {}_{B}+1\right)} \qquad (3)$$

353 where E is the effect (response) stimulated by the allosteric agonist, E_m is the
354 maximum response of the system stimulated by the full agonist ($Ca^{2+}{}_{o}$), τ_B is an
355 operational measure of allosteric agonist efficacy, defined as the inverse of the
356 fraction of receptors that must be occupied by agonist to obtain the half-maximal
357 response, [B] is the allosteric agonist concentration and n is the transducer slope.
358

359 **Results**

360 Rationale for choice of ligands and signalling pathways

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361 The structures of the calcimimetics used in this study are shown in Figure 1. The
362 prototypical phenylalkylamine calcimimetics, cinacalcet and NPS-R568 (3-(2-
363 chlorophenyl)-N-((1R)-1-(3-methoxyphenyl)ethyl)-1-propanamine) have been well
364 characterised in vitro and in vivo (Nemeth et al., 2004; Nemeth et al., 1998). Calindol
365 ((R)-2-[N-(1-(1-naphthyl)ethyl)aminomethyl]indole) was the most potent
366 calcimimetic identified at the Institut de Chimie des Substances Naturelles (ICSN,
367 France) from a series of diamines based around the structure of NPS-R568 (Kessler et
368 al., 2004). R,R-calcimimetic B was the most potent CaSR ligand identified by Amgen
369 in a dibenzylamine series and exhibited ideal in vivo pharmacodynamics. In an IP
370 accumulation assay, R,R-calcimimetic B was estimated to have greater affinity than
371 NPS-R568 (Harrington et al., 2010; Henley et al., 2011). The published synthesis of
372 R,R-calcimimetic B employed a route yielding a diastereomeric ratio (d.r.) of 14:1 of
373 R,R-calcimimetic B and the corresponding S,R-diastereoisomer (S-1-(6-methoxy-4'-
374 (trifluoromethyl)-3-biphenylyl)-N-(R)-1-phenylethyl)ethanamine) respectively, which
375 were then separated via HPLC (Harrington et al., 2010). S,R-calcimimetic B was
376 100-fold less potent than R,R-calcimimetic B (Harrington et al., 2010), comparable to
377 the stereoselectivity of the R- and S-isomers of NPS- 568 and cinacalcet (Hammerland
378 et al., 1998; Nemeth et al., 2004). Given the remarkable difference in potency of the
379 individual diastereoisomers, we sought to isolate and further characterise each one
380 independently. Adapting the synthesis of Harrington et al, we were able to generate a
381 mixture of diastereoisomers with a d.r. of 4:1. These were successfully isolated by
382 either chiral HPLC or preparative layer chromatography (PLC) (see Appendix S1 for
383 full synthetic methods). Structurally, the contrasting pharmacological behaviour of
384 each diastereoisomer can be attributed to the spatial orientation of the methyl group
385 adjacent to the biphenyl and amino moieties. With this in mind, it was of interest to

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386 evaluate the pharmacological activity of the 'nor' calcimimetic B derivative (R-N-((6-
387 methoxy-4'-(trifluoromethyl)-3-biphenylyl)methyl)-1-phenylethanamine), with a
388 methylene group replacing the methyl of interest. This was synthesised in a similar
389 fashion to the R,R- and S,R-calcimimetic B derivatives. AC-265347 was identified in
390 a screen by ACADIA Pharmaceuticals as a potent calcimimetic. It is structurally
391 distinct from the phenylalkylamine calcimimetics and calcimimetic B, and was found
392 to have improved potency over cinacalcet in an IP accumulation assay (Ma et al.,
393 2011).
394
395 We investigated the effects of the calcimimetics in Ca^{2+}_i mobilisation, IP
396 accumulation and pERK1/2 assays because each of these pathways has been
397 undeniably linked to CaSR regulation of PTH release from parathyroid chief cells
398 and/or calcitonin release from thyroid C cells, as outlined in the introduction.
399 Although additional pathways are also involved in the regulation of PTH and
400 calcitonin release, we selected those for which assays can be reliably performed in a
401 high throughput manner to enable robust quantification of allosteric modulation and
402 biased signalling.
403
404 Calcimimetics are biased modulators of CaSR signalling
405 To evaluate the extent to which calcimimetics engender ligand-biased modulation at
406 the CaSR, we first characterised their ability to potentiate the endogenous agonist,
407 Ca^{2+}_o, in IP_1 accumulation, Ca^{2+}_i mobilisation and pERK1/2 assays. These
408 experiments generated Ca^{2+}_o concentration-response curves in the absence and
409 presence of the allosteric modulators.
410

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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

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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

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461 concentrations of AC-265347 can be visualised in the modulator "cooperativity bias
462 plot" as shown in Figure 4D. This illustrates the impact of equivalent AC-265347
463 concentrations on Ca^{2+}_o potency in Ca^{2+}_i mobilisation or IP_1 assays on the y-axis, and
464 pERK1/2 assays on the x-axis. If AC-265347 modulated both pathways equally, the
465 data would converge on the line of identity. However, because it modulates one
466 pathway to a greater degree than the other, the data points are distributed away from
467 the line of identity towards the preferred pathway (i.e. towards IP_1 over pERK1/2 and
468 towards pERK1/2 over Ca^{2+}_i).
469

470 Third generation calcimimetics are agonists at the CaSR

471 In IP_1 accumulation and Ca^{2+}_i mobilisation assays, the calcimimetics stimulated
472 receptor activity in the presence of vehicle (buffer) alone. AC-265347, R,R-
473 calcimimetic B and nor-calcimimetic B also did so in pERK1/2 assays. We
474 previously simulated the effects of cinacalcet on signalling in the presence of an
475 ambient concentration of agonist to reconstruct the experimental conditions under
476 which our Ca^{2+}_i mobilisation and pERK1/2 assays are undertaken (Davey et al.,
477 2012). These simulations suggested that positive modulation of ambient agonists in
478 the buffer (Ca^{2+}_o and Mg^{2+}_o) was expected. Accordingly, when we omitted ambient
479 Ca^{2+}_o and Mg^{2+}_o from the assay buffer, Ca^{2+}_i mobilisation and IP_1 accumulation
480 stimulated by cinacalcet, NPS-R568 and calindol on their own was largely inhibited
481 (Figure 5), indicating that the observed "baseline effect" was primarily due to
482 potentiation of ambient agonist activity. In contrast, AC-265347 and the calcimimetic
483 B analogues retained activity in the absence of ambient Ca^{2+}_o and Mg^{2+}_o (Figure 5).
484 The effects of omitting only Ca^{2+}_o from the buffer can be observed in Supplemental
485 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

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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

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536 related to its lower lipophilicity relative to the other compounds. This parameter can
537 be represented by calculated partition coefficient (CLog P, see Figure 1), which for
538 AC-265347 was found to be considerably lower than for the other allosteric
539 modulators tested. Thus, AC-265347 may have a reduced propensity to cross cell
540 membranes to pharmacochaperone misfolded receptors trapped in the ER and Golgi
541 compartments.
542
543 **Discussion**
544 The present study evaluated the pharmacological activity of structurally related and
545 diverse calcimimetics across multiple measures of receptor activity, identifying
546 distinct ligand-biased profiles for each compound. Importantly, whereas
547 phenylalkylamine modulators are biased towards Ca^{2+}_i mobilisation and IP_1
548 accumulation, S,R-calcimimetic B is biased only towards modulation of IP_1
549 accumulation, and nor-calcimimetic B is unbiased. R,R-calcimimetic B and AC-
550 265347 on the other hand are biased towards pERK1/2 and IP_1 accumulation. Of
551 note, although Ca^{2+}_i mobilisation via Gq-coupled receptors typically stems from the
552 PLC-IP pathway, the divergence in bias between Ca^{2+}_i and IP1 assays observed herein
553 suggests that CaSR-mediated Ca^{2+}_i mobilisation is also facilitated via alternative
554 mechanisms. This is supported by a number of previous findings. In rat medullary
555 thyroid carcinoma cells, Ca^{2+}_o activation of the CaSR resulted in Ca^{2+}_i influx via ion-
556 gated calcium channels in addition to IP3-mediated calcium mobilisation (Thomsen et
557 al., 2012b). Sr^{2+}_o, on the other hand, stimulated CaSR-mediated PLC/IP3/Ca^{2+}_i
558 mobilisation, but did not trigger opening of calcium channels in these cells (Thomsen
559 et al., 2012b). Similarly, although both Ca^{2+}_o and L-phenylalanine stimulated Ca^{2+}_i
560 mobilisation in CaSR-transfected HEK293 cells, only Ca^{2+}_o promoted IP

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561 accumulation and diacylglycerol production (Rey et al., 2005). Finally, we recently
562 showed that truncation of the CaSR after R^{866} resulted in a complete inability of the
563 receptor to stimulate Ca^{2+}_i mobilisation, whereas IP accumulation was reduced, but
564 maintained (Goolam et al., 2014). In the same study, mutations in intracellular loops
565 2 and 3 greatly impaired IP accumulation but had a weaker affect on Ca^{2+}_i
566 mobilisation. These findings suggest Ca^{2+}_i mobilisation stimulated from the CaSR is
567 in part driven via an IP-independent mechanism.
568
569 Intriguingly, although AC-265347 is a positive modulator of CaSR signalling, it is a
570 neutral modulator of receptor trafficking. These findings build on our earlier studies
571 of prototypical CaSR positive and negative allosteric modulators that initially
572 identified bias in modulation by these compounds (Davey et al., 2012; Leach et al.,
573 2013).
574
575 Ligand-biased signalling by CaSR modulators may be driven by ligand-specific
576 stabilisation of distinct receptor states that couple preferentially to particular
577 intracellular signalling pathways. This is suggested by the different functional
578 affinities or cooperativities with the endogenous agonist estimated at each pathway.
579 We introduced this concept several years ago (Leach et al., 2007) and have
580 subsequently observed biased allosteric modulation at the M_4 muscarinic (Leach et
581 al., 2010), A_1 adenosine (Aurelio et al., 2009) and glucagon-like peptide 1 (GLP-1)
582 (Koole et al., 2011) receptors, indicating that pathway selectivity may be achieved
583 with allosteric modulators acting at a number of GPCRs.
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585 AC-265347 exhibited high cooperativity in pERK1/2 assays, maximally enhancing
586 the potency of Ca^{2+}_o nearly 10-fold, in comparison to the 3-fold enhancement in
587 potency observed with cinacalcet. This is consistent with previous findings indicating
588 that AC-265347 is more potent than cinacalcet with respect to IP_1 accumulation
589 assays but has comparable potency with respect to cellular proliferation (Ma et al.,
590 2011). This suggests that AC-265347 exhibits ligand-biased modulation of distinct
591 CaSR signalling pathways. pERK1/2 plays a significant role in the suppression of
592 PTH release (Corbetta et al., 2002; Thomsen et al., 2012b) but may be less important
593 for CaSR-mediated stimulation of calcitonin release (Thomsen et al., 2012b). Thus,
594 compounds that favour pERK1/2 over Ca^{2+}_i mobilisation may have reduced
595 propensity to induce calcitonin-dependent hypocalcaemia when compared to
596 cinacalcet. Accordingly, there is pronounced separation (300-fold) in the
597 concentration of S-AC-265347 required to suppress serum PTH levels versus the
598 concentration that reduces serum Ca^{2+}_o levels in healthy rats (Ma et al., 2011).
599 Similarly, concentrations of calcimimetic B that maximally inhibit PTH secretion in
600 nephrectomised rats have little effect on calcitonin release or serum Ca^{2+}_o levels
601 (Henley et al., 2011). In contrast, cinacalcet concentrations required to maximally
602 suppress PTH secretion also stimulate calcitonin release and reduce serum Ca^{2+}_o
603 levels in rats (Nemeth et al., 2004), suggesting less selectivity of cinacalcet for
604 suppression of PTH release. AC-265347 and R,R-calcimimetic B are thus potentially
605 important lead compounds of value in elucidating the roles of pERK1/2 in CaSR-
606 mediated regulation of PTH and calcitonin release.
607
608 The fact that third generation but not phenylalkylamine calcimimetics are agonists in
609 their own right may also contribute to their parathyroid selectivity. When stimulus-

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610 response coupling is strong, for instance in tissues such as the parathyroid glands
611 where CaSR expression is high, partial agonist effects will become more pronounced.
612
613 The CaSR is promiscuous in its coupling to intracellular signalling pathways, and the
614 influence of individual pathways to physiological outcomes such as regulation of
615 hormone release from chief cells of the parathyroid and parafollicular C cells of the
616 thyroid, and control of ion transport in the kidney, is still being elucidated. Although
617 we have selected to investigate the modulatory effects of calcimimetics on three key
618 signalling pathways that regulate some of the physiological actions of the CaSR, these
619 pathways are not exhaustive. For instance, G12/13-mediated cytoskeletal
620 rearrangements are important for CaSR-mediated suppression of PTH release (Quinn
621 et al., 2007) but experiments that measure G12/13-mediated membrane ruffling, for
622 instance, are not amenable to high throughput screening techniques and have
623 subsequently not been included in the present study. Our ongoing work aims to
624 extend these studies to examine activity across multiple pathways in primary cell
625 lines, to establish the link between signalling bias and in vivo pharmacological and
626 physiological calcimimetic effects.
627
628 It must also be noted that allosterism may be influenced by the kinetics of ligand
629 binding relative to the different time points underlying response generation in each
630 experiment. Thus, an alternative explanation for the observed bias is that each ligand
631 stabilises the same state with different kinetics. However, the same direction of bias
632 towards Ca^{2+}_i mobilisation over pERK1/2 is also observed following preincubation of
633 the CaSR with cinacalcet and NPS-R568 for 30 minutes prior to measurement of
634 agonist-mediated receptor signalling (Davey et al., 2013). Thus, differences in

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

643

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

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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.
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689

690 **AUTHOR CONTRIBUTIONS:**

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

695

696

Accepted Article

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925 **Figure 1 Structure of the CaSR allosteric modulators examined in this**
926 **study.** Calculated partition coefficient (CLog P) obtained from PerkinElmer
927 ChemBioDraw software are shown.
928
929 **Figure 2 Structurally distinct calcimimetics potentiate Ca^{2+}_{o}-mediated**
930 **receptor activation with different potencies.** Ca^{2+}_{o}-mediated IP_1 accumulation in
931 the presence of 0 (●), 0.003 μM (□), 0.01 μM (○), 0.03 μM (○), 0.1 μM (△),
932 0.3 μM (◇), 1 μM (▽), 3 μM (⬡) and 10 μM (⊗) cinacalcet (A), R,R-
933 calcimimetic B (B) and AC-265347 (C). Data are mean + s.e.m from at least 4
934 independent experiments performed in duplicate.
935
936 **Figure 3 Calcimimetics display distinct functional affinities and/or**
937 **cooperativities for CaSR conformations that couple to different signalling**
938 **pathways.** Modulator functional affinities (functional pK_B) and cooperativities (αβ)
939 were determined as described in the Methods, by fitting the Ca^{2+}_{o} pEC_{50} in the
940 absence and presence of modulator determined in Ca^{2+}_{i} mobilisation (white bars),
941 pERK1/2 (grey bars) and IP_1 accumulation (black bars) assays to an allosteric ternary
942 complex model (equation 2). The affinity of the modulator was unconstrained in each
943 pathway. Statistical differences shown by asterisks are demonstrated where an F-test
944 determined that the data were fitted best when the modulator affinities and
945 cooperativies were different between the three pathways. Data are mean + s.e.m from
946 at least 4 independent experiments performed in duplicate.
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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 (▲) 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 (△), 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-

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

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980 **DISCLOSURE STATEMENT:**

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

984

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988 Principal Research Fellows of the NHMRC.

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990 **COMPETEING INTERESTS:**

991 None

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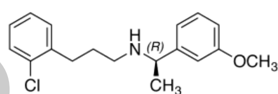
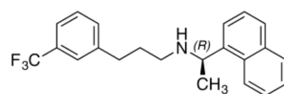


NPS-R568
CLogP: 4.92



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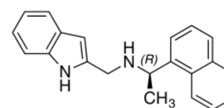

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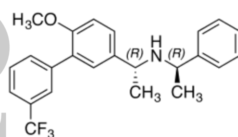

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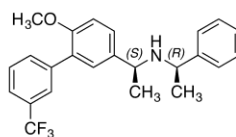
CLogP: 5.47



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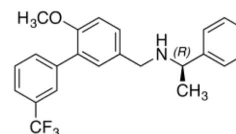
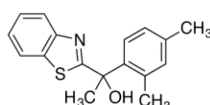


nor-Calcimimetic B
CLogP: 5.16



AC-265347
CLogP: 3.74

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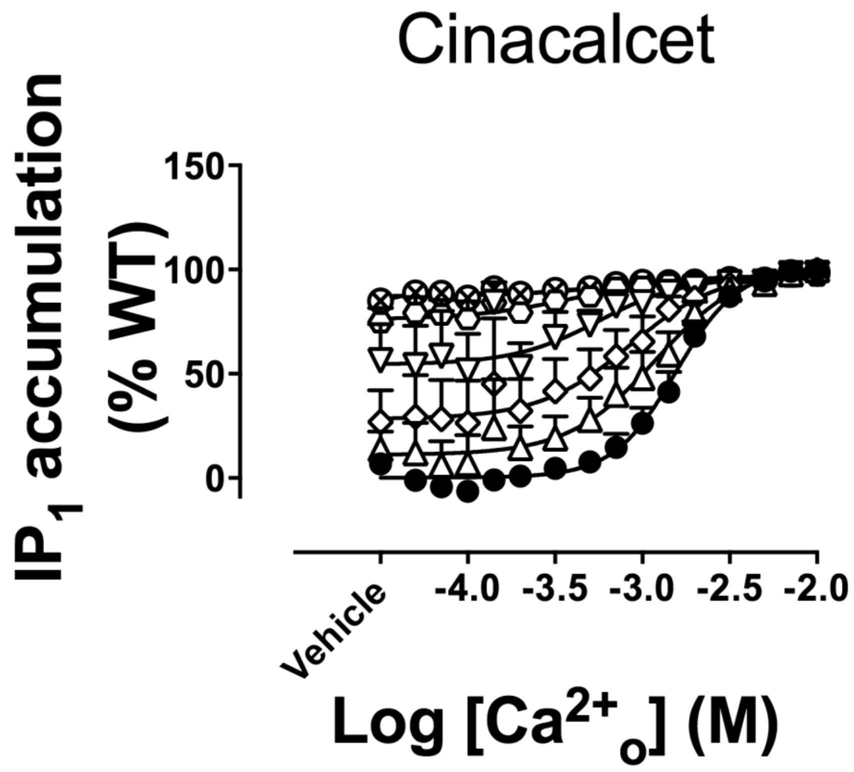

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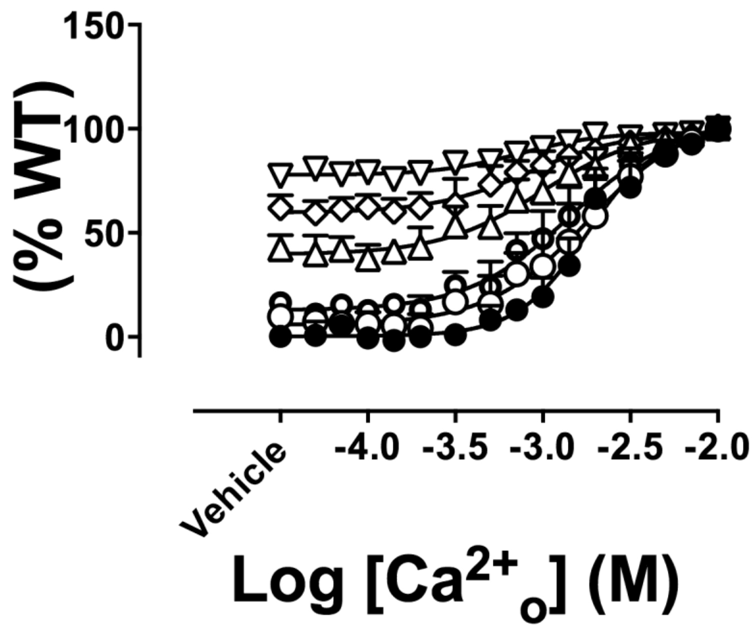

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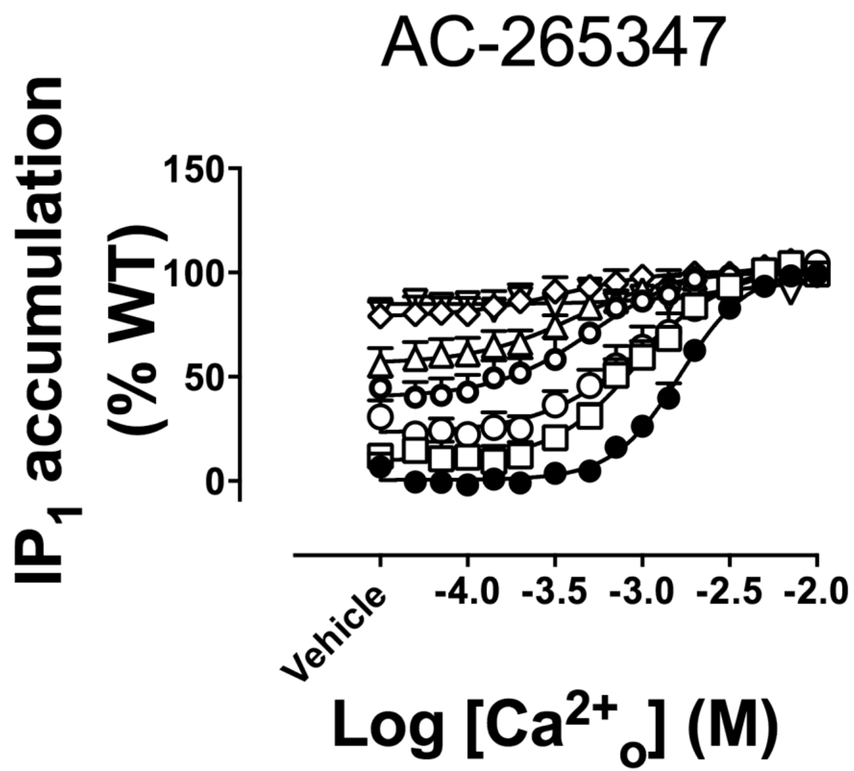

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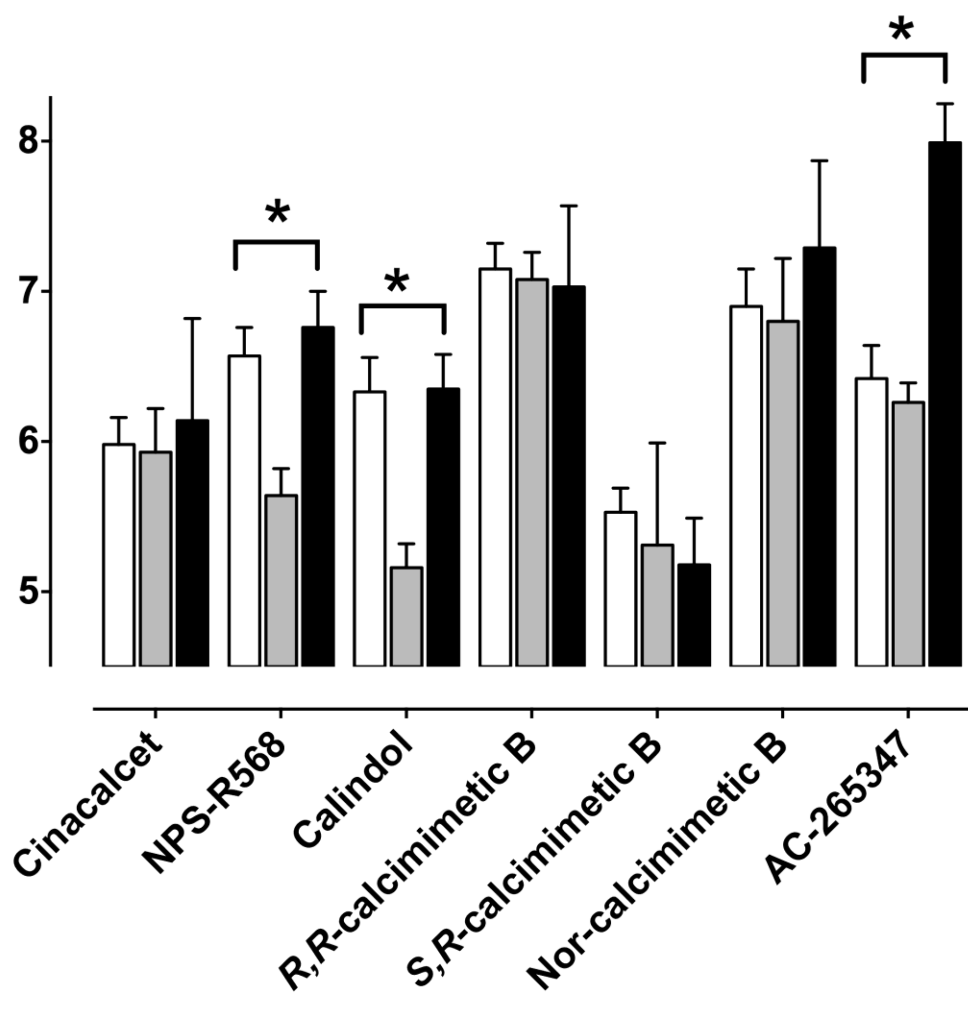

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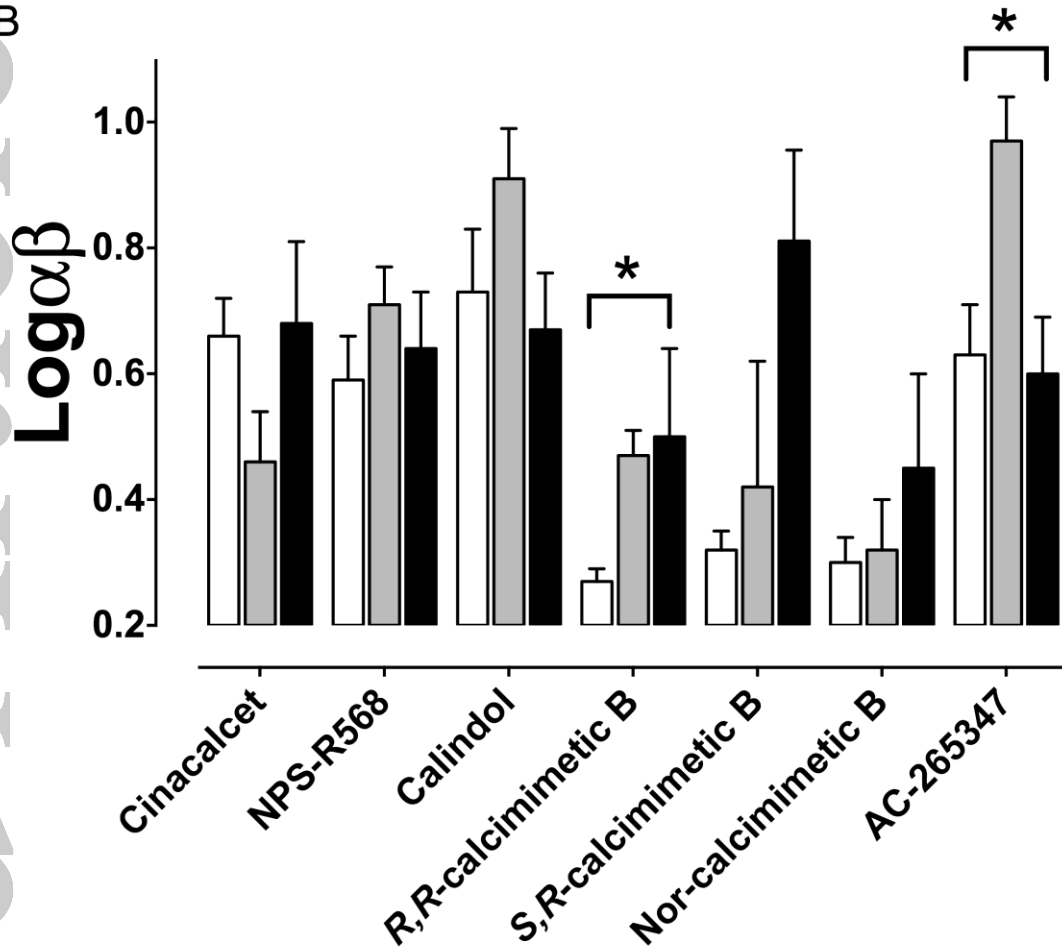

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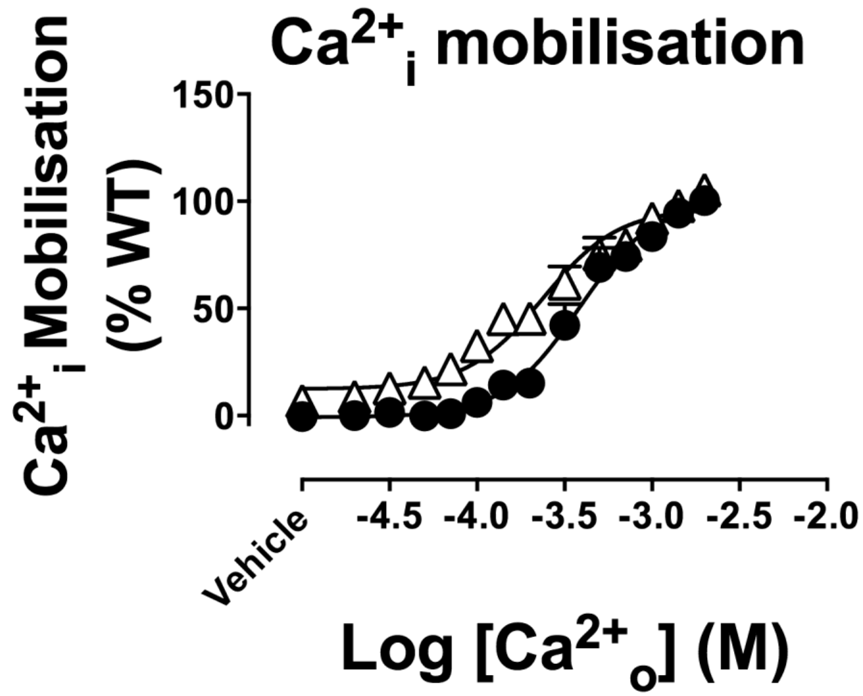

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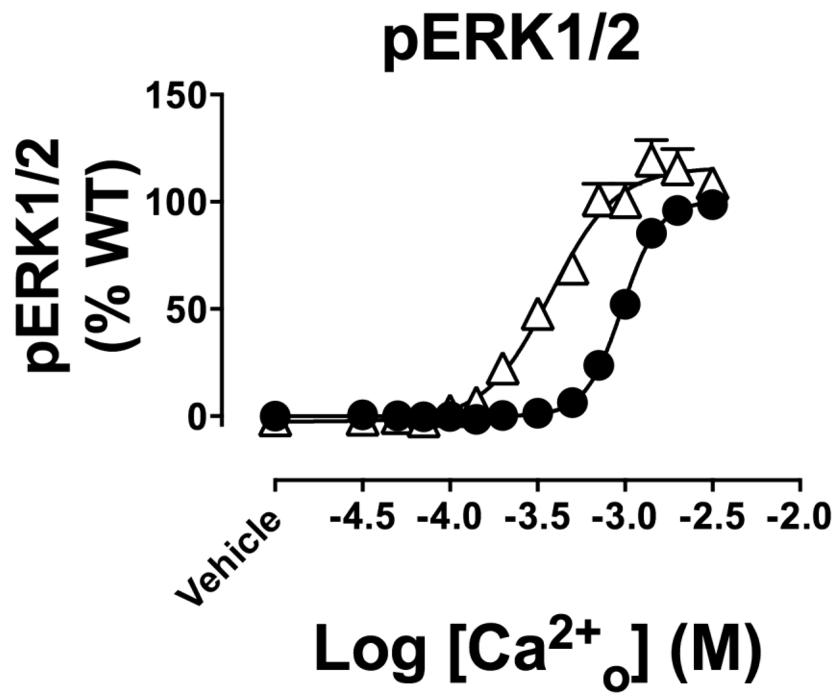

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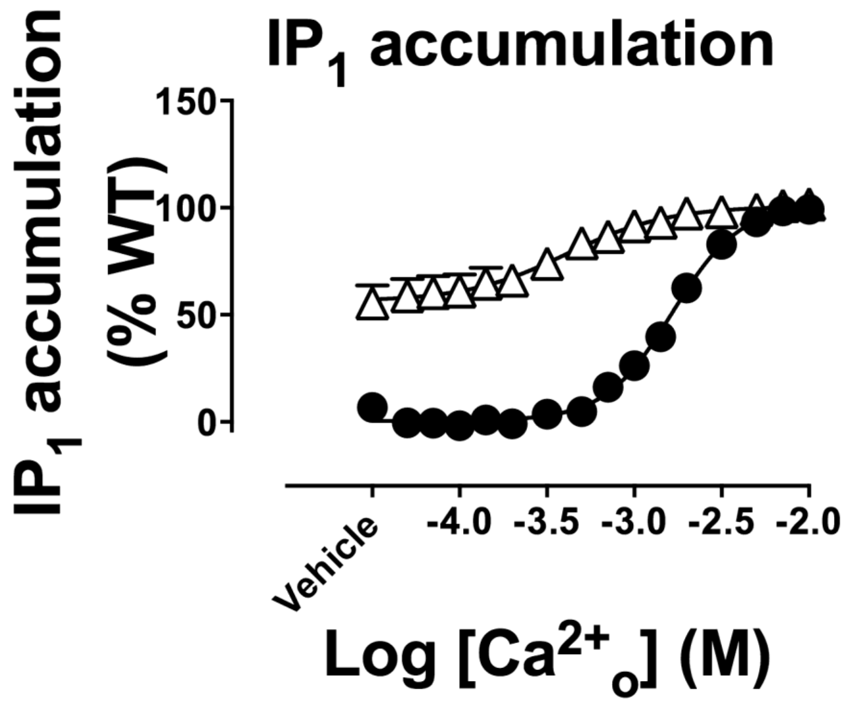

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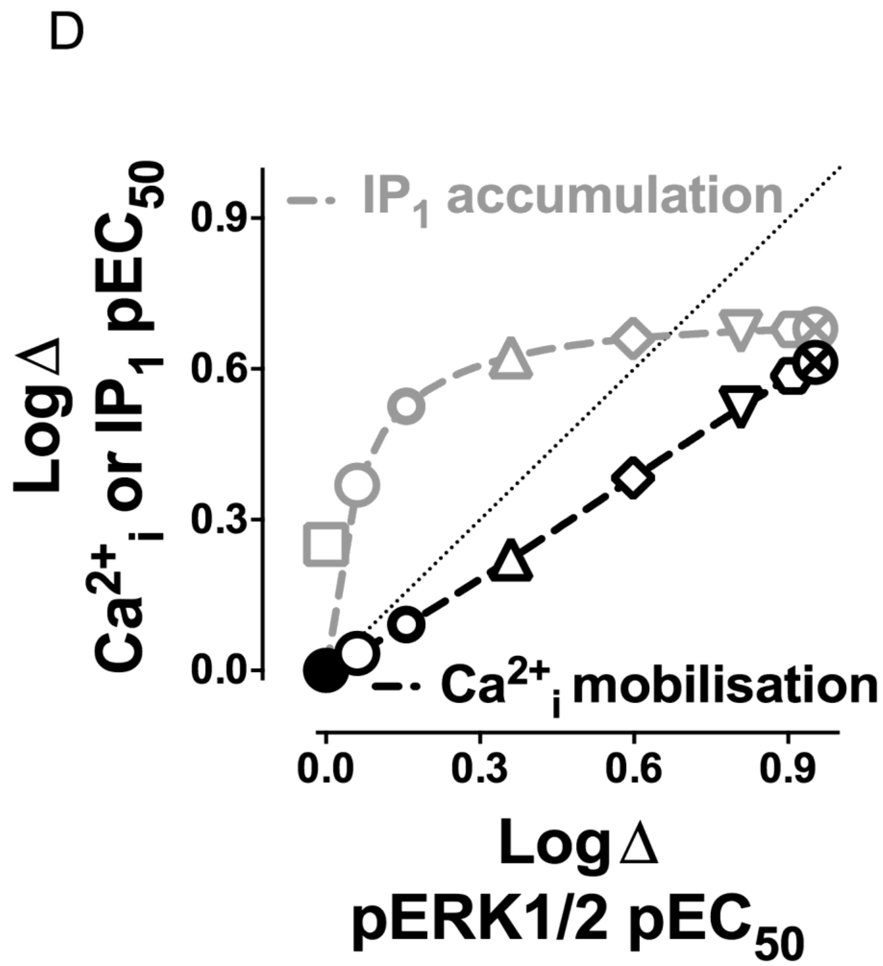

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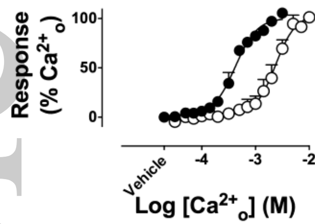

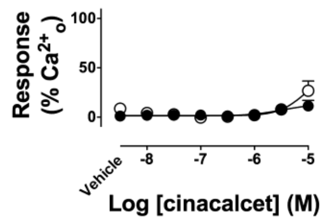

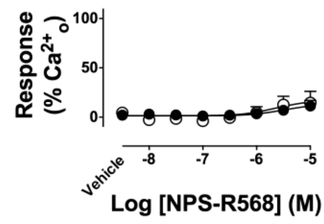

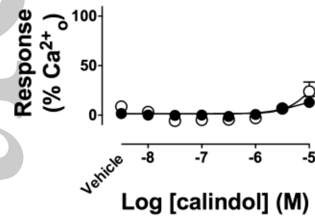

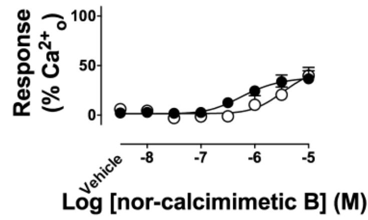

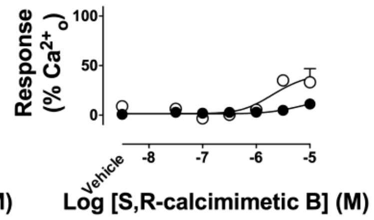

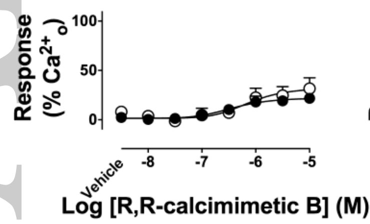

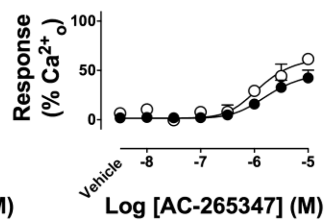

● Ca^{2+}_i mobilisation
○ IP_1 accumulation

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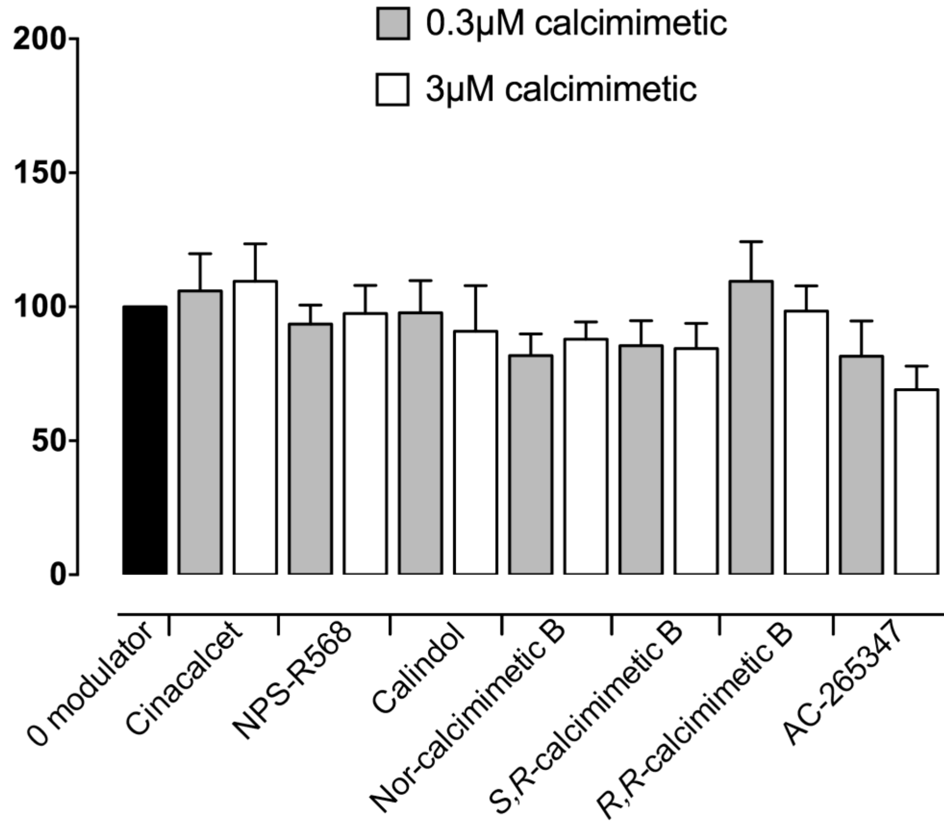

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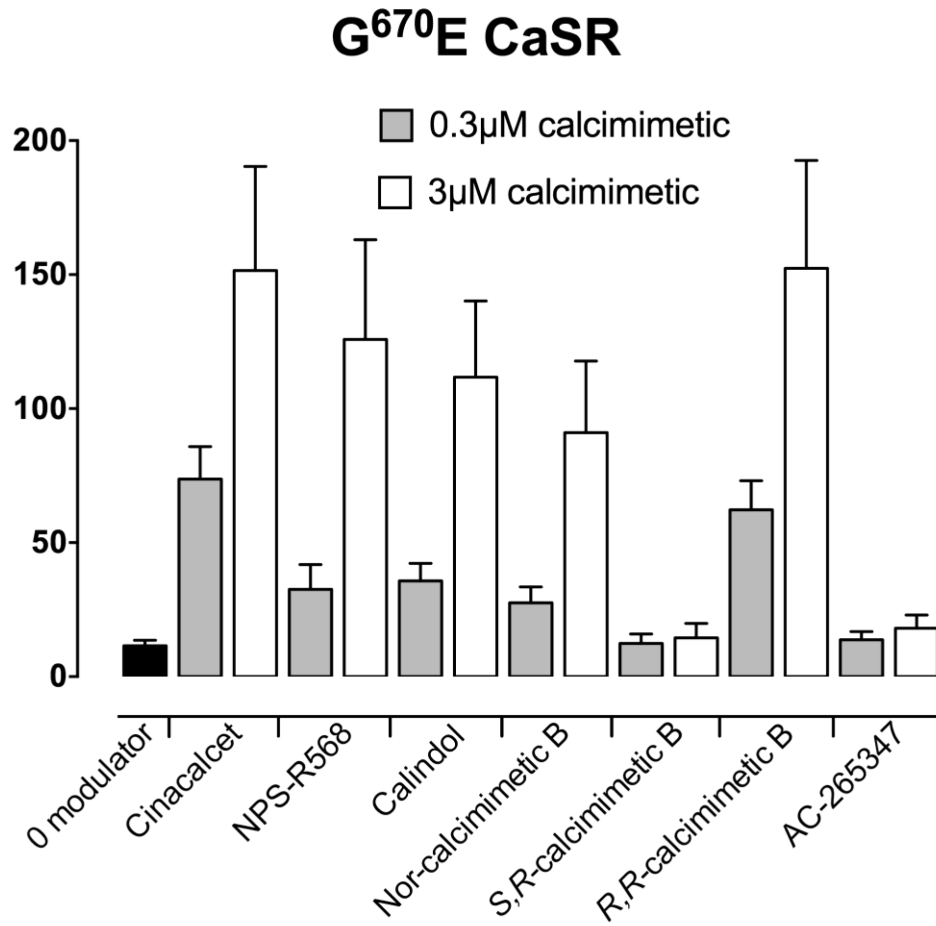

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Table 1. Pharmacological parameters that govern the allosteric activity of CaSR modulators in Ca^{2+}_i mobilisation, pERK1/2 and IP_1 accumulation assays. The potency of Ca^{2+}_o in the presence of increasing concentrations of modulator was fitted 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)

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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)

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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)

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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)