

**Calcimimetic and calcilytic therapies for inherited disorders of the calcium-sensing receptor signalling pathway**

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## Abstract

The calcium-sensing receptor (CaS receptor) plays a pivotal role in extracellular calcium homeostasis and germline loss- and gain-of-function mutations cause familial hypocalciuric hypercalcaemia (FHH) and autosomal dominant hypocalcaemia (ADH), respectively. CaS receptor signal transduction in the parathyroid glands is likely regulated by G-protein subunit  $\alpha_{11}$  ( $G\alpha_{11}$ ) and adaptor-related protein complex-2 sigma subunit (AP2 $\sigma$ ), and recent studies have identified germline mutations of these proteins as a cause of FHH and/or ADH. Calcimimetics and calcilytics are positive and negative allosteric modulators of the CaS receptor that have potential efficacy for symptomatic forms of FHH and ADH. Cellular studies have demonstrated that these compounds correct signalling and/or trafficking defects caused by mutant CaS receptor,  $G\alpha_{11}$  or AP2 $\sigma$  proteins. Moreover, mouse model studies indicate that calcilytics can rectify the hypocalcaemia and hypercalciuria associated with ADH, and patient-based studies reveal calcimimetics to ameliorate symptomatic hypercalcaemia caused by FHH. Thus, calcimimetics and calcilytics represent targeted therapies for inherited disorders of the CaS receptor signalling pathway.

**Abbreviations:** ADH, autosomal dominant hypocalcaemia; ADH1, autosomal dominant hypocalcaemia type 1; ADH2, autosomal dominant hypocalcaemia type 2; ADIS, agonist-driven insertional signalling; AP2 $\sigma$ , adaptor-related protein complex-2 sigma subunit;  $Ca^{2+}_o$ , extracellular calcium;  $Ca^{2+}_i$ , intracellular calcium; CaS receptor, calcium-sensing receptor; DAG, diacylglycerol; *Dsk7*, *Dark skin 7*; ENU, *N*-ethyl-*N*-nitrosourea; ER, endoplasmic reticulum; FHH, familial hypocalciuric hypercalcaemia; FHH1, familial hypocalciuric hypercalcaemia type 1; FHH2, familial hypocalciuric hypercalcaemia type 2; FHH3, familial hypocalciuric hypercalcaemia type 3;  $G\alpha_{11}$ , G-protein subunit  $\alpha_{11}$ ; GPCR, G-protein coupled receptor; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; NSHPT, neonatal severe hyperparathyroidism; *Nuf*, nuclear flecks; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PTH, parathyroid hormone; TMD, transmembrane domain.

## Introduction

The extracellular calcium ( $\text{Ca}^{2+}_o$ )-sensing receptor is a dimeric cell-surface protein that belongs to the glutamate (family C) class of G-protein coupled receptors (GPCRs). The calcium-sensing receptor, also referred to as [CaS receptor](#) (Alexander et al., 2017), is highly expressed in calcitropic tissues such as the parathyroid glands and kidneys, and also in non-calcitropic tissues such as the pancreatic islets (Babinsky et al., 2017; Hannan, Babinsky & Thakker, 2016). The CaS receptor plays a key role in systemic calcium homeostasis by detecting increases in the prevailing  $\text{Ca}^{2+}_o$  concentration, which initiates signalling via multiple intracellular pathways. These include the G-protein subunit  $\alpha_{11}$  ( $G\alpha_{11}$ )-dependent stimulation of phospholipase C (PLC) activity, which leads to an accumulation of inositol 1,4,5-triphosphate together with the rapid mobilisation of intracellular  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}_i$ ), and also activates the mitogen-activated protein kinase (MAPK) pathway (Figure 1) (Hannan, Babinsky & Thakker, 2016). These intracellular events mediate a decrease in parathyroid hormone (PTH) secretion and reduction in renal tubular calcium reabsorption (Figure 1) (Hannan, Babinsky & Thakker, 2016). The sensitivity of parathyroid and renal tubular cells to  $\text{Ca}^{2+}_o$  is likely influenced by the level of CaS receptor cell-surface expression. This in turn is regulated by agonist-driven insertional signalling (ADIS), which enhances anterograde trafficking of newly synthesised CaS receptors to the plasma membrane (Grant, Stepanchick, Cavanaugh & Breitwieser, 2011); and also by clathrin-mediated endocytosis and retrograde trafficking of cell-surface CaS receptors, which is considered to involve the heterotetrameric adaptor-related protein complex-2 (AP2) (Figure 1) (Hannan, Babinsky & Thakker, 2016).

The central role of CaS receptor signalling and trafficking in  $\text{Ca}^{2+}_o$  homeostasis has been demonstrated by the identification of mutations affecting this GPCR and its intracellular partner proteins, which result in alterations in serum calcium and urinary calcium excretion. For example, germline heterozygous loss-of-function mutations affecting the CaS receptor,  $G\alpha_{11}$ , and AP2  $\sigma$ -subunit (AP2 $\sigma$ ) lead to familial hypocalciuric hypercalcaemia (FHH) types 1 to 3, respectively; whereas germline heterozygous gain-of-function mutations of the CaS receptor and  $G\alpha_{11}$  cause

autosomal dominant hypocalcaemia (ADH) types 1 and 2, respectively (Figure 1, Table 1) (Hannan et al., 2012; Nesbit et al., 2013a; Nesbit et al., 2013b). FHH is an autosomal dominant condition characterised by mild-to-moderate elevations of serum calcium concentrations, normal or elevated circulating parathyroid hormone (PTH) concentrations, and inappropriately low urinary calcium excretion (Hannan & Thakker, 2013). FHH is usually an asymptomatic condition, however, some patients may develop symptomatic hypercalcaemia or recurrent pancreatitis, and low bone mineral density values as well as cognitive dysfunction has been noted in some FHH type 3 (FHH3) patients (Table 1) (Hannan et al., 2015a). Moreover, the offspring of parents affected with FHH type 1 (FHH1) may harbour heterozygous, homozygous or compound heterozygous CaS receptor mutations that cause neonatal severe hyperparathyroidism (NSHPT). This life-threatening disorder is characterized by severe hypercalcaemia and hyperparathyroid skeletal disease leading to fractures and respiratory distress (Table 1) (Hannan & Thakker, 2013). Occasionally, patients harbouring heterozygous or homozygous loss-of-function CaS receptor mutations have been reported to develop primary hyperparathyroidism in adulthood (Table 1) (Hannan & Thakker, 2013). Autosomal dominant hypocalcaemia is characterised by the opposite biochemical phenotype to FHH, and patients thus have low serum calcium concentrations, normal or low PTH concentrations, and a relative or absolute hypercalciuria (Hannan, Babinsky & Thakker, 2016). Approximately 50% of ADH1 patients have symptomatic hypocalcaemia and >30% of patients have renal and/or intracerebral calcifications (Table 1) (Hannan, Babinsky & Thakker, 2016). Some ADH1 patients with severe gain-of-function CaS receptor mutations may additionally have Bartter syndrome type V, which is characterised by hypokalaemic alkalosis, renal salt wasting and hyperreninaemic hyperaldosteronism (Table 1) (Watanabe et al., 2002).

The management of NSHPT and severe hypercalcaemia due to FHH generally involves surgical neck exploration and parathyroidectomy, as conventional medical therapies such as bisphosphonates have limited efficacy for these disorders (Waller et al., 2004). Symptomatic ADH patients are commonly managed with calcium and active vitamin D preparations, however their use predisposes patients to the development of marked hypercalciuria, nephrocalcinosis, nephrolithiasis and renal impairment (Hannan, Babinsky & Thakker, 2016; Nesbit et al., 2013a). ADH patients have

also been treated with recombinant PTH (1-34) (teriparatide), however, this peptide therapy may not always prevent hypercalciuric renal complications (Theman et al., 2009). Calcimimetic and calcilytic compounds are positive and negative modulators of the CaS receptor, respectively (Nemeth & Goodman, 2016), and have the potential to correct the underlying pathophysiological alterations in parathyroid and kidney function caused by loss-of-function or gain-of-function mutations affecting this receptor or its intracellular partner proteins. This article will provide an overview of calcimimetic and calcilytic compounds and discuss the cellular, mouse model and patient-based studies that have evaluated their use for the management of calcitropic and non-calcitropic phenotypes associated with FHH, NSHPT and ADH.

## **Classification & structure of calcimimetics & calcilytics**

### *Calcimimetics*

Calcimimetics are ligands that mimic or enhance the effects of  $\text{Ca}^{2+}_o$  at the CaS receptor, and are divided into two types: type I calcimimetics are agonists, which include naturally occurring ligands such as polyvalent cations; and type II calcimimetics are positive allosteric modulators that increase the sensitivity of the CaS receptor to  $\text{Ca}^{2+}_o$ , thereby shifting the concentration-response curve of cells expressing this receptor to the left (Hannan, Babinsky & Thakker, 2016; Nemeth, 2004). Some type II calcimimetics may also have intrinsic agonist actions, and are referred to as allosteric agonists (Leach et al., 2016). The first generation of type II calcimimetics are phenylalkylamine compounds that were derived from the structure of fendiline, which is a voltage-gated calcium channel blocker (Nemeth et al., 1998). NPS R-568 (also known as [tecalcet](#)) is a fendiline analogue (Figure 2) and was the first calcimimetic to be evaluated in clinical trials. This compound was shown to decrease plasma PTH concentrations in patients with primary hyperparathyroidism (Silverberg et al., 1997), secondary hyperparathyroidism due to end-stage renal failure (Antonsen, Sherrard & Andress, 1998), or parathyroid carcinoma (Collins, Skarulis, Bilezikian, Silverberg, Spiegel & Marx, 1998). However, NPS R-568 had a variable pharmacokinetic profile and was superseded by [cinacalcet](#) (also known as

NPS 1493 and AMG073) (Figure 2). Cinacalcet is an NPS R-568 analogue characterised by an improved pharmacokinetic profile with higher bioavailability and more reliable dose-effect relationships in individual patients (Nemeth et al., 2004). Cinacalcet was the first GPCR allosteric modulator to obtain regulatory approval, and is approved as a daily oral therapy for patients with either: secondary hyperparathyroidism due to end-stage renal failure; inoperable forms of primary hyperparathyroidism; or parathyroid carcinoma (Nemeth & Goodman, 2016). More recently, a type II calcimimetic known as [AC265347](#), which has a novel benzothiazole structure (Figure 2) has been identified (Ma et al., 2011). *In vitro* studies involving cultured fibroblasts indicate AC265347 to have greater potency and efficacy than cinacalcet (Ma et al., 2011). Furthermore, AC265347 has been shown to be more potent than cinacalcet at lowering serum PTH when administered subcutaneously, but less potent when administered orally in studies involving wild-type rats (Ma et al., 2011). Whereas, the efficacy of these two compounds on serum PTH concentrations is similar by either route of administration (Ma et al., 2011; Nemeth et al., 2004). In addition, AC265347 has been shown to have greater intrinsic agonist properties than cinacalcet, and to enhance CaS receptor signalling responses in the absence of  $\text{Ca}^{2+}_o$  to a larger extent (Ma et al., 2011). [Etelcalcetide](#) (also known as AMG416) is a synthetic polycationic peptide (Figure 2) that forms a transient covalent disulphide bond with the Cys482 residue located in the CaS receptor extracellular domain, and acts as a type II calcimimetic and agonist of this GPCR (Alexander et al., 2015; Nemeth & Goodman, 2016). Etelcalcetide is administered intravenously and has a longer circulating half-life than cinacalcet in renal failure patients, and has recently been approved for the management of secondary hyperparathyroidism in adult patients on haemodialysis (Block et al., 2017).

### *Calcilytics*

Calcilytics are negative allosteric modulators that reduce the sensitivity of the CaS receptor to  $\text{Ca}^{2+}_o$ , thereby shifting the concentration-response curve of cells expressing this receptor to the right (Nemeth & Goodman, 2016). Calcilytics comprise two main classes of orally active compounds, which are the amino alcohols (e.g. [NPS 2143](#), [ronacaleret](#), NPSP795 (also known as [SB-423562](#)) and JTT-305/MK-5442 (also known as [encaleret](#))) and quinazolinones (e.g. [ATF 936](#) and AXT 914)

(Figure 2) (Nemeth & Goodman, 2016). Calcilytics were originally investigated as potential therapies for osteoporosis, as these compounds stimulated transient PTH secretion, which had the potential to induce anabolic effects on bone mass (Nemeth et al., 2001). NPS 2143 was the first potent and selective calcilytic to be identified, and pre-clinical studies revealed this calcilytic to lead to sustained increases in PTH secretion, which did not alter bone mass (Gowen et al., 2000). NPS 2143 was subsequently modified to generate compounds such as ronacaleret and NPSP795 (Figure 2), which induced a more short-lived rise in PTH secretion (Kumar et al., 2010). However, a phase II clinical trial involving ronacaleret showed a lack of efficacy for post-menopausal osteoporosis (Fitzpatrick et al., 2011). The JTT-305/MK-5442 and AXT 914 calcilytic compounds have also been evaluated in postmenopausal subjects, and been shown not to increase bone mineral density or markers of bone formation, respectively (Halse et al., 2014; John et al., 2014).

### **Mechanism of action of calcimimetics & calcilytics**

Calcimimetics and calcilytics are predicted to bind within a cavity located between the mid-portion and extracellular aspect of the CaS receptor transmembrane domain (TMD) (Leach et al., 2016). Within this binding cavity, the Glu837 residue has been shown to be critical for binding both phenylalkylamine calcimimetics and amino alcohol calcilytics, such that a loss of this negatively charged Glu837 residue attenuated the effects of these compounds (Jacobsen, Gether & Brauner-Osborne, 2017; Leach et al., 2016; Miedlich, Gama, Seuwen, Wolf & Breitwieser, 2004; Petrel, Kessler, Dauban, Dodd, Rognan & Ruat, 2004). At the entrance to this binding cavity, the Glu767 and Arg680 residues have also been demonstrated to bind cinacalcet and NPS 2143, respectively (Leach et al., 2016). Thus, these compounds bind to similar regions within the TMD cavity, whereas the structurally distinct AC265347 compound has been shown to bind deeper within this cavity. This finding may help to explain differences in the pharmacological actions of AC265347 compared to the phenylalkylamines and amino alcohols (Leach et al., 2016). Calcimimetics are considered to act by stabilising the TMD in a conformation that facilitates G-protein coupling, whereas calcilytics stabilise

the TMD in an inactive conformation (Jacobsen, Gether & Brauner-Osborne, 2017). However, the mechanisms by which cinacalcet and NPS 2143 induce opposing effects on CaS receptor function despite binding to almost identical regions within the TMD remain to be elucidated. Recent mutagenesis studies have begun to delineate TMD residues such as Trp818 and Tyr825, which may mediate the potentiating effects of cinacalcet, and also identified that the Leu776 residue may mediate the inhibitory effects of NPS 2143 (Leach et al., 2016). Moreover, it has been shown that calcimimetics are required to bind to only one monomer of the dimeric CaS receptor to potentiate GPCR function, whereas calcilytics are required to bind to both monomers to achieve full inhibition of CaS receptor function (Jacobsen, Gether & Brauner-Osborne, 2017).

Calcimimetic and calcilytic compounds have been shown to influence CaS receptor-mediated  $\text{Ca}^{2+}_i$  and MAPK signalling responses in a concentration-dependent manner (Davey, Leach, Valant, Conigrave, Sexton & Christopoulos, 2012; Nemeth et al., 2001; Nemeth et al., 1998). Furthermore, the structurally different classes of allosteric modulators have distinct effects on biased signalling (Davey, Leach, Valant, Conigrave, Sexton & Christopoulos, 2012). Thus, the phenylalkylamine calcimimetic and amino alcohol calcilytic compounds show greater positive and negative allosteric modulation of  $\text{Ca}^{2+}_i$  mobilization, respectively, compared to their effects on MAPK responses (Davey, Leach, Valant, Conigrave, Sexton & Christopoulos, 2012). Whereas, the AC265347 calcimimetic, which is a benzothiazole compound, biases signalling towards the MAPK cascade (Leach et al., 2016). These biased signalling responses may arise from the ability of structurally distinct modulators to stabilise CaS receptor conformations that activate different signalling pathways (Davey, Leach, Valant, Conigrave, Sexton & Christopoulos, 2012). In addition to the effects of calcimimetics and calcilytics on signal transduction, these compounds influence both the total cellular expression and plasma membrane expression of the CaS receptor. Thus, one study showed the NPS R-568 calcimimetic to increase expression of wild-type and loss-of-function mutant CaS receptors, whilst demonstrating that the NPS 2143 calcilytic decreased the expression of wild-type and gain-of-function mutant receptors (Huang & Breitwieser, 2007). Whereas, another study showed that NPS 2143 had no significant effect on the cell-surface expression of wild-type or gain-of-function mutant CaS receptors, but increased the cell-surface expression of loss-of-function mutant receptors (Leach,



Wen, Cook, Sexton, Conigrave & Christopoulos, 2013). The mechanism underlying the effects of allosteric modulators on CaS receptor expression may involve these compounds binding to the TMD of newly synthesised receptors within the ER (Figure 3). Thus, the allosteric modulators may act as pharmacochaperones to facilitate correct protein folding and biosynthesis, thereby ensuring that the nascent receptors are not targeted for proteasomal degradation, but instead undergo post-translational modifications such as glycosylation, and are trafficked from the ER to the Golgi and plasma membrane (Figure 3) (Breitwieser, 2014). Calcimimetics may additionally increase CaS receptor expression by enhancing signalling from plasma membrane-localized receptors, which act via ADIS to increase receptor biosynthesis and trafficking to the cell surface (Figure 3) (Grant, Stepanchick & Breitwieser, 2012). In keeping with these findings, *in vivo* studies have shown calcimimetic treatment to upregulate parathyroid CaS receptor expression in a rat model for secondary hyperparathyroidism (Mizobuchi et al., 2004), and also in patients on dialysis (Sumida et al., 2013).

### **Calcimimetic treatment for hypercalcaemic disorders of the CaS receptor signalling pathway**

#### *Familial hypocalciuric hypercalcaemia type 1 (FHH1) and neonatal severe hyperparathyroidism (NSHPT)*

Calcimimetic drugs represent a targeted therapy for symptomatic forms of FHH1 and NSHPT (Table 2), and have been demonstrated to improve the EC<sub>50</sub> values and maximal responses of loss-of-function mutant CaS receptors *in vitro* (Lu, Yang, Gnacadja, Christopoulos & Reagan, 2009; Rus et al., 2008). However, acute exposure to the NPS R-568 calcimimetic had no effect on receptors that were truncated and/or had reduced cell-surface expression (Rus et al., 2008). Whereas, prolonged exposure of CaS receptor-expressing cells to NPS R-568 or cinacalcet increased the cell-surface expression of the majority of loss-of-function mutant CaS receptors, indicating that these calcimimetics are likely acting as pharmacochaperones to enhance mutant receptor biosynthesis and trafficking (Huang & Breitwieser, 2007; Leach, Wen, Cook, Sexton, Conigrave & Christopoulos, 2013; White, McKenna, Cavanaugh & Breitwieser, 2009). However, the AC265347 calcimimetic compound did not influence mutant CaS receptor cell-surface expression, most likely due to its

reduced membrane permeability, which will prevent its actions as a pharmacochaperone (Cook et al., 2015). Thus, AC265347 may be more suitable for the treatment of loss-of-function CaS receptor mutations that selectively impair signal transduction, whilst cinacalcet treatment may be more appropriate for loss-of-function mutations that impair receptor signalling and trafficking (Cook et al., 2015; Leach, Wen, Cook, Sexton, Conigrave & Christopoulos, 2013).

Cinacalcet has been used to treat symptomatic hypercalcaemia in FHH1 patients (Table 2), and the acute effects of cinacalcet on serum PTH and calcium concentrations in these patients have been characterized (Festen-Spanjer, Haring, Koster & Mudde, 2008; Timmers, Karperien, Hamdy, de Boer & Hermus, 2006). Thus, a single 30 mg oral dose of cinacalcet was found to maximally lower serum PTH concentrations at 2 hours post-dose, with the PTH concentrations returning to baseline by 12 hours post-dose (Festen-Spanjer, Haring, Koster & Mudde, 2008; Timmers, Karperien, Hamdy, de Boer & Hermus, 2006). However, this single 30 mg cinacalcet dose had no effect on serum calcium concentrations (Festen-Spanjer, Haring, Koster & Mudde, 2008; Timmers, Karperien, Hamdy, de Boer & Hermus, 2006), whereas a single 60 mg dose of cinacalcet did decrease gradually the serum calcium concentrations over a 12 hour period after administration (Timmers, Karperien, Hamdy, de Boer & Hermus, 2006). Moreover, repetitive daily dosing with 30 mg cinacalcet administered once or twice daily has been shown to result in a sustained lowering of serum calcium concentrations in FHH1 patients, and also to increase in urinary calcium excretion (Festen-Spanjer, Haring, Koster & Mudde, 2008; Rasmussen, Jorgensen & Schwarz, 2011). In addition, long-term cinacalcet treatment has been reported to improve hypercalcaemic symptoms such as muscle aches, anorexia, polydipsia and constipation (Alon & VandeVoorde, 2010; Rasmussen, Jorgensen & Schwarz, 2011; Sethi, Nagesh, Kelwade, Parekh & Dukle, 2017). In one patient with FHH1 and recurrent pancreatitis, cinacalcet had a dose-dependent effect on the frequency of hospital admissions due to pancreatitis with 90mg/day cinacalcet (administered as 30 mg three-times daily) leading to a cessation of acute pancreatitis episodes for >2 years (Gunganah, Grossman & Druce, 2014). Cinacalcet therapy has also been reported to aid post-surgical healing in a child with FHH1 who underwent a tympanoplasty for chronic otitis media, which was complicated by tympanosclerosis due to calcium deposition (Alon & VandeVoorde, 2010). Long-term cinacalcet treatment has been reported to be effective at lowering

serum calcium concentrations in all FHH1 patients, and without major adverse effects such as hypocalcaemia. In contrast, the response of NSHPT-associated hypercalcaemia to cinacalcet is variable and appears to depend on the underlying CaS receptor mutation. Thus, oral cinacalcet therapy has been reported to be effective at ameliorating hypercalcaemia and hyperparathyroidism, and in improving the skeletal mineralisation in NSHPT patients harbouring a heterozygous Arg185Gln CaS receptor mutation (Fisher, Cabrera & Imel, 2015; Gannon, Monk & Levine, 2014; Reh, Hendy, Cole & Jeandron, 2011). Whereas cinacalcet was ineffective in NSHPT patients harbouring bilallelic truncating CaS receptor mutations (Atay et al., 2014; Garcia Soblechero, Ferrer Castillo, Jimenez Crespo, Dominguez Quintero & Gonzalez Fuentes, 2013), and this may be due to the truncated mutant receptor being unable to bind cinacalcet and/or couple with downstream signalling proteins.

#### *Familial hypocalciuric hypercalcaemia type 2 (FHH2)*

The effectiveness of cinacalcet in rectifying the FHH2-associated loss-of-function abnormalities caused by the downstream  $G\alpha_{11}$  protein has been assessed by *in vitro* and *in vivo* studies. Thus, recent *in vitro* studies have shown that cinacalcet improves the signalling responses of HEK293 cells stably expressing the CaS receptor (HEK-CaS receptor), which have been transiently transfected with FHH2-associated  $G\alpha_{11}$  mutants (Babinsky et al., 2016; Gorvin et al., 2017a). Indeed, nanomolar doses of cinacalcet were demonstrated to rectify the impaired  $Ca^{2+}_i$  responses associated with loss-of-function  $G\alpha_{11}$  mutations (Table 2) (Babinsky et al., 2016; Gorvin et al., 2017a). Moreover, siRNA knockdown studies showed that cinacalcet enhanced the signalling mediated by the FHH2 mutant  $G\alpha_{11}$  proteins rather than by exerting indirect effects on endogenously expressed wild-type  $G\alpha_{11}$  proteins (Babinsky et al., 2016). However, the response of FHH2-associated mutant cells to cinacalcet treatment may be influenced by the location of the mutation within the  $G\alpha_{11}$  protein. Thus, FHH2-causing mutations located within regions of the  $G\alpha_{11}$  GTPase domain involved in GPCR binding (Ile200del) or PLC coupling (Phe220Ser) have been shown to require higher (40-100 nM) cinacalcet concentrations for rectifying responses (Babinsky et al., 2016; Gorvin et al., 2017a), than the FHH2-

causing Leu135Gln mutation, which is located in the guanine nucleotide-stabilising  $G\alpha_{11}$  helical domain and which responded to treatment with 10 nM cinacalcet (Babinsky et al., 2016).

A patient with FHH2 due to a heterozygous germline Phe220Ser  $G\alpha_{11}$  mutation has been treated with cinacalcet (Table 2). The patient had hypercalcaemia in association with headaches, constipation and pruritus, and was initially commenced on cinacalcet 30 mg daily, which failed to normalize his elevated serum calcium concentrations over a period of 3 months (Gorvin et al., 2017a). However, the hypercalcaemia of this FHH2 patient did normalize following treatment with 60 mg daily of cinacalcet (Gorvin et al., 2017a). These findings are in keeping with cellular studies, which showed that a higher (100 nM) cinacalcet concentration was required to rectify the loss-of-function of the Phe220Ser  $G\alpha_{11}$  mutation *in vitro* compared with other FHH2-causing mutations, which have responded to 10-40 nM concentrations of cinacalcet (Babinsky et al., 2016; Gorvin et al., 2017a). Although the cinacalcet was effective in ameliorating the hypercalcaemia, the calcimimetic did not improve the symptoms and it was therefore discontinued after a period of 4 months (Gorvin et al., 2017a).

#### *Familial hypocalciuric hypercalcaemia type 3 (FHH3)*

Cinacalcet has also been evaluated as a therapy for symptomatic hypercalcaemia associated with FHH3. *In vitro* studies revealed this calcimimetic to rectify the loss-of-function associated with all three reported FHH3-causing AP2 $\sigma$  mutations (Table 2) (Howles et al., 2016). Indeed, administration of 10 nM cinacalcet corrected the impaired  $Ca^{2+}_i$  and MAPK signalling responses of HEK-CaS receptor cells expressing Arg15Cys, Arg15His or Arg15Leu AP2 $\sigma$  mutant proteins (Howles et al., 2016). *In vivo*, cinacalcet has been administered to three FHH3 patients, who each harboured a heterozygous Arg15Cys, Arg15His or Arg15Leu AP2 $\sigma$  mutation, and had symptoms such as fatigue, musculoskeletal pain and headaches (Howles et al., 2016). Treatment with cinacalcet 30-60mg daily led to >20% reductions in serum calcium concentrations in all three FHH3 patients, and also lowered serum PTH concentrations, which remained within the normal range (Howles et al., 2016). Cinacalcet, which was administered for >30 months in these patients, also led to a symptomatic improvement and did not cause adverse effects such as nausea, vomiting or hypocalcaemia (Howles et

al., 2016). Cinacalcet (30-60mg daily) lowered serum calcium concentrations into the lower half of the normal range in a child with hypercalcaemia due to an Arg15Leu AP2 $\sigma$  mutation and a chromosome 22q11.2 deletion syndrome (Tenhola et al., 2015). However, the child developed hypocalcaemic symptoms such as paraesthesia and numbness, and the calcimimetic was therefore stopped after three years of treatment (Tenhola et al., 2015). These studies show that cinacalcet-mediated allosteric modulation of the CaS receptor can rectify the loss-of-function and symptomatic hypercalcaemia that are associated with the three types of FHH3-causing Arg15 AP2 $\sigma$  mutations (Table 2). However, long-term surveillance of cinacalcet-treated FHH patients is required to assess the safety of this calcimimetic and prevent life-threatening hypocalcaemia (Howles et al., 2016).

### **Calcilytic treatment for hypocalcaemic disorders of the CaS receptor signalling pathway**

#### *Autosomal dominant hypocalcaemia type 1*

Calcilytic compounds have been assessed for the management of ADH1 (Table 2), and *in vitro* studies have shown the calcilytic, NPS 2143, to rectify the increased signalling responses associated with ADH1-causing CaS receptor mutations (Table 2) (Hannan et al., 2015b; Leach, Wen, Cook, Sexton, Conigrave & Christopoulos, 2013; Letz et al., 2010). However, NPS 2143 may have limited efficacy for severe gain-of-function ADH1 mutants, and was shown not to alter the Ca<sup>2+</sup><sub>i</sub> responses of the constitutively active Ala843Glu mutant (Leach, Wen, Cook, Sexton, Conigrave & Christopoulos, 2013), which causes Bartter syndrome type V. Interestingly, the responsiveness of severe gain-of-function ADH1 mutants to NPS 2143 could be improved by co-expressing them with wild-type CaS receptors (Letz et al., 2010). The *in vitro* efficacy of NPS 2143 can be also reduced by some mutations affecting the TMD binding cavity (Letz et al., 2010), although the quinazolinone-derived calcilytic drugs (ATF 936 and AXT 914) have been reported to ameliorate the excessive signalling responses of all ADH1 mutants, including those mutations leading to constitutive activation and/or Bartter syndrome type V (Letz et al., 2014).

The *in vivo* effects of calcilytics and their ability to rectify the hypocalcaemia associated with ADH1 have been assessed in mouse models that harbour germline gain-of-function CaS receptor

mutations (Table 2). In one study, NPS 2143 was administered as a single dose to a mutant mouse model known as *Nuclear flecks (Nuf)*, which was generated by chemical mutagenesis involving the isopropyl methane sulfonate (iPMS) alkylating agent (Hough et al., 2004). *Nuf* mice have hypocalcaemia, reduced plasma PTH concentrations and ectopic calcification in association with a germline gain-of-function mutation, Leu723Gln, located within the CaS receptor TMD (Hannan et al., 2015b; Hough et al., 2004). A single intraperitoneal injection of NPS 2143 significantly increased plasma calcium and PTH concentrations in heterozygous and homozygous *Nuf* mice at 1-hour after administration, with the values returning to baseline after 4-hours. The elevations in plasma calcium concentrations induced by NPS 2143, were not associated with any increase in urinary calcium excretion (Hannan et al., 2015b). Longer-term *in vivo* studies involving the JTT-305/MK-5442 calcilytic compound have been undertaken in two knock-in mouse models, which harbour ADH1-causing germline heterozygous Cys129Ser and Ala843Glu gain-of-function CaS receptor mutations (Dong et al., 2015). Administration of JTT-305/MK-5442 by daily oral gavage over a 12-week period led to sustained increases in serum calcium concentrations with significant reductions in urinary calcium excretion (Dong et al., 2015). Moreover, treatment with JTT-305/MK-5442 in mouse models harbouring the ADH1-causing Cys129Ser or Ala843Glu CaS receptor mutations prevented the development of nephrocalcinosis, which was observed in mice treated with the drug vehicle or recombinant PTH (1-34) (Dong et al., 2015). The NPSP795 calcilytic compound has also been evaluated in a phase IIa clinical trial involving five ADH1 patients harbouring germline CaS receptor mutations (Ramnitz et al., 2015). Intravenous administration of NPSP795 significantly increased plasma PTH concentrations and reduced urinary calcium excretion (Table 2) (Ramnitz et al., 2015). However, circulating calcium concentrations were not altered in this study, and the optimal dosing regimen for NPSP795 remains to be established in ADH1 patients (Ramnitz et al., 2015).

#### *Autosomal dominant hypocalcaemia type 2 (ADH2)*

*In vitro* studies have shown that NPS 2143 can rectify the increased signalling responses of HEK-CaS receptor cells expressing the ADH2-associated Arg181Gln or Phe341Leu  $G\alpha_{11}$  mutant proteins (Table 2) (Babinsky et al., 2016). However, in keeping with studies involving the FHH2-associated  $G\alpha_{11}$

mutant proteins, the response to NPS 2143 appeared to be related to the location of the mutation within the  $G\alpha_{11}$  protein (Babinsky et al., 2016). Thus, the Phe341Leu mutation, which is located within the C-terminal region of the  $G\alpha_{11}$  GTPase domain that is involved in GPCR binding, required a 3-fold increase in the NPS 2143 dose to rectify the gain-of-function compared to the Arg181Gln mutation, which is located at the interface between the  $G\alpha_{11}$  helical and GTPase domains (Babinsky et al., 2016).

The effect of NPS 2143 on the hypocalcaemia associated with ADH2 has been evaluated in two mouse models (Gorvin et al., 2017b; Roszko et al., 2017). In one mouse model, which is known as *Dark skin 7 (Dsk7)* and was generated by *N*-ethyl-*N*-nitrosourea (ENU) chemical mutagenesis, NPS 2143 was administered as a single dose by oral gavage (Gorvin et al., 2017b). Treatment with NPS 2143 rectified the hypocalcaemia and increased plasma PTH concentrations of the *Dsk7* mice, which harbour a germline gain-of-function Ile62Val  $G\alpha_{11}$  mutation (Gorvin et al., 2017b). Indeed, this calcilytic induced 4-5 fold elevations in plasma PTH concentrations of heterozygous and homozygous *Dsk7* mice, and this was associated with a 0.25-0.50 mmol/L increase in plasma calcium concentrations (Gorvin et al., 2017b). In the other ADH2 mouse model, which was generated by CRISPR-Cas9 gene editing and harbours a human ADH2-causing germline Arg60Cys  $G\alpha_{11}$  mutation, intraperitoneal injection of a single dose of NPS 2143 significantly increased blood ionised calcium and PTH concentrations (Roszko et al., 2017). Moreover, NPS 2143 significantly lowered urinary calcium excretion in mice harbouring the Arg60Cys  $G\alpha_{11}$  mutation (Roszko et al., 2017). Thus, these single dose *in vivo* studies have demonstrated that calcilytics can rectify the hypocalcaemia caused by ADH2 without leading to hypercalciuria (Table 2) (Gorvin et al., 2017b; Roszko et al., 2017).

### **Effect of calcilytic treatment on the impaired glucose tolerance of an ADH1 mouse model**

The CaS receptor is highly expressed in pancreatic islet  $\beta$ -cells and  $\alpha$ -cells, where it is considered to regulate insulin and glucagon secretion, respectively (Gray et al., 2006). Moreover, studies involving the *Nuclear flecks (Nuf)* mouse model have highlighted a role for this GPCR in systemic glucose homeostasis (Babinsky et al., 2017). Thus, *Nuf* mice, which harbour a germline gain-of-function CaS

receptor mutation, were found to have significantly impaired glucose tolerance in association with hypoinsulinaemia and also a lack of glucose-mediated suppression of glucagon secretion (Babinsky et al., 2017). These findings demonstrate that germline CaS receptor mutations may give rise to non-calcitropic phenotypes as well as influencing  $Ca^{2+}_o$  homeostasis. To determine whether calcilytic treatment may rectify the impaired glucose tolerance of *Nuf* mice, ronacaleret was administered by twice-daily oral gavage over a period of 5 days. This calcilytic was shown to ameliorate the impaired glucose tolerance of heterozygous (*Nuf/+*) and homozygous (*Nuf/Nuf*) mice (Table 2) (Babinsky et al., 2017). However, the mechanisms underlying the glucose-lowering effects of ronacaleret remains to be fully elucidated, as this calcilytic only improved insulin secretion in *Nuf/+* mice, but not in *Nuf/Nuf* mice, and also had no effect on glucagon secretion (Babinsky et al., 2017). One possibility is that ronacaleret acting via the CaS receptors, which are expressed in skeletal muscle and adipose tissue, may have sensitised these peripheral tissues to the actions of insulin, and thereby improved glucose tolerance (Babinsky et al., 2017).

## Conclusions

Calcimimetic and calcilytic therapies provide a targeted approach to rectifying the molecular and pathophysiological alterations caused by germline mutations of the CaS receptor and its intracellular partner proteins. Thus, cellular studies indicate that these compounds can rectify signalling and/or trafficking defects caused by mutant CaS receptor,  $G\alpha_{11}$  or  $AP2\sigma$  proteins. Calcimimetics have been shown to be effective at lowering serum calcium and PTH concentrations in patients with FHH types 1-3, and also in some patients with NSHPT. In addition, calcilytics can rectify the hypocalcaemia and hypercalciuria in mouse models for ADH types 1 and 2. Moreover, these allosteric modulators have potential benefit for non-calcitropic phenotypes such as the impaired glucose tolerance caused by a germline gain-of-function CaS receptor mutation.



### **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 (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

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**Table 1.** Hypercalcaemic and hypocalcaemic disorders of the calcium-sensing receptor (CaS receptor) and partner proteins ( $G\alpha_{11}$  and AP2 $\sigma$ ).

Disorder	OMIM <sup>a</sup>	Inheritance	Gene <sup>b</sup>	Chromosomal localisation	Clinical features
<b>Hypercalcaemic disorders</b>					
Familial hypocalciuric hypercalcaemia type 1 (FHH1)	145980	Autosomal dominant	<i>CASR</i>	3q21.1	Asymptomatic in majority of patients
Familial hypocalciuric hypercalcaemia type 2 (FHH2)	145981	Autosomal dominant	<i>GN111</i>	19p13.3	Asymptomatic in majority of patients
Familial hypocalciuric hypercalcaemia type 3 (FHH3)	600740	Autosomal dominant	<i>AP2SI</i>	19q13.3	Hypercalcaemic symptoms in >20% of patients Low bone mineral density in >50% of patients Childhood cognitive deficits in >75% of patients
Neonatal severe hyperparathyroidism (NSHPT)	239200	Autosomal recessive or dominant	<i>CASR</i>	3q21.1	Hyperparathyroid bone disease Hypercalcaemic symptoms
Adult-onset primary hyperparathyroidism (PHPT)	-	Autosomal recessive or dominant	<i>CASR</i>	3q21.1	Nephrolithiasis in >40% of patients Low bone mineral density in >25% of patients
<b>Hypocalcaemic disorders</b>					
Autosomal dominant hypocalcaemia type 1 (ADH1)	601198	Autosomal dominant	<i>CASR</i>	3q21.1	Hypocalcaemic symptoms in ~50% of patients Ectopic calcifications in ~35% of patients
Autosomal dominant hypocalcaemia type 2 (ADH2)	615361	Autosomal dominant	<i>GN111</i>	19p13.3	Hypocalcaemic symptoms in >75% of patients Short stature reported in two kindreds
Bartter syndrome type V	601198	Autosomal dominant	<i>CASR</i>	3q21.1	Renal salt wasting and hypokalaemia Hypocalcaemic symptoms in >75% of patients

<sup>a</sup>Online Mendelian Inheritance in Man. <sup>b</sup>*CASR* encodes the CaS receptor; *GN111* encodes  $G\alpha_{11}$ ; and *AP2SI* encodes AP2 $\sigma$ .

Adapted from Hannan FM, Babinsky VN, Thakker RV. Disorders of the calcium-sensing receptor and partner proteins: insights into the molecular basis of calcium homeostasis. *J Mol Endocrinol.* 2016; 57(3): R127-42.

**Table 2.** Summary of key studies assessing effectiveness of calcimimetics and calcilytics for FHH, NSHPT and ADH.

Disorder	<i>In-vitro</i> studies	<i>In-vivo</i> studies
<b>Hypercalcaemic disorders</b>		
FHH1/NSHPT	NPS R-568 and cinacalcet enhance the signalling responses and cell-surface expression of loss-of-function FHH1/NSHPT-causing mutant CaS receptors (Huang & Breitwieser, 2007; Leach, Wen, Cook, Sexton, Conigrave & Christopoulos, 2013; Rus et al., 2008; White, McKenna, Cavanaugh & Breitwieser, 2009)	Cinacalcet lowers serum calcium and PTH concentrations, and improves hypercalcaemic symptoms in FHH1 patients (Festen-Spanjer, Haring, Koster & Mudde, 2008; Rasmussen, Jorgensen & Schwarz, 2011; Timmers, Karperien, Hamdy, de Boer & Hermus, 2006)  Cinacalcet lowers serum calcium and PTH concentrations in NSHPT patients harbouring a heterozygous Arg185Gln CaS receptor mutation, but is ineffective for NSHPT caused by biallelic truncating CaS receptor mutations (Atay et al., 2014; Fisher, Cabrera & Imel, 2015; Gannon, Monk & Levine, 2014; Reh, Hendy, Cole & Jeandron, 2011)
FHH2	Cinacalcet enhances the signalling responses of cells expressing loss-of-function FHH2-causing $G\alpha_{11}$ mutants (Babinsky et al., 2016)	Cinacalcet normalises serum calcium concentrations in an FHH2 patient (Gorvin et al., 2017a)
FHH3	Cinacalcet enhances the signalling responses of cells expressing loss-of-function FHH3-causing Arg15Cys, Arg15His, or Arg15Leu AP2 $\sigma$ mutations (Howles et al., 2016)	Cinacalcet lowers serum calcium and PTH concentrations, and improves hypercalcaemic symptoms in FHH3 patients with Arg15Cys, Arg15His or Arg15Leu AP2 $\sigma$ mutations (Howles et al., 2016)
<b>Hypocalcaemic disorders</b>		
ADH1	NPS 2143 reduces the signalling responses of cells expressing gain-of-function ADH1-causing CaS receptor mutants, but has limited efficacy for constitutively active CaS receptor mutants (Leach, Wen, Cook, Sexton, Conigrave & Christopoulos, 2013; Letz et al., 2010)  ATF 936 and AXT 914 rectify the gain-of-function caused by constitutively active CaS receptor mutants (Letz et al., 2014)	Acute administration of NPS 2143 and JTT-305/MK-5442 increases serum calcium and PTH concentrations in mouse models for ADH1 (Dong et al., 2015; Hannan et al., 2015b)  Administration of JTT-305/MK-5442 over 12 weeks reduces urinary calcium excretion and prevents nephrocalcinosis in mouse models for ADH1 (Dong et al., 2015)  Administration of ronacalceret over 5 days rectifies the impaired glucose tolerance in a mouse model for ADH1 (Babinsky et al., 2017)  Intravenous infusion of NPS795 increases serum PTH concentrations and reduces urinary calcium excretion in ADH1 patients (Ramnitz et al., 2015)
ADH2	NPS 2143 reduces the signalling responses of cells expressing gain-of-function ADH2-causing $G\alpha_{11}$ mutants (Babinsky et al., 2016; Gorvin et al., 2017b; Roszko et al., 2017)	NPS 2143 increases serum calcium and PTH concentrations in mouse models for ADH2 (Gorvin et al., 2017b; Roszko et al., 2017)



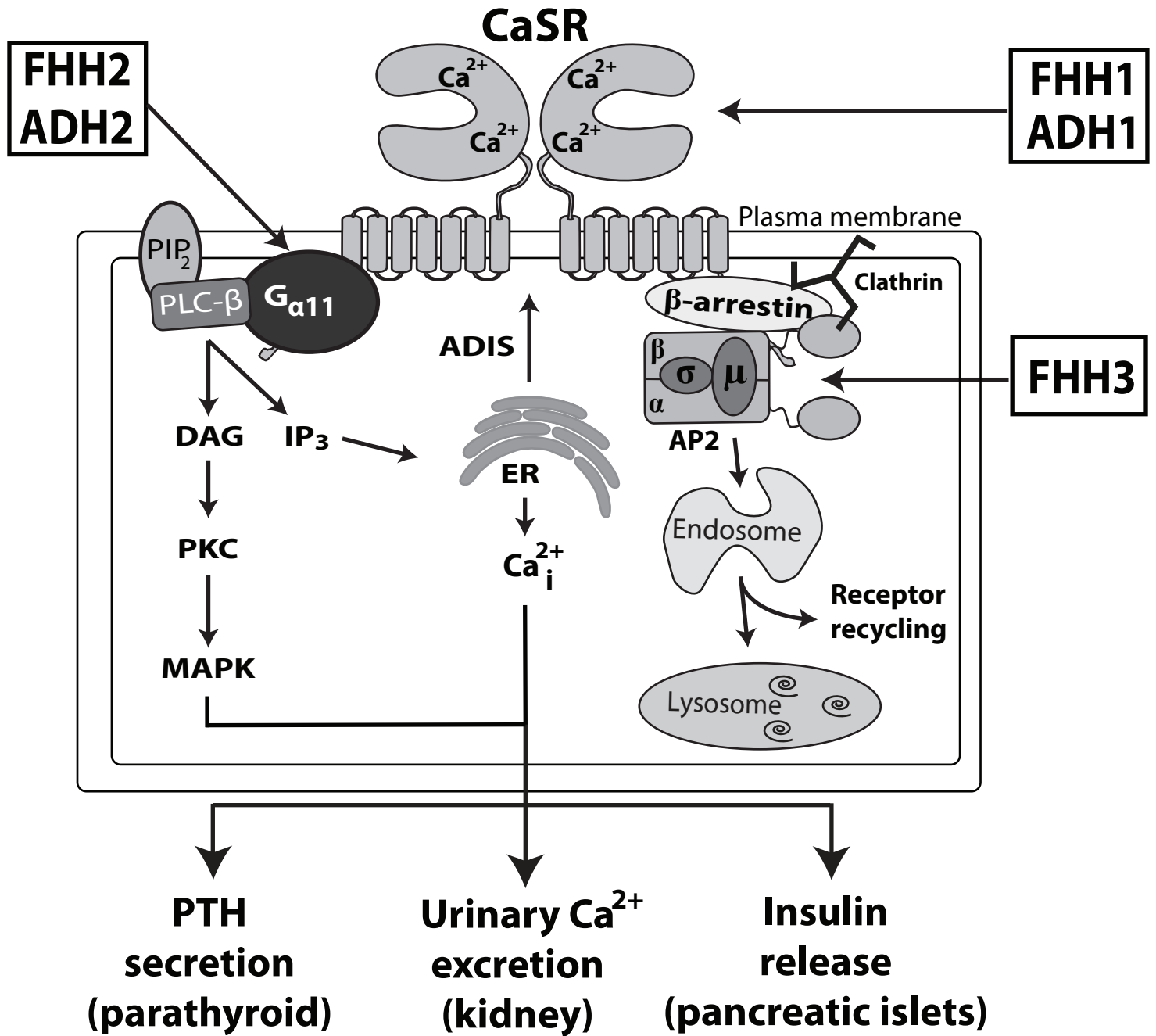
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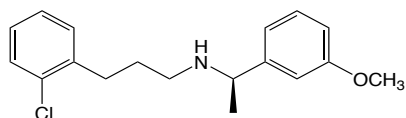
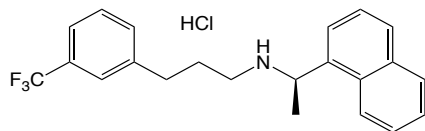
**Figure 1.** Overview of calcium-sensing receptor signalling and trafficking. The binding of calcium ( $\text{Ca}^{2+}$ ) to the CaS receptor extracellular domain leads to  $\text{G}\alpha_{11}$ -dependent activation of phospholipase C- $\beta$  (PLC- $\beta$ ) and the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) from membrane bound phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ). The increase in intracellular  $\text{IP}_3$  levels facilitates the release of  $\text{Ca}^{2+}$  from intracellular stores such as the endoplasmic reticulum (ER). DAG activates protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway. These signalling events lead to a decrease in parathyroid hormone (PTH) secretion and reduction in renal tubular calcium reabsorption, and also enhance insulin release from pancreatic islets. CaS receptor cell-surface expression is regulated by agonist-driven insertional signalling (ADIS) (Grant, Stepanchick, Cavanaugh & Breitwieser, 2011) and also by an endocytic complex comprising clathrin,  $\beta$ -arrestin and the AP2 complex, which may traffic this GPCR to the endosomal-lysosomal degradation pathway or recycle the CaS receptor back to the cell-surface (Breitwieser, 2014). Loss- and gain-of function mutations of the CaS receptor lead to FHH1 and ADH1, respectively; loss- and gain-of function mutations of the  $\text{G}\alpha_{11}$  subunit are associated with FHH2 and ADH2, respectively; and loss-of-function mutations of the  $\text{AP2}\sigma$  subunit lead to FHH3.

**Figure 2.** Structure of synthetic calcimimetic and calcilytic compounds. A. Synthetic type II calcimimetics comprise a structurally diverse group of compounds, which include phenylalkylamines such as: NPS R-568 and cinacalcet; AC265347, which has a benzothiazole structure; and Etelcalcetide, which is a polycationic peptide. B. All calcilytics described to-date are synthetic compounds and are divided into two classes: amino alcohols such as NPS 2143, ronacaleret, NPSP795 and JTT-305/MK-5442; and the quinazolinone-derived calcilytics such as AXT 914 and ATF 936.

**Figure 3.** Effect of calcimimetics and calcilytics on calcium-sensing receptor signalling and trafficking. Calcimimetic (red) and calcilytic (blue) compounds bind to the transmembrane domain of the plasma membrane-expressed CaS receptor and modulate  $\text{Ca}^{2+}_o$ -mediated signalling responses. These signalling responses may influence CaS receptor biosynthesis via the agonist-driven insertional signalling (ADIS) mechanism (Grant, Stepanchick, Cavanaugh & Breitwieser, 2011). The hydrophobic nature of most CaS receptor allosteric modulators may allow these drugs to enter the endoplasmic reticulum (ER), where they act as pharmacochaperones to influence protein folding, post-translational modifications and proteasomal degradation of newly formed CaS receptors. Thus, calcimimetics and calcilytics may influence the proportion of receptors that pass the conformational checkpoints for ER release and are trafficked to the cell surface (Breitwieser, 2014).

Figure 1



**Figure 2****A. Type II calcimimetic compounds****NPS R-568****Cinacalcet (NPS 1493/AMG073)****Etelcalcetide (AMG 416)**

H-L-Cys-OH

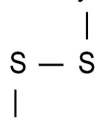
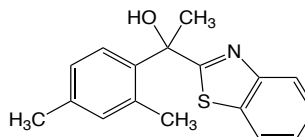
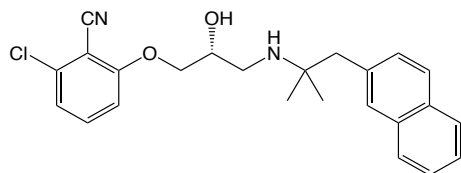
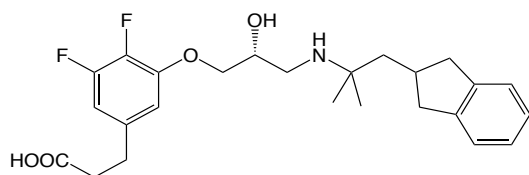
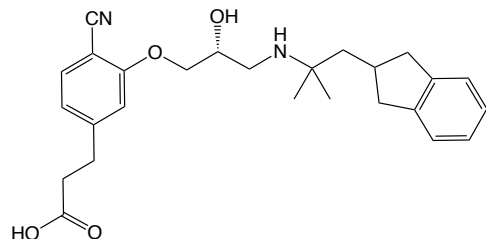
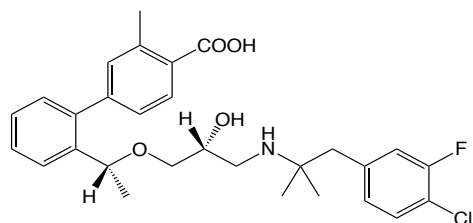
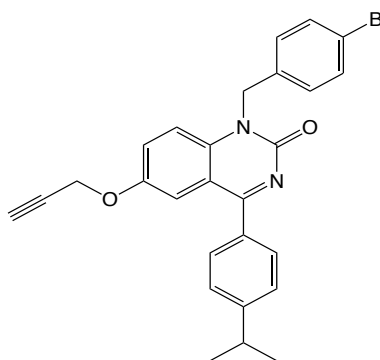
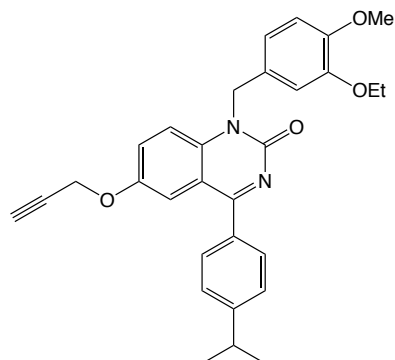
Ac-D-Cys-D-Ala-D-Arg-D-Arg-D-Arg-D-Ala-D-Arg-NH<sub>2</sub>**AC-265347****B. Calcilytic compounds****Amino alcohols****NPS 2143****Ronacaleret****NPSP795****JTT-305/MK-5442****Quinazolinones****AXT914****ATF936**

Figure 3

