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RESEARCH PAPER

Divergent effects of strontium and calcium-sensing receptor positive allosteric modulators (calcimimetics) on human osteoclast activity

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BACKGROUND AND PURPOSE

Strontium ranelate, a drug approved and until recently used for the treatment of osteoporosis, mediates its effects on bone at least in part *via* the calcium-sensing (CaS) receptor. However, it is not known whether bone-targeted CaS receptor positive allosteric modulators (PAMs; calcimimetics) represent an alternative (or adjunctive) therapy to strontium (Sr_o^{2+}).

EXPERIMENTAL APPROACH

We assessed three structurally distinct calcimimetics [cinacalcet, AC-265347 and a benzothiazole tri-substituted urea (BTU-compound 13)], alone and in combination with extracellular calcium (Ca_o^{2+}) or Sr_o^{2+}, in G protein-dependent signalling assays and trafficking experiments in HEK293 cells and their effects on cell differentiation, tartrate-resistant acid phosphatase (TRAP) activity and hydroxyapatite resorption assays in human blood-derived osteoclasts.

KEY RESULTS

Sr_o^{2+} activated CaS receptor-dependent signalling in HEK293 cells in a similar manner to Ca_o^{2+}, and inhibited the maturation, TRAP expression and hydroxyapatite resorption capacity of human osteoclasts. Calcimimetics potentiated Ca_o^{2+}- and Sr_o^{2+}-mediated CaS receptor signalling in HEK293 cells with distinct biased profiles, and only cinacalcet chaperoned an endoplasmic reticulum-retained CaS mutant receptor to the cell surface in HEK293 cells, indicative of a conformational state different from that engendered by AC-265347 and BTU-compound 13. Intriguingly, only cinacalcet modulated human osteoclast function, reducing TRAP activity and profoundly inhibiting resorption.

CONCLUSION AND IMPLICATIONS

Although AC-265347 and BTU-compound 13 potentiated Ca_o^{2+}- and Sr_o^{2+}-induced CaS receptor activation, they neither replicated nor potentiated the ability of Sr_o^{2+} to inhibit human osteoclast function. In contrast, the FDA-approved calcimimetic, cinacalcet, inhibited osteoclast TRAP activity and hydroxyapatite resorption, which may contribute to its clinical effects on bone mineral density

LINKED ARTICLES

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Abbreviations

BMD, bone mineral density; CaS receptor, calcium-sensing receptor; Ca_o^{2+}, extracellular calcium; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; PAM, positive allosteric modulator; PTH, parathyroid hormone; RANK-L, receptor activator of NF-κB ligand; $Sr^{2+}o$, strontium; TRAP, tartrate-resistant acid phosphatase

Introduction

Bone remodelling is essential for bone integrity and primarily involves the interaction between bone-resorbing osteoclasts and bone-forming osteoblasts. This process is tightly regulated at many levels, including by osteocyte-, osteoblast- and osteoclast-derived factors, mediators released by the bone matrix upon resorption by osteoclasts and other local and systemic hormones (Sims and Martin, 2014; Sims and Martin, 2015). Osteoporosis reflects an imbalance of bone remodelling, favouring resorption over formation, and is typified by low bone mineral density (BMD) and increased fracture risk (Watts *et al.,* 2013). Many osteoporosis therapeutics are based on reducing osteoclast-mediated bone resorption (Marcus, 2013). Strontium ($Sr^{2+}o$) ranelate has been used clinically for the treatment of osteoporosis and reduces morphometric vertebral fractures in postmenopausal women (Meunier *et al.,* 2004; Reginster *et al.,* 2005). Unfortunately, it is also associated with an increased incidence of cardiac disorders and thromboembolic events, leading to its reclassification for restricted use by the European Medicines Agency in 2014 (EMA, 2014). Currently, it is prescribed only for the treatment of severe osteoporosis in postmenopausal women and adult men at high risk of fracture. One advantage of strontium ranelate is its proposed dual action as an anti-resorptive and anabolic agent, further aided by its high incorporation into bone (Dahl *et al.,* 2001). This remained a driver for continued use in Europe (EMA, 2014), although the restrictions in place have led to cessation of its marketing in 2017 for commercial reasons (Servier, 2017).

Sr_o^{2+} inhibits the maturation and activity of osteoclast cultures (Bonnelye *et al.,* 2008; Caudrillier *et al.,* 2010), induces apoptosis (Hurtel-Lemaire *et al.,* 2009) and promotes osteoblast differentiation and recruitment (Chattopadhyay *et al.,* 2007; Brennan *et al.,* 2009). However, the anabolic activity of Sr_o^{2+} has been questioned; improved bone strength associated with incorporation of Sr_o^{2+} into the bone matrix and the anti-resorptive actions on osteoclasts may have been sufficient to produce the increase in BMD observed in clinical trials (Bain *et al.,* 2009; Chavassieux *et al.,* 2014). Whilst the exact mechanism of action of Sr_o^{2+} remains unclear, the calcium-sensing receptor (**CaS receptor**) may be partly responsible (Chattopadhyay *et al.,* 2007; Fromigué *et al.,* 2009; Hurtel-Lemaire *et al.,* 2009; Caudrillier *et al.,* 2010). Thus, a combination therapy, comprising Sr_o^{2+} and a CaS receptor positive allosteric modulator (PAM), in a bone-targeted formulation, could reduce the required dose of Sr_o^{2+} and therefore the risk of adverse effects.

The CaS receptor is a Class C GPCR that pleiotropically couples to G proteins and is expressed in many tissues that regulate extracellular calcium (Ca_o^{2+}) levels, including the parathyroid gland [where it inhibits **parathyroid hormone** (PTH) release], osteoblasts/osteoclasts (where it regulates bone turnover), thyroid parafollicular C cells (where it stimulates **calcitonin** release) and the kidney (where it regulates renal Ca_o^{2+} re-absorption) (Chang *et al.,* 1999; Kantham *et al.,* 2009; Thomsen *et al.,* 2012; Goltzman and Hendy, 2015; Riccardi and Valenti, 2016). However, the CaS receptor is also expressed in many tissues not directly involved in Ca_o^{2+} homeostasis, including cardiovascular cells (where calcimimetics can display hypertensive effects) (Bonomini *et al.,* 2012) and mesenchymal stem cells (where it contributes to osteogenic differentiation) (Pipino *et al.,* 2014), amongst others. The CaS receptor is responsive to Ca_o^{2+} and other cations (including Sr_o^{2+}) that can preferentially activate distinct signalling pathways; in HEK293 cells expressing the bovine CaS receptor, Sr_o^{2+} is less potent than Ca_o^{2+} in Ca_i^{2+} mobilization and IP_1 accumulation assays but is equipotent to Ca_o^{2+} in ERK1/2 phosphorylation assays (Chattopadhyay *et al.,* 2007). Intriguingly, Sr_o^{2+} is more potent than Ca_o^{2+} at stimulating calcitonin release from rat medullary thyroid carcinoma 6–23 cells, highlighting the context-dependence of agonist potency at the CaS receptor (Thomsen *et al.,* 2012).

Small molecule PAMs have been identified for the CaS receptor (Jian-Nong *et al.,* 2011; Deprez *et al.,* 2013), most notably **cinacalcet**, which is used clinically for the treatment of secondary hyperparathyroidism (Cunningham *et al.,* 2005; Davey *et al.,* 2012; Cook *et al.,* 2015). Cinacalcet has been extensively characterized for its ability to modulate CaS receptor-mediated signalling (Leach *et al.,* 2013; Leach *et al.,* 2016). The structurally distinct calcimimetic, **AC-265347**, which reduces serum PTH levels in rats, has also been relatively well characterized as an allosteric modulator (Smajilovic *et al.,* 2011;Cook *et al.,* 2015 ; Leach *et al.,* 2016). These studies reveal that, like positively charged cations, cinacalcet and AC-265347 stabilize receptor conformations that differentially regulate CaS receptor-signalling pathways, a phenomenon termed biased modulation (Cook *et al.,* 2015; Leach *et al.,* 2016). This bias extends to actions as pharmacochaperones, where cinacalcet, but not AC-265347, traffics a naturally occurring endoplasmic reticulum-retained mutant CaS receptor (Cook *et al.,* 2015). In contrast to cinacalcet and AC-265347, relatively little is known about the molecular pharmacology of other calcimimetics, such as the structurally distinct benzothiazole tri-substituted urea series (Deprez *et al.,* 2013).

In the present study, we have extended our previous observations of PAM-mediated bias at the CaS receptor to include effects on agonist-mediated luciferase transcription under the control of serum response factor response element (SRF-RE)-Luc transcription and a surrogate of RhoA-mediated signalling. We also profiled the structurally distinct PAM, BTU-compound 13 (Deprez *et al.,* 2013) (Figure 1) and, to test the propensity for 'probe-dependence' (Valant *et al.,* 2012), characterized the ability of each calcimimetic to modulate both Ca_o^{2+}- and Sr_o^{2+}-activity in a potentially differential manner. Furthermore, to establish whether Sr_o^{2+} and calcimimetic

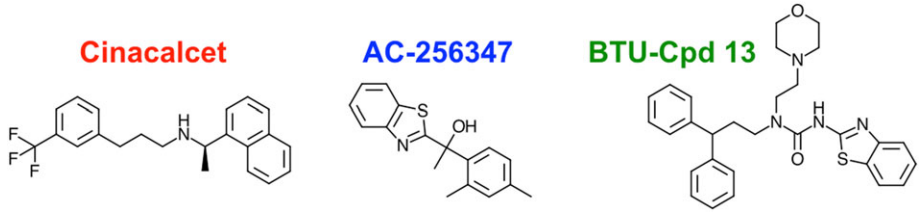

Figure 1
Structures of the calcimimetics used in this study.

combination treatment could be considered for the treatment of osteoporosis, we evaluated how calcimimetic pharmacology translates into human CD14+ monocyte-derived osteoclasts. Using a quantitative, high throughput, multistaining method (Diepenhorst *et al.,* 2017), we assessed osteoclast tartrate-resistant acid phosphatase (TRAP) activity and nucleation state. Coupled with hydroxyapatite resorption assays, we show that cinacalcet, but not AC-265347 or BTU-compound 13, inhibits osteoclast-mediated resorption without impacting osteoclast differentiation and with only a small reduction in TRAP activity, in contrast to Sr_o^{2+}, which robustly reduces TRAP activity, osteoclast maturation and resorption.

Methods

Recombinant cell culture

Flp-In HEK293-TREx-c-*myc*-CASR cells containing the human CaS receptor gene (*CASR*) were used in all recombinant cell assays and were maintained in DMEM and 10% FBS (Invitrogen as previously described; Davey *et al.,* 2012). For ERK1/2 phosphorylation and Ca_i^{2+} mobilization assays, cells were seeded at 80 000 cells per well into Corning 96-well plates coated with 2.5 μg per well poly-D lysine. *CASR* expression was induced by incubation with 100 ng·mL^{-1} tetracycline overnight at 37°C in an atmosphere of 5% CO_2.

Ca_i^{2+} mobilization assay

Ca_i^{2+} mobilization assays were performed as previously described (Davey *et al.,* 2012). Seeded, tetracycline-induced cells were washed twice in assay buffer (150 mM NaCl, 2.6 mM KCl, 0.1 mM $CaCl_2$, 1.18 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, 4 mM probenecid, 0.5% w·v^{-1} BSA, pH 7.4) and loaded with 0.9 μM Fluo-4-AM for 1 h at 37°C. Cells were washed twice with 100 μL per well assay buffer before co-addition (in duplicate) of the calcimimetics and Ca_o^{2+} or Sr_o^{2+}. Intracellular calcium mobilization was determined by the peak change in fluorescence using a Flexstation-1 microplate reader (Molecular Devices, Sunnyvale, CA, USA) using 485 nm excitation and 525 nm emission filters.

IP_1 accumulation assay

Induced Flp-In HEK293-TREx-c-*myc*-CASR cells were harvested and resuspended in assay buffer (150 mM NaCl, 2.6 mM KCl, 0.1 mM $CaCl_2$, 1.18 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, 50 mM $LiCl_2$, pH 7.4); 1 × 10^4 cells in 7 μL were added to wells of a 384-well white Proxiplate (PerkinElmer) in addition to 7 μL agonist (Ca_o^{2+} or Sr_o^{2+}) with or without calcimimetic (in duplicate). Plates were briefly centrifuged and allowed to incubate at 37°C for 45 min. The reaction was stopped and cells lysed by the addition of 6 μL per well lysis buffer (CisBio Bioassays). Lysates were analysed for IP_1 accumulation using the IP-One Tb™ assay kit (CisBio Bioassays) according to the manufacturer's protocol as previously described (Cook *et al.,* 2015).

ERK1/2 phosphorylation assay

ERK1/2 phosphorylation assays were performed on 4 h serum-starved cells in 0.1 mM Ca^{2+}-containing serum-free DMEM, as described above. Concentration responses to Ca_o^{2+} or Sr_o^{2+} in the presence and absence of calcimimetic were performed (in duplicate) at 37°C and were normalized to the maximal Ca_o^{2+}- or Sr_o^{2+}-responses. Cells were co-stimulated for 2.5 min with calcimimetics and Ca_o^{2+} or Sr_o^{2+}. The medium was removed and the cells lysed with the 100 μL SureFire lysis buffer (TGR Biosciences). Lysates were frozen prior to analysis with the SureFire™ ERK1/2 (Thr201/Tyr204) phosphorylation Assay Kit according to the manufacturer's protocol (PerkinElmer) using an EnVision multilabel plate reader (PerkinElmer) for detection.

Serum response factor response element (SRF-RE)-Luc reporter gene assay

As a surrogate for CaS receptor coupling to RhoA activity, a reporter gene assay was used to measure luciferase transcription under the control of SRF-RE-Luc. Briefly, Flp-In HEK293-TREx-c-*myc*-CASR, at 80 000 cells per well, were transfected with 0.1 μg per well pGL4.34[luc2P/SRF-RE/Hygro] (Promega) DNA using a ratio of 1:3 DNA:PEI, in Corning 96-well white plates coated with 2.5 μg per well poly-D lysine. CaS receptor expression was simultaneously induced with 100 ng·mL^{-1} tetracycline. After incubation at 37°C in an atmosphere of 5% CO_2 for 5 h, the media were exchanged for 100 μL per well serum-free, low Ca_o^{2+} (0.1 mM) DMEM with 100 ng·mL^{-1} tetracycline, and cells were incubated overnight at 37°C in an atmosphere of 5% CO_2. The following day, DMEM was aspirated and cells were washed once with PBS, and 100 μL assay buffer (150 mM NaCl, 2.6 mM KCl, 0.1 mM $CaCl_2$, 1.18 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4) was added to each well. Cells were stimulated with agonist with or without calcimimetic (in duplicate) for 6 h at 37°C. The plate was equilibrated to room temperature prior to the addition of Bright-Glo™ (Promega) reagent at a final dilution of 1:2. Following incubation at room temperature for 2 min, luminescence was measured (EnVision multilabel plate reader,

PerkinElmer). Data were normalized to the maximal response stimulated by Ca_o^{2+} or Sr_o^{2+} in the absence of any calcimimetic.

Source of human blood

Collection and use of human blood samples (buffy coat and whole blood) was conducted according to the guidelines and approval of Monash University (Clayton, Australia) and the Monash University Research Ethics Committee (Clayton, Australia). Blood (100 mL) was obtained from human volunteers from the Victorian Blood Donor Registry or from buffy coat preparations from the Australian Red Cross blood service (Melbourne, Australia, under the Monash University Research Ethics Committee approval; HREC CF14/999-2014000425).

Osteoclast differentiation and culture

Osteoclasts were differentiated from CD14+ monocytes isolated from human blood as previously described (Nicholson *et al.*, 2000). Briefly, buffy coat or whole blood was diluted 1:1 in 1 × PBS; 30 mL of diluted blood was layered over 15 mL Ficoll (GE Healthcare) in a Leucosep tube and centrifuged at 800× *g* for 20 min, at room temperature (21°C) with slow deceleration. The peripheral blood mononuclear cells (PBMC) layer was aspirated and washed three times in PBS by centrifugation at 300× *g* for 10 min at room temperature. CD14+ PBMCs were isolated using human CD14 MACS MicroBeads (Miltenyl Biotec) as per the manufacturer's protocol.

Isolated CD14+ PBMCs were seeded at 200 000 cells per well in 96-well plates in α-MEM (Life Technologies, 1.8 mM $CaCl_2$) supplemented with 10% heat-inactivated FBS (Life Technologies) and cultured overnight at 37°C in 5% CO_2. Cells were differentiated with the addition of 20 ng·mL^{-1} macrophage colony-stimulating factor (M-CSF) (Sigma-Aldrich) 24 h after isolation and 20 ng·mL^{-1} M-CSF and 20 ng·mL^{-1} receptor activator of NF-κB ligand (**RANK-L**) (Merck) 72 h later. Cells were cultured for up to 2 weeks at 37°C, 5% CO_2 with media refreshed every 3 days.

Reverse transcriptase PCR

CD14+ monocytes were seeded at 2 × 10^6 cells per well in a six-well plate and were incubated with 20 ng·mL^{-1} M-CSF for 3 days. Cells were then treated with 20 ng·mL^{-1} M-CSF or 20 ng·mL^{-1} M-SCF and 20 ng·mL^{-1} RANK-L for a further 7 days. Cells were lysed using 350 μL lysis buffer with 3.5 μL β-mercaptoethanol, and RNA was extracted using the Isolate II RNA Mini Kit (BioLine) according to the manufacturer's protocol. RNA yields were determined using a NanoDrop 2000 and A260/280 and A260/230 ratios recorded for quality assessment. Immediately, 2 μg of RNA was converted to cDNA using Tetro cDNA Synthesis Kit (BioLine) according to the manufacturer's protocol using the random hexamer primer mix, in duplicate. Subsequent cDNA was diluted 1:5 in nuclease-free water and samples stored at −20°C. RT-PCR reactions were carried out using 2 μL cDNA, 2 μL forward and reverse primers at 5 μM (Supporting Information Table S1) and SybrGreen (Roche) as per the manufacturer's protocol and reactions were run on a LightCycler ® 480 Instrument II (Roche). Primer efficiency was calculated using the standard curve method. Data were analysed using the Pfaffl method (Pfaffl, 2001), expressed as ΔΔCt normalized to a calibrator sample and to both reference genes, plotted as mean ± SEM and represent five independent donors with samples run in duplicate.

Western blot for human CaS receptor

CD14+ human monocytes were seeded on six-well plates and incubated for the indicated time in the presence or absence of 20 ng·mL^{-1} RANK-L. Cells were then washed with PBS, 50 μL of SDS lysis buffer + DTT + β-mercaptoethanol + protein inhibitor added to each well and cells recovered using a scraper. Samples were sonicated at 40% amplitude for 30 s and loaded into a 6% polyacrylamide separating gel. After running samples for 1 h at 100 V, gel was transferred overnight at 30 V onto a nitrocellulose membrane using a wet transfer system at 4°C. Membrane was then blocked using PBS-T 1% milk for 1 h and incubated with 1 μg·mL^{-1} of CaS receptor antibody (5C10, ADD clone, ThermoFisher) overnight at 4°C. Membrane was then washed three times with PBS-T 1% milk and incubated with anti-mouse HRP (1:1000) secondary antibody for 1 h at room temperature. Following three washes with PBS-T, the membrane was imaged using the Chemidoc Touch imager (Bio-rad) and developed with Luminata™ Forte Western HRP Substrate (Merck Millipore) according to the manufacturer's instructions.

Tartrate-resistant acid phosphatase (TRAP) activity assay

TRAP activity was quantified as previously described (Diepenhorst *et al.*, 2017). Briefly, CD14+ PBMCs were isolated and seeded at 200 000 cells per well into 96-well plates and were incubated with 20 ng·mL^{-1} M-CSF for 3 days followed by differentiation with 20 ng·mL^{-1} M-CSF and 20 ng·mL^{-1} RANK-L in the presence or absence of 5, 10 or 20 mM $SrCl_2$ and/or 3 μM calcimimetic for 8 days. Cells were fixed, stained and imaged as previously described (Diepenhorst *et al.*, 2017) with additional staining for the calcitonin receptor. Briefly, staining for the calcitonin receptor involved blocking with 0.5% BSA/PBS for 1 h at room temperature, overnight incubation with 1:500 mouse anti-human calcitonin receptor antibody (Welcome receptor) in PBS at 4°C and incubation with anti-mouse Alexa-594 (1:1000, Life Technologies) prior to imaging cells on the In Cell 2000 (GE Healthcare), four fields of view per well in duplicate wells using a 10× objective. TRAP activity is presented as the mean number of TRAP foci per cell ± SEM for 10 individual experiments (each with four fields of view analysed), binned by osteoclast maturation state (number of nuclei; Diepenhorst *et al.*, 2017).

Resorption assay

CD14+ PBMCs were isolated and seeded as previously described into Corning® Osteo Assay Surface 96-well plates and incubated with 20 ng·mL^{-1} M-CSF for 3 days. Cells were then differentiated into osteoclasts in the presence or absence of 5, 10, 20 mM $SrCl_2$ or 3 μM calcimimetic (in duplicate) with 20 ng·mL^{-1} M-CSF, 20 ng·mL^{-1} RANK-L for a further 8 days. The area of the Osteo Assay surface resorbed was determined using Von Kossa staining at the end of 8 days of differentiation. Briefly, cells were removed by incubation with 10% bleach solution at 22°C for 5 min. Wells were washed twice with water before

allowing to air-dry overnight. Wells were then stained with 5% (w.v^{-1}) aqueous silver nitrate at 22°C in the dark for 30 min prior to a 5 min wash in water. Wells were then incubated with 5% (w·v^{-1}) sodium carbonate in formalin for 4 min. Wells were imaged using an In Cell 2000 (GE Healthcare) and images analysed in Fiji to determine the proportion of hydroxyapatite Osteo Assay surface resorbed. Data were collated from 10 donors, expressed as mean percentage area resorbed ± SEM.

Flow cytometry for the detection of cell-surface receptor expression

Flow cytometry was used to measure changes in the cell surface expression of either WT CaS receptor or the G670E mutant CaS receptor as previously described (Cook *et al.*, 2015). Briefly, Flp-In HEK293-TREx-c-*myc*-CASR and CaS receptor-G670E cells were seeded at 80 000 cells per well into a 96-well plate and expression was induced with 100 ng·mL^{-1} tetracycline in the presence of vehicle or 3 μM calcimimetic overnight at 37°C. The following day, cells were harvested, washed in 1 × PBS with 0.1% BSA and 2 mM EDTA (wash buffer) by centrifugation (350× *g*, 4°C for 3 min) before resuspension and 30 min incubation in 100 μL blocking buffer (1 × PBS, 5% BSA and 2 mM EDTA); 1 μg·mL^{-1} AF647-conjugated 9E10 made in-house as previously described was then added and incubated for 1 h. Cells were washed as previously described and resuspended in wash buffer containing Sytox blue stain (Invitrogen). Fluorescence was measured using a FACS Canto (Becton Dickinson). Data are mean ± SEM of five independent experiments.

Data analysis and statistics

All non-linear regression analysis was performed in GraphPad Prism 7. Interaction experiments between Ca_o^{2+} or Sr_o^{2+} and cinacalcet, AC-265347 or BTU-compound 13 were fitted globally to the following operational model of allosterism and agonism (Leach *et al.*, 2007; Aurelio *et al.*, 2009) where the transducer slope was constrained to be less than four:

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

EC_{50} is the agonist concentration that elicits a half maximal response; τ_B denotes the efficacy of the allosteric ligand; α and β denote allosteric effects on orthosteric ligand binding affinity and efficacy, respectively; K_B represents the functional affinity of the allosteric ligand; [A] and [B] represent orthosteric and allosteric ligand concentrations; E_m denotes the maximal possible response of the system; and n is the slope of the transducer function linking agonist receptor occupancy to response. Affinity, cooperativity and efficacy parameters were estimated as logarithms (Christopoulos, 1998).

To determine whether the calcimimetics engendered *biased modulation* of CaS receptor signalling, the statistical differences between either pK_B or log αβ values across different pathways for a given PAM were determined by one-way ANOVA with Tukey's multiple comparisons test for effects in combination with either Ca_o^{2+} or Sr_o^{2+}. In order to determine any *probe-dependent* effects, variations in calcimimetic log αβ determined from interaction with either Ca_o^{2+} or Sr_o^{2+} at a given signalling pathway were compared by two-way ANOVA (with orthosteric probe and signalling endpoints as factors) with Sidak's multiple comparisons post test. TRAP expression data were analysed by repeated measures two-way ANOVA (with Sr_o^{2+} treatment and osteoclast size as variables) followed by Dunnett's multiple comparisons test.

Due to the inherent variability in the resorptive capacity of human donor-derived osteoclasts, hydroxyapatite resorption data were subject to Grubb's test for outliers ($\alpha = 0.05$). For the Sr_o^{2+} dataset, two data points (out of a total of 44; $n = 11$, four treatment conditions) were excluded; the data were analysed by one-way ANOVA with Dunnett's multiple comparisons compared to vehicle. For the calcimimetics ± Sr_o^{2+}, the data from one donor were excluded ($n = 10$ remain); the data were analysed by repeated measures two-way ANOVA with calcimimetic and Sr_o^{2+} treatment as variables and Dunnett's multiple comparisons compared to vehicle. Flow cytometry data were analysed by two-way ANOVA with drug treatment and genotype as variables and Dunnett's multiple comparisons compared to vehicle. The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The data and statistical analyses were performed in GraphPad Prism 7. Group sizes and number of independent experiments appropriately reflect the variability in the datasets and magnitude of the signal size. A difference was considered significant when $P < 0.01$ and was marked on figures accordingly.

Compounds

Cinacalcet was synthesized in-house as described previously (Davey *et al.*, 2012); AC-265347 was purchased from Sigma-Aldrich; BTU-compound 13 was synthesized by Institut de Recherches Servier using published methods (Deprez *et al.*, 2013).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Calcimimetic effects on Ca_o^{2+}-mediated CaS receptor signalling

The interaction between cinacalcet or AC-265347 and Ca_o^{2+} in Ca_i^{2+}-mobilization, IP_1 accumulation and ERK1/2 phosphorylation assays has been previously described (Cook *et al.*, 2015; Leach *et al.*, 2016) and suggests that cinacalcet is biased towards Ca_i^{2+} mobilization and away from ERK1/2 phosphorylation, as evidenced by a significantly lower cooperativity with Ca_o^{2+} in ERK1/2 phosphorylation versus Ca_i^{2+} mobilization assays. Using SRF-RE-Luc transcription as a surrogate for RhoA activity, we now show that cinacalcet also exhibits bias towards RhoA versus ERK1/2 phosphorylation, as evidenced by a significantly greater pK_B for the receptor conformation that couples to this pathway (Table 1; Figure 2; Supporting Information Figure S1). AC-265347 has been

Table 1

Affinity (pK_B) and cooperativity (log αβ) estimates from operational model analysis of cinacalcet, AC-265347 and BTU-compound 13-mediated allosteric modulation of Ca_o^{2+}- and Sr_o^{2+}- stimulated Ca_i^{2+} mobilization, IP_1 accumulation, ERK1/2 phosphorylation and the SRF-RE-Luc transcription in HEK293 cells induced to express CaS receptors

		Cinacalcet				AC-265347				BTU-compound 13			
		Ca_o^{2+}	*n*	Sr_o^{2+}	*n*	Ca_o^{2+}	*n*	Sr_o^{2+}	*n*	Ca_o^{2+}	*n*	Sr_o^{2+}	*n*
Ca_i^{2+}	pK_B	6.29 ± 0.04	29	6.12 ± 0.08	5	6.21 ± 0.07	13	6.11 ± 0.17	5	6.66 ± 0.14	5	6.76 ± 0.11	5
	log αβ (αβ)	0.42 ± 0.03 (2.6)		0.67 ± 0.07 (4.6)		0.44 ± 0.08 (2.5)		0.92 ± 0.23 (8.3)		0.21 ± 0.15 (3.2)		0.20 ± 0.11 (1.6)	
IP_1	pK_B	6.09 ± 0.10	5	6.17 ± 0.06	5	7.34 ± 0.08	5	7.38 ± 0.14	5	7.15 ± 0.11	5	7.24 ± 0.08	7
	log αβ (αβ)	0.41 ± 0.15 (2.6)		0.40 ± 0.09 (2.5)		0.67 ± 0.10 (4.7)		0.45 ± 0.23 (2.8)		0.46 ± 0.16 (2.9)		0.23 ± 0.13 (1.7)	
ERK1/2	pK_B	6.52 ± 0.07	5	6.42 ± 0.18	5	6.06 ± 0.11	10	6.00 ± 0.20	5	6.18 ± 0.18	5	5.96 ± 0.18	5
	log αβ (αβ)	0.12 ± 0.05 (1.3)		0.41 ± 0.05 (2.5)		1.07 ± 0.11 (10)		1.12 ± 0.23 (13)		0.08 ±0.19 (1.2)		0.18 ± 0.11 (1.5)	
SRF-RE	pK_B	7.12 ± 0.19	5	6.53 ± 0.17	5	6.19 ± 0.13	5	5.97 ± 0.23	5	6.47 ± 0.21	5	5.69 ± 0.37	5
	log αβ (αβ)	0.65 ± 0.11 (4.5)		0.82 ± 0.16 (6.6)		1.13 ± 0.15 (13)		1.28 ± 0.26 (19)		1.23 ± 0.23 (17)		1.74 ± 0.41 (55)	

All data are mean ± SEM of the stated number of independent experiments.

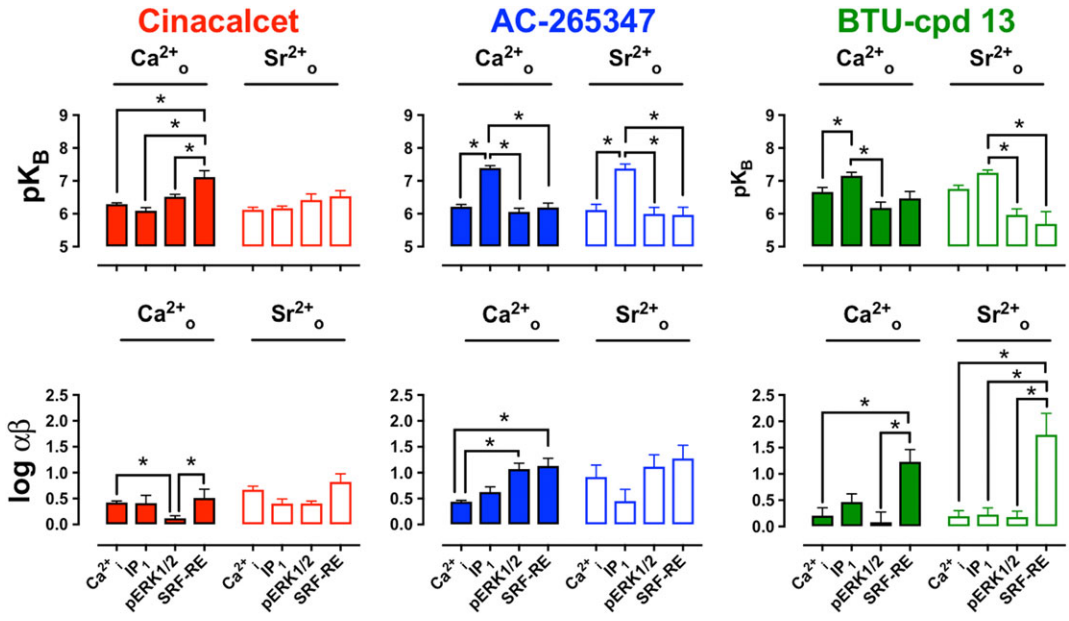

Figure 2

Cooperativity (log αβ) and affinity (pK_B) values for the modulation by cinacalcet, AC-265347 and BTU-compound 13 of Ca_o^{2+}- and Sr_o^{2+}-mediated activation of multiple signalling endpoints in Flp-In HEK293-TREx-c-*myc*-CASR cells (graphed by calcimimetic). Affinity and cooperativity estimates were derived from analysis of grouped datasets using an operational model of allosterism and agonism (see Methods). Data are mean ± SEM for 5–29 individual experiments performed in duplicate (individual '*n*' numbers are shown in Table 1). *$P < 0.01$, one-way ANOVA with Tukey's multiple comparisons test for differences between pathways.

shown to exhibit the greatest positive modulation of Ca_o^{2+} in ERK1/2 phosphorylation assays versus Ca_i^{2+} mobilization assays and also binds with higher affinity to the receptor conformation that couples to IP_1 accumulation (Table 1; Figure 2). Accordingly, we now reveal that AC-265347, like cinacalcet, is a strong positive modulator of Ca_o^{2+}-mediated RhoA activity in comparison to Ca_i^{2+} mobilization (Table 1; Figure 2; Supporting Information Figure S2).

Since there has been no quantitative evaluation of the pharmacology of BTU-compound 13 as a calcimimetic (Deprez *et al.*, 2013), we assessed its ability to modulate Ca_o^{2+}-mediated Ca_i^{2+} mobilization, IP_1 accumulation, ERK1/2 phosphorylation and

SRF-RE-Luc transcription in Flp-In HEK293-TREx-c-*myc*-CASR cells. BTU-compound 13 potentiated Ca_o^{2+}-mediated signalling responses in all assays, confirming its classification as a calcimimetic (Table 1; Figure 2; Supporting Information Figure S3). Like cinacalcet, BTU-compound 13 was biased towards modulation of SRF-RE-Luc transcription assay and away from ERK1/2 phosphorylation (Table 1; Figure 2), but unlike cinacalcet, BTU-compound 13 was also biased away from Ca_i^{2+} mobilization, exhibiting relatively weak potentiation in this assay. Similar to AC-265347, BTU-compound 13 bound with the highest affinity to the receptor conformation that couples to IP_1 accumulation.

Collectively, interaction studies with calcimimetics and Ca_o^{2+} suggest that each calcimimetic engenders its own profile of biased modulation, providing evidence for different conformational states favoured by structurally distinct calcimimetics.

Calcimimetics potentiate Sr_o^{2+}-mediated CaS receptor signalling

Having established that all three calcimimetics potentiated Ca_o^{2+}-mediated CaS receptor signalling, we sought to determine whether they displayed similar pharmacology when using Sr_o^{2+} as the orthosteric agonist probe. Sr_o^{2+} stimulates CaS receptor-mediated Ca_i^{2+} mobilization, IP_1 accumulation, activation of non-selective cation channels and ERK1/2 activity in HEK293 cells transiently transfected with bovine CaS receptor (Chattopadhyay *et al.,* 2007) in addition to calcitonin release, IP_1 accumulation, Ca_i^{2+} mobilization, inhibition of cAMP and ERK1/2 phosphorylation in rat medullary thyroid carcinoma 6–23 cells (Thomsen *et al.,* 2012). Here, we demonstrate that Sr_o^{2+} robustly stimulates Ca_i^{2+}-mobilization, IP_1 accumulation, ERK1/2 phosphorylation and SRF-RE-Luc transcription in Flp-In HEK293-TREx-c-*myc*-CASR cells (Figure 2; Supporting Information Figures S1–S3). Consequently, the same panel of signalling assays were used to evaluate calcimimetic-mediated modulation of Sr_o^{2+}-stimulated CaS receptor signalling (Supporting Information Figures S1–S3). Broadly speaking, all calcimimetics potentiated Sr_o^{2+}-mediated signalling across all pathways. Analogous to observations using Ca_o^{2+} as the agonist, both AC-265347 and BTU-compound 13 bound with highest affinity to the receptor conformation that coupled to Sr_o^{2+}-mediated IP_1 accumulation, and BTU-compound 13 was strongly biased towards Sr_o^{2+}-mediated SRF-RE-Luc transcription. However, in contrast to the Ca_o^{2+} dataset, cinacalcet showed no bias when Sr_o^{2+} was the agonist, and AC-265347 was not significantly biased towards Sr_o^{2+}-mediated ERK1/2 phosphorylation or SRF-RE-Luc transcription (Figure 2; Table 1). Interestingly, although cinacalcet bound with higher affinity to the receptor conformation that coupled to Ca_o^{2+}-mediated SRF-RE-Luc transcription, it showed no preference for the conformation that coupled to Sr_o^{2+}-mediated SRF-RE-Luc transcription.

Collectively, these data suggest that all CaS receptor calcimimetics potentiate Ca_o^{2+} and Sr_o^{2+}-mediated signalling, though there is evidence for both biased modulation and probe-dependence.

Cinacalcet, but not AC-265347 or BTU-compound 13, pharmacochaperones an ER-retained CaS receptor variant

In addition to biased modulation, calcimimetics differentially pharmacochaperone a naturally occurring loss-of-expression CASR-G670E variant (Cook *et al.,* 2015). Thus, whereas cinacalcet rescued G670E cell surface expression, AC-265347 had no pharmacochaperone effect (despite retaining affinity for the mutated receptor; Cook *et al.,* 2015). The divergent pharmacochaperone abilities of cinacalcet versus AC-265347 is either further evidence that these two calcimimetics preferentially stabilize different receptor conformations, or it suggests that AC-265347 is unable to access intracellular receptors, a form of 'location bias' (Irannejad *et al.,* 2017). Therefore, as the three calcimimetics in CaS receptor signalling assays displayed subtle differences in their biased profile, we next explored their potential to differentially regulate CaS receptor trafficking. As wild-type CaS receptor is robustly cell-surface expressed in HEK293 cells (and hence, it is not possible to measure chaperoning; Figure 3), we once again exploited the naturally occurring CaS receptor-G670E variant, that is, predominantly retained in the endoplasmic reticulum and only has low cell surface expression (White *et al.,* 2009; Leach *et al.,* 2012). Cinacalcet (3 μM), but not AC-265347 or BTU-compound 13, significantly enhanced CaS receptor-G670E cell-surface expression as measured by flow cytometry. This suggests that cinacalcet can access and stabilize a conformation of the CaS receptor-G670E variant that is trafficked to the cell surface, but AC-265347 and BTU-compound 13 cannot (Figure 3). All three compounds are lipophilic and are predicted to have high cell permeability (Supporting Information Table S2; Broccatelli *et al.,* 2016; Pires *et al.,* 2015), suggesting that the effects could be due to distinct receptor conformations stabilized by the calcimimetics. However, we cannot rule out that divergent active transport or permeability of the drugs leads to differential access to the intracellular CaS receptor-G670E variant.

Characterization of human CD14+ monocyte derived osteoclasts

Since cinacalcet, AC-265347 and BTU-compound 13 all differentially potentiate Ca_o^{2+} and Sr_o^{2+} signalling and have distinct pharmacochaperone activity, we evaluated the behaviour of all three calcimimetics, alone and in combination with Sr_o^{2+}, in phenotypic assays of human osteoclast function.

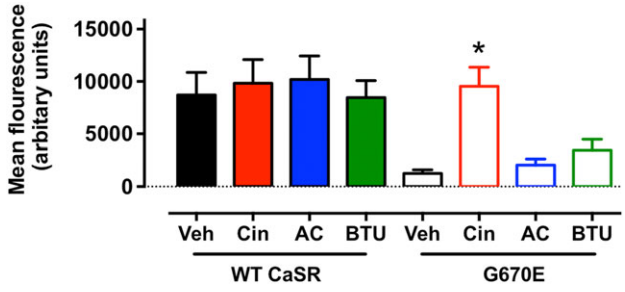

Figure 3

Effect of calcimimetics (3 μM) on the cell surface expression of either c-myc-tagged wild-type or mutant (G670E) CaS receptors expressed in HEK293 cells. Cinacalcet, but not AC-256347 or BTU-compound 13, was able to chaperone the intracellular-retained G670E mutant to the cell surface as detected by flow cytometry (*$P < 0.01$ vs. respective vehicle control; two-way ANOVA with Dunnett's multiple comparisons test, for five individual experiments).

Osteoclasts are large, multinucleated cells responsible for mediating bone resorption. They are sensitive to Sr_o^{2+} *in vivo,* resulting in decreased differentiation, reduced resorption activity and increased apoptosis (Baron and Tsouderos, 2002; Takahashi *et al.,* 2003;Bonnelye *et al.,* 2008 ; Caudrillier *et al.,* 2010). Osteoclasts were differentiated from human CD14+ monocytes isolated from the blood of 10 donors by incubation with M-CSF and RANK-L and used to assess the phenotypic and functional effects of Sr_o^{2+} and calcimimetics. These cultures comprise a heterogeneous population, from mono-nucleated cells through to osteoclasts containing 20+ nuclei, consistent with previous studies (Diepenhorst *et al.,* 2017).

Our osteoclast cultures were characterized based on morphology and the expression of key markers, determined by qPCR and imaging. Osteoclast cultures expressed mRNA for osteoclastic genes, including TRAP (*ACAP5*), *RANK* (the receptor for RANK-L), TNF receptor-associated factor 6 (*TRAF6*; involved in RANK-L signalling), calcitonin receptor (*CALCR*), cathepsin K (*CTSK*) and integrin subunit alphaV (*ITGAV*) (Figure 4A), the latter being a commonly used marker for osteoclasts owing to its role in the formation of the sealing zone. Importantly, these cultures also expressed *CASR* mRNA (Figure 4A). Western blotting studies confirmed CaS receptor protein expression in osteoclast cultures (Figure 4B), with a similar glycosylation state to the CaS receptor protein expressed in the HEK293-TREx-c-*myc*-CASR cells (Figure 4 C). Protein expressions of calcitonin receptor and TRAP were confirmed by immunofluorescence and ELF-97® staining, respectively (Figure 4D, E). A large number of cells were multinucleated with the distinct actin ring staining of osteoclasts (which facilitates formation of the sealing zone for bone resorption; Figure 4F). Figure 4G, H shows Von Kossa staining of hydroxyapatite surface with cells cultured in the absence (G) and presence (H) of 20 ng·mL^{-1} RANK-L, demonstrating its key role in the differentiation of functional osteoclasts.

Sr_o^{2+} inhibits osteoclast differentiation and resorptive activity

Given the mixed cell populations obtained in cultures of differentiated osteoclasts, ranging from mono-nucleated cells to osteoclasts containing 20+ nuclei, a method for single cell analysis of osteoclast activity and maturation was developed (Diepenhorst *et al.,* 2017), taking into account the nucleation state of an osteoclast, which is indicative of its activity and maturation (Piper *et al.,* 1992; Boissy *et al.,* 2002). This method uses TRAP as a marker of osteoclast activity based on its central role in bone matrix resorption and use as a diagnostic measure of bone turnover (Halleen *et al.,* 2006). Originally, this approach was developed to monitor the effect of RANK-L on a differentiating osteoclast population (Diepenhorst *et al.,* 2017). Here, we quantitatively assess the effects of Sr_o^{2+} and calcimimetics on osteoclast differentiation and activity.

Previous studies show that Sr_o^{2+} inhibits osteoclast differentiation and resorptive activity (Bonnelye *et al.,* 2008; Caudrillier *et al.,* 2010). Here, we determine the extent of Sr_o^{2+}-mediated inhibition of osteoclast maturation and activity (Sr_o^{2+} was added to the cells upon initiation of differentiation with RANK-L, 8 days prior to assay). Sr_o^{2+} did not significantly change osteoclast maturation (the proportion of multinucleated cells in RANK-L-differentiated osteoclast cultures; Figure 5A). However, Sr_o^{2+} treatment caused a concentration-dependent and significant reduction in osteoclast activity (TRAP activity) in higher-order osteoclasts (10+ nuclei; TRAP foci per cell reduced from 125.4 ± 12.5 in vehicle-treated cells to 79.5 ± 18.2 with 20 mM Sr_o^{2+}; Figure 5B).

The Sr_o^{2+}-mediated reduction in TRAP activity in higher-order osteoclasts was associated with a significant impairment in the resorptive capacity of osteoclasts; Sr_o^{2+} (20 mM) inhibited osteoclast-mediated resorption of hydroxyapatite artificial bone matrix in a concentration-dependent manner

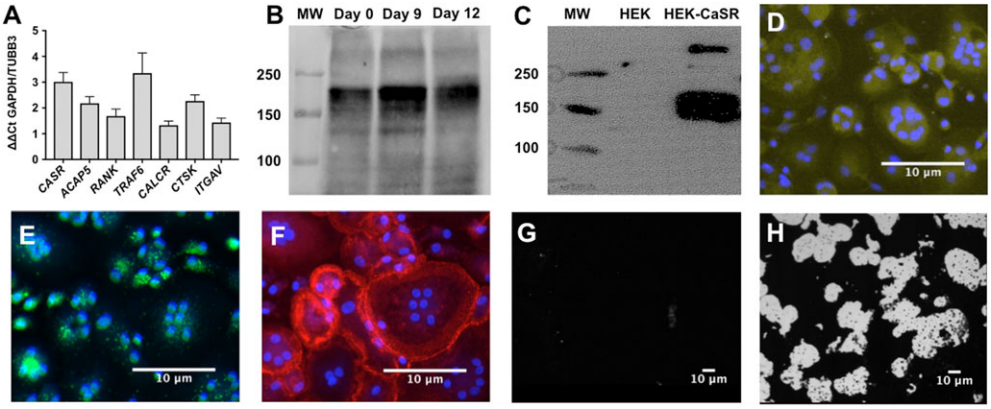

Figure 4

Characterization of human osteoclast cultures. (A) mRNA expression of markers of mature osteoclasts including the CaS receptor (*CASR*), TRAP (*ACAP5*), the receptor for RANK-L (*RANK*), TNF receptor associated factor-6 (*TRAF6*), calcitonin receptor (*CALCR*), cathepsin K (*CTSK*) and integrin subunit α V (*ITGAV*). (B) Expression of CaS receptor protein as detected by Western blotting in osteoclasts with varying days of culture with RANK-L and (C) comparison with human CaS receptors expressed in HEK293 cells. Human osteoclasts are characteristically multinucleated (Hoechst, blue) and express calcitonin receptors (mouse-anti human calcitonin receptor 1° with anti-mouse IgG-Alexa 594 2°, yellow; D) and TRAP (ELF-97®, green; E) and have distinct actin ring morphology typical of active, resorbing osteoclasts (Phaloidin-647, red; F). Von Kossa staining of osteoclast-mediated hydroxyapatite surface resorption after culture in the absence (G) and presence (H) of RANK-L.

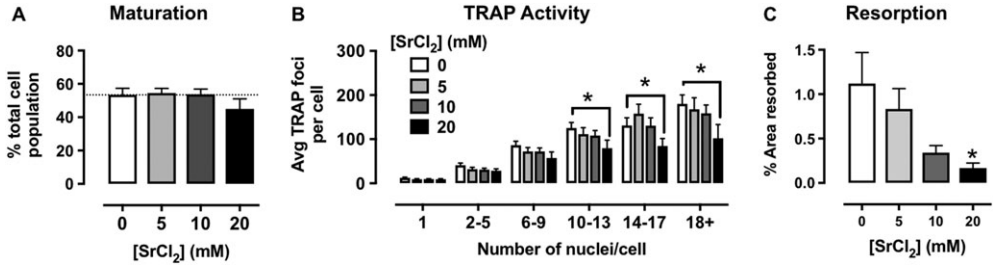

Figure 5

Sr_o^{2+} (20 mM) inhibits osteoclast differentiation and activity. (A) Sr_o^{2+} had no significant effect on the proportion of multi-nucleated cells within the population and (B) significantly decreased TRAP activity (fewer TRAP foci per cell) in higher-order osteoclasts (bins of cells containing greater than 10 nuclei; $*P < 0.01$ vs. vehicle; repeated measures two-way ANOVA with Dunnett's multiple comparisons test). (C) Unsurprisingly, osteoclast-mediated hydroxyapatite surface resorption was impaired in the presence of increasing concentrations of Sr_o^{2+} ($*P < 0.01$ vs. vehicle, one-way ANOVA with Dunnett's multiple comparisons test). Data are mean ± SEM from 11 individual experiments with analysis of four fields of view per experiment per treatment.

(Figure 5C). These results correlate well with previous studies of Sr_o^{2+} on bone cell function (Bonnelye *et al.*, 2008; Caudrillier *et al.*, 2010) and provide a framework to investigate the effects of calcimimetics on osteoclasts and, by extension, their therapeutic potential for the treatment of osteoporosis.

Cinacalcet, but not AC-265347 or BTU-compound 13, potentiates Sr_o^{2+}-mediated inhibition of TRAP activity

To determine the effects of each calcimimetic or in combination with a sub-effective concentration of Sr_o^{2+}, CD14+ monocytes from 11 donors were differentiated to osteoclasts in the presence of 3 μM cinacalcet, AC-265347 or BTU-compound 13 in the absence or presence of 5 mM Sr_o^{2+}. After 8 days, osteoclast cultures were analysed for differentiation and TRAP activity as previously described (Diepenhorst *et al.*, 2017).

None of the calcimimetics adversely affected the appearance of osteoclast cultures (data not shown). Despite robust effects on Sr_o^{2+}-mediated signalling, none of the calcimimetics changed the proportion of multinucleated cells in culture (either alone or in the presence of 5 mM Sr_o^{2+}; Figure 6).

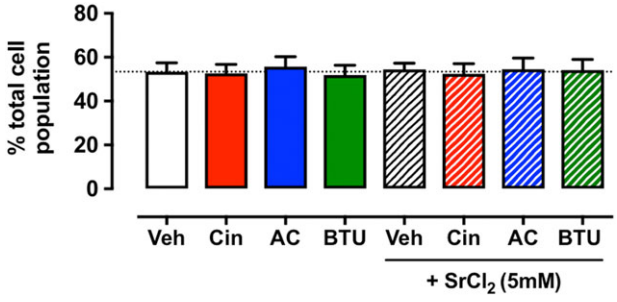

Figure 6

Calcimimetics do not modulate osteoclast differentiation, either alone (3 μM) or in combination with a sub-effective (5 mM) concentration of Sr_o^{2+}. Data are mean ± SEM from 10 individual experiments with four fields of view analysed per experiment per treatment.

However, a combination of cinacalcet plus Sr_o^{2+} significantly inhibited TRAP activity in the most active (18+ nuclei) osteoclasts (Figure 7A), whereas changes in TRAP activity were absent in cultures containing AC-2654347 or BTU-compound 13 (alone or in the presence of 5 mM Sr_o^{2+}; Figure 7B, C). Although BTU-compound 13 appeared to *enhance* TRAP activity in osteoclasts with 18+ nuclei, this effect was absent in the presence of 5 mM Sr_o^{2+} (Figure 7C).

Cinacalcet robustly inhibits osteoclast-mediated hydroxyapatite resorption

Given that cinacalcet inhibited TRAP activity in the mature 18+ nuclei-containing cells, the effect of calcimimetics on osteoclast-mediated resorption was assessed. CD14+ monocytes from 10 donors were differentiated to osteoclasts on hydroxyapatite artificial bone matrix in the presence of calcimimetic (3 μM) in the absence or presence of 5 mM Sr_o^{2+}. After 8 days, the resorbed area was visualized by Von Kossa staining and quantified in Fiji. Remarkably, cinacalcet profoundly inhibited osteoclast-mediated resorption of the hydroxyapatite surface, both alone and in the presence of Sr_o^{2+} (Figure 8). Although BTU-compound 13 marginally reduced resorption when applied alone, no effect was observed in the presence of 5 mM Sr_o^{2+}, and AC-2654347 was without effect.

Discussion

In this study, we showed that three structurally distinct calcimimetics, including the clinically used agent cinacalcet, positively modulate Sr_o^{2+}-mediated CaS receptor signalling in Flp-In HEK293-TREx-c-*myc*-CASR cells. Across a broad range of G protein-dependent signalling events, cinacalcet-mediated positive modulation of Sr_o^{2+} was unbiased, in contrast to its modulation of Ca_o^{2+}, where it was biased away from ERK1/2 phosphorylation and displayed the highest affinity for the receptor conformation that couples to a signalling readout downstream of RhoA activity (SRF-RE-Luc transcription). These latter data are consistent with and extend previous findings (Davey *et al.*, 2012; Leach *et al.*, 2013; Cook *et al.*, 2015; Leach *et al.*, 2016). AC-265347 and

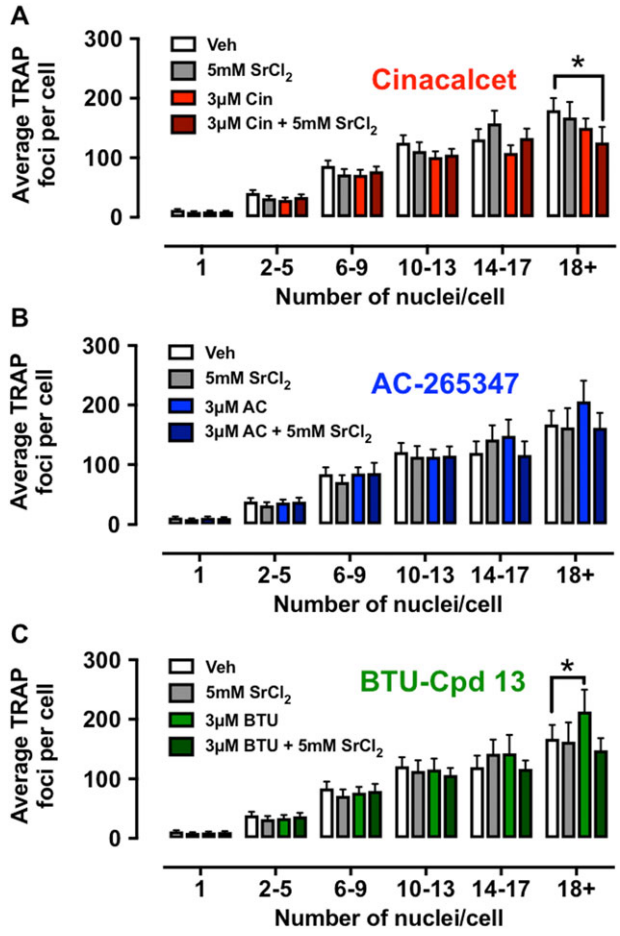

Figure 7

Quantitative analysis of TRAP activity (average number of TRAP foci per cell) in osteoclasts treated with calcimimetics alone (3 μM) or in the presence of a sub-effective concentration of Sr^{2+} (5 mM), binned by nuclei number. Cinacalcet (A; in the presence of Sr_o^{2+}) significantly reduced TRAP activity in the highest-order osteoclasts (18+ nuclei), whereas AC-265347 (B) was without effect. BTU-compound 13 (C) paradoxically appeared to increase TRAP activity alone in osteoclasts with 18+ nuclei, although there was no effect when combined with Sr^{2+}. Data are mean ± SEM from 10 individual experiments with four fields of view analysed (*$P < 0.01$ vs. vehicle; repeated measures two-way ANOVA with Dunnett's multiple comparisons test).

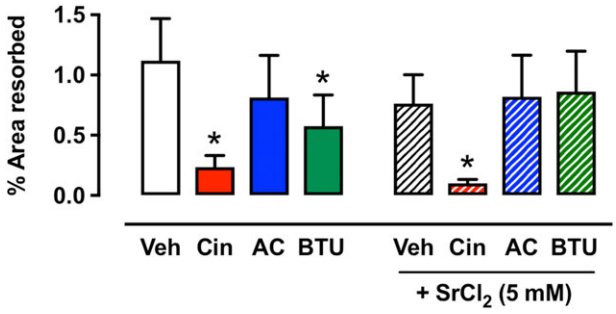

Figure 8

Osteoclasts were differentiated with calcimimetics alone (3 μM) or in the presence of a sub-effective concentration of Sr_o^{2+} (5 mM) for 8 days. Cinacalcet alone (and in combination with Sr_o^{2+}) significantly inhibited osteoclast-mediated hydroxyapatite surface resorption; BTU-compound 13 had a small inhibitory effect alone (which was not seen in combination with Sr_o^{2+}) and AC-256347 was without significant effect. Data are mean ± SEM from 10 individual experiments performed in duplicate (*$P < 0.01$ vs. vehicle; repeated measures two-way ANOVA with Dunnett's multiple comparisons test).

BTU-compound 13 also engender distinct biased modulation of CaS receptor signalling pathways depending on which orthosteric agonist is used as the probe (at the level of both affinity and efficacy). Notably, AC-265347 and BTU-compound 13 are biased towards ERK1/2 phosphorylation and/or SRF-RE-Luc transcription when Ca_o^{2+} is the probe, but only BTU-compound 13 shows bias in the presence of Sr_o^{2+}. Nonetheless, both compounds preferentially bind to the receptor conformation that couples to IP_1 accumulation.

Intriguingly, cinacalcet, but not AC-265347 or BTU-compound 13, pharmacochaperoned the endoplasmic reticulum-retained CaS receptor-G670E mutant to the cell surface. Although we cannot rule out that these compounds differentially access intracellular compartments, they are all predicted to be cell permeable (pkCSM predictor; Broccatelli *et al.*, 2016; Pires *et al.*, 2015), Collectively, these data indicate that the different calcimimetics preferentially stabilize different conformational states of the CaS receptor and thus engender biased modulation and/or exhibit location bias (Irannejad *et al.*, 2017) through access to different receptor pools.

In agreement with previous findings, we show that Sr_o^{2+} reduces human osteoclast maturation, TRAP activity and osteoclast-mediated artificial bone matrix resorption with the most profound effects on the highest-order (and most active) osteoclasts (Figure 5). However, due to its clinical side-effect profile (Compston, 2014; Reginster, 2014), the use of Sr^{2+}o ranelate as a therapeutic was limited to treatment of severe osteoporosis in postmenopausal women and adult men at high risk for fracture (it was withdrawn from the market for commercial reasons in August 2017; Servier, 2017). In order to verify whether bone-targeted calcimimetics alone, or in combination with low-dose Sr^{2+}o ranelate, might represent an alternative therapeutic approach in osteoporosis, we evaluated their effect on osteoclast maturation and activity. In broad terms, none of the calcimimetics, either alone or in combination with a sub-effective concentration of Sr_o^{2+}, reduced osteoclast maturation or TRAP activity, although cinacalcet (when combined with 5 mM Sr_o^{2+}) did significantly reduce TRAP activity in the highest-order osteoclasts. Whereas cinacalcet profoundly inhibited osteoclast-mediated artificial bone matrix resorption (even without Sr_o^{2+}), BTU-compound 13 had a small inhibitory effect (that was absent in the presence of Sr_o^{2+}) and AC-265347 was without effect. This suggests that a cinacalcet/Sr_o^{2+} combination might represent a useful approach in targeting osteoclasts; however, as neither AC-265347 nor BTU-compound 13 shared the same profile on TRAP activity and hydroxyapatite resorption, it appears that this effect may be specific to cinacalcet, rather than calcimimetics *per se*.

One previous study showed that cinacalcet (1 μM) did not alter the differentiation, TRAP activity or bone resorption of human osteoclasts (Shalhoub *et al.*, 2003) or potentiate the

effects of Ca_o^{2+} (1.6–6.1 mM). However, this lack of efficacy may reflect an absence of *CASR* mRNA in the human monocyte-derived osteoclast cultures, in contrast to our findings. Nonetheless, the differences between the effect of cinacalcet and AC-265347 or BTU-compound 13 in our osteoclast cultures are perhaps surprising, although they could be explained by the differences in biased modulatory profile of the calcimimetics.

The expression and trafficking of the CaS receptor is highly regulated at many levels, including within the endoplasmic reticulum, where mutated and misfolded receptors can be ubiquitinated and targeted for degradation (Huang *et al.,* 2006). Calcimimetics and calcilytics have been shown to act as pharmacological chaperones for CaS receptor mutants (Huang and Breitwieser, 2007; Peacock *et al.,* 2009; White *et al.,* 2009; Leach *et al.,* 2013; Cook *et al.,* 2015); it is possible that the calcimimetics herein may differentially regulate cell-surface receptor expression. By examining the trafficking of the loss-of-expression, naturally occurring, G670E point mutation CaS receptor (Leach *et al.,* 2013; Cook *et al.,* 2015), we showed that cinacalcet, but not AC-265347 or BTU-compound 13, rescues cell-surface receptor expression. Whilst these data are from studies in recombinant cells (and using a polymorphic variant), they suggest that the different modulators stabilize distinct conformational states to permit (or not) receptor trafficking. Thus, these different conformations could contribute to phenotypic differences between the calcimimetics in regulating osteoclast function. This observation requires further investigation in a physiologically relevant context; unfortunately, due to the lack of well-validated antibodies, it was not possible to determine whether cinacalcet was able to differentially traffic the endogenous CaS receptor in human osteoclasts.

We cannot discount that the differences between Sr_o^{2+}, cinacalcet, AC-265347 and BTU-compound 13 in osteoclasts are due to off-target effects of Sr_o^{2+} or cinacalcet acting beyond the CaS receptor. Sr_o^{2+} can interact with other molecular targets of Ca_o^{2+}, including gap-junction hemi-channels (Hofer and Brown, 2003), a ryanodine receptor-like protein that is expressed in osteoclasts (Zaidi *et al.,* 1995) and GPRC6, a close relative of the CaS receptor that also plays an important role in bone physiology (Pi *et al.,* 2000; Pi *et al.,* 2005); indeed, Sr_o^{2+}-mediated activation of the GPRC6 receptor is reported as partially responsible for its effects on osteoblasts (Pi *et al.,* 2005; Rybchyn *et al.,* 2009). Collectively, it is likely that both CaS receptor and non-CaS receptor mechanisms mediate the Sr_o^{2+} effects; therefore, it might be useful to examine these effects on osteoclasts derived from patients with naturally occurring CaS receptor mutations. Off-target effects of cinacalcet have not been well-defined, though results from a pan-target screening panel of various neuronal GPCRs, ion channels and transporters (coupled with the relatively high lipophilicity of the compound) suggest the propensity for a number of additional interactions, though most of the targets identified in this study (e.g. monoamine transporters) are not involved in osteoclast physiology (Wu-Wong *et al.,* 2013). Furthermore, these data would need to be coupled with knowledge of the broader pharmacology of AC-265347 or BTU-compound 13 to identify a causative factor in the effects of cinacalcet.

The broad lack of interaction between Sr_o^{2+} and calcimimetics at the level of osteoclast function indicates that a bone-targeted combination therapy approach for osteoporosis may not be viable; nonetheless, the effect of cinacalcet alone on osteoclast function (irrespective of aetiology) is intriguing and potentially important from a clinical perspective. There are mixed reports on the impact of cinacalcet on BMD in the clinic (Peacock *et al.,* 2005; Malluche *et al.,* 2008; Peacock *et al.,* 2009; Shigematsu *et al.,* 2009; Tsuruta *et al.,* 2013), most likely reflecting modulation of the CaS receptor in multiple cell types and organs (e.g. parathyroid, osteoclasts and osteoblasts). Nevertheless, these data suggest that an inhibitory effect on osteoclasts may contribute to this overall profile.

In summary, we showed that three structurally diverse calcimimetics potentiate Sr_o^{2+}-mediated activation of the CaS receptor in HEK293 cells in a similar manner to Ca_o^{2+}; however, for AC-265347 and BTU-compound 13, these effects do not generally translate to potentiation of Sr_o^{2+} to inhibit human osteoclast maturation and function, suggesting that Sr^{2+}o ranelate may mediate its effects *via* both CaS receptor- and non-CaS receptor-dependent pathways. However, the clinically approved agent, cinacalcet, did inhibit osteoclast function (both TRAP activity and resorption), although it is not clear to what degree these effects are related to its allosteric modulation of the CaS receptor or an unappreciated off-target activity (or a combination thereof). Nonetheless, an evaluation of direct effects on bone cell function may well be warranted for the development of any future calcimimetic or calcilytic agents.

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Author contributions

N.A.D., K.L., A.N.K., P.R., A.E.C., T.L.P. performed all experimental work. N.A.D., K.L., C.N. and C.J.L. analyzed data. N.A.D., K.L. and C.J.L. wrote the manuscript. P.P., M.S., P.R., K.L., R.J.S., W.N.C., P.M.S., A.C. and C.J.L. conceived the studies and reviewed the manuscript draft(s).

Conflict of interest

P.P. and M.S. are employed by Servier, which until 2017 marketed strontrium ranelate for the treatment of osteoporosis. N.A.D., K.L., A.N.K., P.R., A.E.C., T.L.P., C.N., R.J.S., W.N.C., P.M.S., A.C. and C.J.L. are (or were) employed by Monash University and receive financial support from Servier for G protein-coupled receptor drug discovery.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure S1 Concentration response curves for Ca_o^{2+}- and Sr_o^{2+}-mediated CaS receptor signalling across multiple signalling pathways in the presence of increasing concentrations of cinacalcet in Flp-In HEK293-TREx-c-*myc*-CASR cells. Data are grouped from 5–29 individual experiments performed in duplicate; the curves represent model fits to the data according to the operational model of allosterism and agonism (see Methods).

Figure S2 Concentration response curves for Ca_o^{2+}- and Sr_o^{2+}-mediated CaS receptor signalling across multiple signalling pathways in the presence of increasing concentrations of AC-265347 in Flp-In HEK293-TREx-c-*myc*-CASR cells. Data are grouped from 5–13 individual experiments performed in duplicate; the curves represent model fits to the data according to the operational model of allosterism and agonism (see Methods).

Figure S3 Concentration response curves for Ca_o^{2+}- and Sr_o^{2+}-mediated CaS receptor signalling across multiple signalling pathways in the presence of increasing concentrations of BTU compound 13 in Flp-In HEK293-TREx-c-*myc*-CASR cells. Data are grouped from five individual experiments performed in duplicate; the curves represent model fits to the data according to the operational model of allosterism and agonism (see Methods).

Table S1 Primers for the characterization of mature osteoclast cultures using RT-PCR.

Table S2 Calculated physicochemical and predicted cell permeability parameters for calcimimetics.