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

Jian-Nong Ma, Michelle Owens, Magnus Gustafsson, Jacob Jensen, Ali Tabatabaei, Kara Schmelzer, Roger Olsson, and Ethan S. Burstein

ACADIA Pharmaceuticals, Inc., San Diego, California

Received December 14, 2010; accepted January 12, 2011

ABSTRACT

We discovered structurally novel human calcium-sensing receptor (CaSR) allosteric agonists and compared their pharmacology to phenylalkylamine calcimimetics. 1-Benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol (AC-265347) activated CaSR signaling in cellular proliferation and phosphatidylinositol (PI) hydrolysis assays with potencies of 30 and 10 nM, respectively. (S)-1-Benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol [(S)-AC-265347], the S-enantiomer of AC-265347, was approximately 10- to 20-fold more potent than (R)-1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol [(R)-AC-265347]. The phenylalkylamines cinacalcet and calindol had activity similar to that of AC-265347 in cellular proliferation assays but less activity in PI assays. All compounds had reduced activity when extracellular Ca\(^{2+}\) was removed, indicating that they cooperate with Ca\(^{2+}\) to activate CaSRs, and all activated CaSR isoforms with the N-terminal extracellular domain deleted, indicating that they interact with the transmembrane domains. In both cases, AC-265347 and therefore (S)-AC-265347 were significantly more efficacious than the phenylalkylamines. Mutations E837A7.39 and I841A7.43 strongly reduced phenylalkylamine-induced signaling, but not AC-265347- or (S)-AC-265347-induced signaling, suggesting different modes of binding. AC-265347 and (S)-AC-265347 stimulated significantly greater responses than cinacalcet or calindol at each of four loss-of-function human polymorphic CaSR variants. AC-265347 did not inhibit the CYP2D6 cytochrome P450 isozyme, unlike cinacalcet, which is a potent CYP2D6 inhibitor. In rats, AC-265347, (S)-AC-265347, and (R)-AC-265347 each reduced serum parathyroid hormone (PTH) with a rank order potency correlated with their in vitro potencies. AC-265347 and (S)-AC-265347 also reduced plasma ionizable calcium ([Ca\(^{2+}\)])\(_{in}\). AC-265347 was orally active, and its plasma concentrations correlated well with its effects on serum PTH. Thus, these highly efficacious CaSR allosteric agonists represent leads for developing therapeutic agents with potential advantages over existing therapies.

Introduction

Extracellular calcium ([Ca\(^{2+}\)\(_{in}\)]\(_o\)) is able to function as a “first” messenger, affecting a wide array of cellular processes, and therefore blood levels of [Ca\(^{2+}\)\(_i\)]\(_o\) are subject to extremely tight control (Brown and MacLeod, 1991). This regulation is mediated primarily by a calcium-sensing receptor (CaSR), first cloned from bovine parathyroid cells (Brown et al., 1993). The CaSR is a member of the G protein-coupled receptor (GPCR) superfamily and belongs to the group C family of GPCRs, which also includes the GABAB receptor and the metabotropic glutamate receptors. These receptors contain a 10- to 20-fold more potent than (R)-1-(3-methoxyphenyl)ethyl]propan-1-amine. vice versa, their in vitro potencies. AC-265347 and (S)-AC-265347 each reduced serum parathyroid hormone (PTH) with a rank order potency correlated with their in vitro potencies. AC-265347 and (S)-AC-265347 also reduced plasma ionizable calcium ([Ca\(^{2+}\)])\(_{in}\). AC-265347 was orally active, and its plasma concentrations correlated well with its effects on serum PTH. Thus, these highly efficacious CaSR allosteric agonists represent leads for developing therapeutic agents with potential advantages over existing therapies.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.110.178194.

ABBREVIATIONS: CaSR, calcium-sensing receptor; GPCR, G protein-coupled receptor; PTH, parathyroid hormone; HEK, human embryonic kidney; AC-265037, 1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol; (S)-AC-265037, (S)-1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol; (R)-AC-265347, (R)-1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol; R-SAT, Receptor Selection and Amplification Technology; PI, phosphatidylinositol; LC, liquid chromatography; NPS R-568, 3-(2-chlorophenyl)-N-[(1R)-1-(3-methoxyphenyl)ethyl]propan-1-amine.
stasis including the parathyroid, thyroidal C cells, kidney, bone (including osteoclasts, osteoblasts, and osteocytes), chondrocytes (cartilage-forming cells), intestine (including duodenum and ileum), and placenta. However, the CaSR is also found in organs not involved in maintaining [Ca\(^{2+}\)]\(_o\) homeostasis such as brain with the highest levels in the subfornical organ (hypothalamic thirst center); neurons, astrocytes, and microglia; pituitary gland; bone marrow and peripheral blood (including platelets and monocytes); keratinocytes; the gastrointestinal system including the esophagus, stomach, small intestine, and colon; and the pancreas where it may affect insulin and glucagon secretion (Brown and MacLeod, 2001). Thus, besides its well documented role in maintaining [Ca\(^{2+}\)]\(_o\) homeostasis, the CaSR may also function as a nutrient sensor, an osmolarity regulator, and a regulator of hormone secretion, cellular chemotaxis, proliferation, differentiation, apoptosis, and gene expression.

The physiological roles of the CaSR have been further validated through correlation of human CaSR polymorphisms with diseases of [Ca\(^{2+}\)]\(_o\) homeostasis. Inactivating mutations in the human CaSR lead to familial hypocalciuric hypercalcemia (or familial benign hypercalcemia), and neonatal severe hyperparathyroidism, whereas activating mutations in the human CaSR cause autosomal dominant hypocalciuric hypercalcemia with hypercalciuria and Bartter syndrome (Thakker, 2004). Autoimmune antibodies to the CaSR cause autoimmune hypocalciuric hypercalcemia and acquired hyperparathyroidism (Thakker, 2004).

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

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

**Materials and Methods**

**Ligands.** MgCl\(_2\) and CaCl\(_2\) were from Sigma-Aldrich (St. Louis, MO). Pharmacy-grade cinacalcet hydrochloride (Sensipar) tablets were dissolved in dimethyl sulfoxide stock solutions immediately before use. Calindol hydrochloride was from Toronto Research Chemicals (North York, ON, Canada). 1-Benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol (AC-275347) and its resolved enantiomers (\(S\)-1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol) [(\(S\))-AC-265347] and (\(R\)-1-benzothiazol-2-yl-1-(2,4-dimethyl-phenyl)-ethanol) [(\(R\))-AC-265347] were synthesized at ACADIA Pharmaceuticals, Inc. (San Diego, CA) (Gustafsson et al., 2010). Compound structure was verified by NMR. Purity was greater than 99% as measured by high-performance liquid chromatography and gas chromatography.

**Cell Culture.** NIH 3T3 cells (American Type Culture Collection, Manassas, VA) were incubated at 37°C in a humidified atmosphere (5% CO\(_2\)) in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 U/ml penicillin G, 50 U/ml streptomycin (Invitrogen), and 10% calf serum (Sigma-Aldrich) or 25% UltraCULTURE synthetic supplement (Lonza Walkersville Inc., Walkersville, MD). HEK 293T (American Type Culture Collection) cells were cultured similarly except that 10% fetal calf serum was substituted for 10% calf serum.

**Materials and Methods**
Phosphatidylinositol Hydrolysis Assays. Phosphatidylinositol (PI) hydrolysis assays were performed using diluted SPA beads (80 μl; GE Healthcare, Little Chalfont, Buckinghamshire, UK), followed by 30 μl of each cell lysate/well of Pico plates (PerkinElmer Life and Analytical Sciences, Waltham, MA) as described previously (Gardell et al., 2008). The "high Ca²⁺" buffer was identical to the buffer used for the cellular proliferation assays with the addition of 10 mM LiCl as described previously. The "no Ca²⁺" buffer was composed of Hanks’ buffered salt solution (HyClone, Logan, UT) plus 0.5 mM MgCl₂, 0.2% bovine serum albumin, and 10 mM LiCl and did not contain antibiotics.

Data Analysis. Concentration-response graphs for all functional assays were plotted, and EC₅₀ values were determined by nonlinear regression analysis using Prism software (version 4.0; GraphPad Software, Inc., San Diego, CA) according to the following equation:
where $X$ is the logarithm of concentration and $Y$ is the response; $Y$ starts at Bottom and goes to Top with a sigmoid shape.

PTH-Releasing Assays. Male Sprague-Dawley rats (~150–200 g) were housed with free access to rat chow and water at two animals per cage for at least 2 days before use. Drugs were dissolved in 5% dimethyl sulfoxide-5% water-90% PEG 400 and dosed either subcutaneously or orally. The controls were given the same volume of vehicle as the test groups. Blood samples were obtained at the indicated times, and the plasma fraction was separated and stored at −80°C until further use. PTH levels were analyzed using a rat intact PTH radioimmunoassay kit according to the manufacturer’s instructions (Immutopics International, San Clemente, CA).

Analysis of Plasma Phosphate and Ca$^{2+}$ Concentrations. Plasma phosphate levels were analyzed using a phosphate assay kit with an improved Malachite Green dye from BioAssay Systems (Hayward, CA) according to the manufacturer’s instructions. Plasma levels of ionizable Ca$^{2+}$ were determined using an ion-selective electrode blood-gas analyzer (IDEXX Laboratories, Inc., West Sacramento, CA).

Analysis of AC-265347 Concentrations. Plasma levels of AC-265347 were measured in rats dosed orally with 10 or 30 mg/kg. Plasma samples were collected at 0, 0.5, 1, 2, 4, 6, and 24 h and analyzed by LC-tandem mass spectrometry. The LC-tandem mass spectrometry analysis was performed using a 4000 QTRAP (Applied Biosciences, Foster City, CA) hybrid triple quadrupole linear ion trap mass spectrometer equipped with electrospray ionization and operated in multiple reaction monitoring mode. The AC-265347 ion pair was 284.2/266. The mass spectrometer was coupled to a high-performance liquid chromatography system consisting of two LC-20AD high-performance pumps interfaced with a CBM-20A controller (Shimadzu, Columbia, MD) and a CTC HTC PAL (LEAP Technologies, Carrboro, NC) autosampler. Separation was performed using a 50 × 2.1-mm Hypersil GOLD aQ (Thermo Fisher Scientific, Waltham, MA) reverse-phase C18 column equipped with a guard column. LC solvent A was water and solvent B was acetonitrile, each containing 1% formic acid. Data collection and processing were performed using Analyst software (version 1.4.2).

Results

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

AC-265347-activated CaSR signaling in cellular proliferation and PI hydrolysis assays with potencies of 30 and 10 nM, respectively (Fig. 2; Tables 1 and 2). The enantiomers of AC-265347 were each active, with (S)-AC-265347 being approximately 10- to 20-fold more potent than (R)-AC-265347. The phenylalkylamine calcimetics cinacalcet (Nemeth et al., 2004) and calindol had activity similar to that of AC-265347 in cellular proliferation assays, but less activity in PI hydrolysis assays. Under assay conditions of high ambient Ca$^{2+}$, all of these compounds stimulated 85 to 100% of the maximal functional response to MgCl$_2$ in cellular prolifera-

### Table 1

**Agonist activation of WT and mutant CaSRs by CaSR ligands in cellular proliferation assays**

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Ligands</th>
<th>pEC$_{50}$</th>
<th>Efficacy vs. WT</th>
<th>Hill No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>AC-265347</td>
<td>7.6 ± 0.6</td>
<td>97 ± 8</td>
<td>1.10 ± 0.06</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>7.5 ± 0.2</td>
<td>99 ± 9</td>
<td>1.19 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>7.0 ± 0.2</td>
<td>89 ± 12</td>
<td>1.12 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>7.4 ± 0.2</td>
<td>103 ± 9</td>
<td>1.01 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>7.6 ± 0.1</td>
<td>86 ± 6</td>
<td>1.09 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>2.4 ± 0.1</td>
<td>100 ± 4</td>
<td>2.90 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>R66C</td>
<td>AC-265347</td>
<td>5.7 ± 0.3</td>
<td>16 ± 13</td>
<td>N.C.</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>5.6 ± 0.3</td>
<td>10 ± 3</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>5.6 ± 0.3</td>
<td>3 ± 0</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>4 ± 1</td>
<td>N.C.</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>11 ± 6</td>
<td>N.C.</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1.3 ± 0.5</td>
<td>28 ± 16</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>T138M</td>
<td>AC-265347</td>
<td>6.2 ± 0.2</td>
<td>99 ± 12</td>
<td>1.39 ± 0.14</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>6.4 ± 0.2</td>
<td>100 ± 13</td>
<td>1.16 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>5.5 ± 0.1</td>
<td>58 ± 17</td>
<td>0.94 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>6.6 ± 0.1</td>
<td>57 ± 1</td>
<td>1.13 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>6.8 ± 0.1</td>
<td>53 ± 7</td>
<td>1.05 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1.2 ± 0.2</td>
<td>107 ± 13</td>
<td>2.90 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>R185Q</td>
<td>AC-265347</td>
<td>6.1 ± 0.2</td>
<td>77 ± 11</td>
<td>1.83 ± 0.18</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>6.4 ± 0.1</td>
<td>76 ± 9</td>
<td>1.77 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>&lt;5.0</td>
<td>30 ± 4</td>
<td>1.69 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>5.6 ± 0.3</td>
<td>31 ± 3</td>
<td>1.28 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>6.0 ± 0.3</td>
<td>25 ± 5</td>
<td>0.97 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1.7 ± 0.2</td>
<td>13 ± 5</td>
<td>2.48 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>R795W</td>
<td>AC-265347</td>
<td>&lt;5.0</td>
<td>44 ± 10</td>
<td>N.C.</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>5.4 ± 0.2</td>
<td>62 ± 7</td>
<td>1.31 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>5.6 ± 0.2</td>
<td>63 ± 7</td>
<td>1.31 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>5.6 ± 0.3</td>
<td>31 ± 3</td>
<td>1.28 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>6 ± 1</td>
<td>N.C.</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.8 ± 0.2</td>
<td>53 ± 8</td>
<td>2.01 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>E837A</td>
<td>AC-265347</td>
<td>6.4 ± 0.2</td>
<td>66 ± 14</td>
<td>1.24 ± 0.13</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>6.5 ± 0.3</td>
<td>72 ± 18</td>
<td>1.17 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>5.6 ± 0.4</td>
<td>48 ± 20</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>&lt;5.0</td>
<td>18 ± 4</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>&lt;5.0</td>
<td>29 ± 8</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>2.5 ± 0.2</td>
<td>71 ± 9</td>
<td>3.07 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>I814A</td>
<td>AC-265347</td>
<td>7.5 ± 0.3</td>
<td>103 ± 9</td>
<td>0.98 ± 0.19</td>
</tr>
<tr>
<td>(S)-AC-265347</td>
<td>7.4 ± 0.2</td>
<td>101 ± 10</td>
<td>1.34 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>(R)-AC-265347</td>
<td>5.6 ± 0.6</td>
<td>69 ± 13</td>
<td>1.29 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td>6.9 ± 0.3</td>
<td>25 ± 5</td>
<td>0.98 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>7.8 ± 0.3</td>
<td>24 ± 7</td>
<td>0.74 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>2.5 ± 0.1</td>
<td>104 ± 9</td>
<td>2.02 ± 0.16</td>
<td></td>
</tr>
</tbody>
</table>

WT, wild type; N.C., not calculated.

![Downloaded from JASPER Journals.org on July 7, 2017](http://www.aspetjournals.org)
TABLE 2
Agonist activation of WT and mutant CaSRs by CaSR ligands in PI hydrolysis assays

The compounds indicated were tested in PI hydrolysis using transiently transfected HEK 293T cells as described under Materials and Methods. Assays were conducted in buffer systems containing either 1.6 mM CaCl₂, 0.8 mM MgCl₂ (Ca²⁺ medium), or 0.5 mM MgCl₂ (no Ca²⁺ medium). Data were normalized to the responses to CaCl₂, and 100% represents 5- to 10-fold responses over baseline in typical experiments. Data represent the means ± S.E.M. of three or more independent experiments.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>pEC₅₀ Efficacy vs. WT</th>
<th>Hill No.</th>
<th>pEC₅₀ Efficacy vs. WT</th>
<th>Hill No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>AC-265347</td>
<td>8.1 ± 0.3</td>
<td>84 ± 24</td>
<td>0.72 ± 0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>8.3 ± 0.3</td>
<td>86 ± 27</td>
<td>0.64 ± 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R)-265347</td>
<td>7.0 ± 0.5</td>
<td>75 ± 2</td>
<td>0.70 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>6.9 ± 0.3</td>
<td>80 ± 13</td>
<td>0.74 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>7.3 ± 0.3</td>
<td>75 ± 14</td>
<td>0.74 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>6.5 ± 0.3</td>
<td>44 ± 21</td>
<td>0.63 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>AC-265347</td>
<td>6.6 ± 0.4</td>
<td>52 ± 31</td>
<td>0.65 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>6.0 ± 0.1</td>
<td>27 ± 18</td>
<td>0.85 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>6.0 ± 0.2</td>
<td>35 ± 16</td>
<td>0.85 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>6.2 ± 0.2</td>
<td>39 ± 18</td>
<td>0.61 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>5.6 ± 0.1</td>
<td>28 ± 12</td>
<td>1.22 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>R66C</td>
<td>AC-265347</td>
<td>6.4 ± 0.1</td>
<td>21 ± 3</td>
<td>0.95 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>6.4 ± 0.0</td>
<td>24 ± 3</td>
<td>0.88 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>5.6 ± 0.2</td>
<td>15 ± 3</td>
<td>1.13 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>5.5 ± 0.2</td>
<td>16 ± 1.5</td>
<td>0.92 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>2.4 ± 0.2</td>
<td>27 ± 2</td>
<td>2.53 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>T138M</td>
<td>AC-265347</td>
<td>6.2 ± 0.0</td>
<td>64 ± 11</td>
<td>0.95 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>6.3 ± 0.1</td>
<td>68 ± 4</td>
<td>0.84 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>5.7 ± 0.4</td>
<td>32 ± 5</td>
<td>1.02 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>5.3 ± 0.5</td>
<td>32 ± 3</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>2.3 ± 0.2</td>
<td>111 ± 16</td>
<td>2.82 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>R185Q</td>
<td>AC-265347</td>
<td>6.1 ± 0.1</td>
<td>55 ± 12</td>
<td>1.29 ± 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>6.2 ± 0.1</td>
<td>64 ± 14</td>
<td>0.98 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>5.6 ± 0.2</td>
<td>16 ± 6</td>
<td>1.13 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>5.0 ± 0.</td>
<td>21 ± 3</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>1.8 ± 0.2</td>
<td>18 ± 3</td>
<td>2.23 ± 0.67</td>
<td></td>
</tr>
<tr>
<td>R795W</td>
<td>AC-265347</td>
<td>5.7 ± 0.1</td>
<td>15 ± 5</td>
<td>1.42 ± 0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>5.8 ± 0.1</td>
<td>18 ± 4</td>
<td>1.17 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>N.C.</td>
<td>6 ± 3</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>N.C.</td>
<td>9 ± 3</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>2.4 ± 0.2</td>
<td>14 ± 2</td>
<td>3.57 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>E837A</td>
<td>AC-265347</td>
<td>6.4 ± 0.2</td>
<td>55 ± 6</td>
<td>0.96 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>6.6 ± 0.5</td>
<td>47 ± 4</td>
<td>0.78 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>&lt;5.0</td>
<td>27 ± 1</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>&lt;5.0</td>
<td>30 ± 4</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>2.7 ± 0.1</td>
<td>65 ± 10</td>
<td>1.84 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>I841A</td>
<td>AC-265347</td>
<td>7.6 ± 0.4</td>
<td>44 ± 8</td>
<td>0.59 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(S)-265347</td>
<td>8.0 ± 0.7</td>
<td>43 ± 7</td>
<td>0.56 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Calindol</td>
<td></td>
<td>N.C.</td>
<td>17 ± 4</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>Cinacalcet</td>
<td></td>
<td>N.C.</td>
<td>16 ± 4</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>2.9 ± 0.2</td>
<td>54 ± 4</td>
<td>1.73 ± 0.47</td>
<td></td>
</tr>
</tbody>
</table>

WT, wild type; N.C., not calculated.

CYP2D6 inhibitor (IC₅₀, 87 nM; Nakashima et al., 2007), and both cinacalcet and calindol have been shown to block L-type Ca²⁺ channels (Thakore and Ho, 2010). All of the calciumics described to date including cinacalcet allosterically increase the sensitivity and responsiveness of CaSR to [Ca²⁺]₀ (Jensen and Brüuner-Osborne, 2007). Therefore, we retested all of these compounds in PI hydrolysis assays in the absence of extracellular Ca²⁺ (Fig. 2, E and F; Table 2). The maximum responses and potencies of all of the compounds were greatly reduced in the absence of extracellular Ca²⁺, indicating that they cooperate with Ca²⁺ to activate CaSRs. Under these conditions, significant differences in the efficacy of these compounds became apparent, with AC-265347 and (S)-AC-265347 stimulating significantly greater responses than cinacalcet, calindol, or (R)-AC-265347. Besides these two buffer systems, we tested these ligands in PI assays in buffers lacking penicillin and streptomycin but containing a range of Ca²⁺ concentrations (0.5–2 mM) and observed that removal of these antibiotics did not alter the rank order activity of these compounds (data not shown).

The natural ligands for family C GPCRs bind to the large, extracellular N-terminal domain characteristic of this receptor family, whereas drugs and other artificial compounds targeting family C GPCRs interact primarily with the transmembrane helical domains of these receptors (Brüuner-Osborne et al., 2007). Therefore, we tested all of the compounds on cells expressing a CaSR isoform with the N-terminal domain deleted (AN); however, AC-265347 and (S)-AC-265347 stimulated significantly greater responses than did cinacalcet, calindol, or (R)-AC-265347. As expected, deletion of the N terminus of the CaSR abolished activation by CaCl₂. We retested all the compounds at AN CaSRs in the absence of extracellular Ca²⁺ and observed that they still retained agonist activity. Under these conditions, maximal responses to
all compounds were further reduced; however, the potencies were not significantly different from the potencies at CaSRs in the presence of extracellular Ca$^{2+}$. These results are in agreement with previous studies suggesting that CaSRs may contain more than one Ca$^{2+}$-binding site (Ray et al., 2005) and indicate that both classes of compounds interact with the transmembrane domains of CaSRs and both have intrinsic agonist activity, but AC-265347 and (S)-AC-265347 have greater intrinsic activity than the phenylalkylamine calcimimetics.

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

**Fig. 3.** AC-265347 activates CaSRs through the transmembrane domains. The compounds indicated were tested for responses in PI hydrolysis assays as described under Materials and Methods using HEK 293T cells transiently transfected with CaSRs with the N-terminal extracellular domain deleted. Assays were conducted in a buffer system containing 1.6 mM CaCl$_2$ and 0.8 mM MgCl$_2$ (A) or no CaCl$_2$ and 0.5 mM MgCl$_2$ (B). Data points represent the means of duplicate determinations. Graphs are representative of at least three independent experiments. Responses were normalized to the responses to CaCl$_2$ by wild-type CaSRs. ■, (S)-AC-265347; ○, cinacalcet; △, calindol; ◆, CaCl$_2$. ND, no drug.

**Fig. 4.** AC-265347 activates CaSRs through a novel binding site. The compounds indicated were tested for functional responses in cellular proliferation (A and B) or PI hydrolysis (C and D) assays as described under Materials and Methods using cells transiently transfected with CaSRs mutated as follows: E837A (A and C); I841A (B and D). Data points represent the means of duplicate determinations. Graphs are representative of at least three independent experiments. Responses were normalized to the responses to MgCl$_2$ (A and B) or CaCl$_2$ (C and D) by wild-type CaSRs. ■, (S)-AC-265347; ○, cinacalcet; △, calindol; ◆, CaCl$_2$. ND, no drug.
Fig. 5. CaSR allosteric agonists differentially activate loss-of-function polymorphic variants. The indicated compounds were tested for functional responses in cellular proliferation (A–D) or PI hydrolysis (E–H) assays as described above; using cells transiently transfected with CaSRs mutated as follows: T138M (A and E), R185Q (B and F), R795W (C and G), and R66C (D and F). Data points represent the means of duplicate determinations. Graphs are representative of at least three independent experiments. Responses were normalized to the responses to MgCl₂ (A–D) or CaCl₂ (E–H) by wild-type CaSRs. ■, (S)-AC-265347; ○, cinacalcet; ◇, MgCl₂ (A–D) or CaCl₂ (E–H). ND, no drug.
CaSRs by positive and negative modulators (Hu et al., 2002, 2005, 2006; Petrel et al., 2004). In agreement with previous studies, the activity of cinacalcet was strongly reduced at E837A, and its efficacy was significantly reduced at I841A7.43 (Fig. 4; Tables 1 and 2). Calindol activity was similarly affected. In contrast, the activities of AC-265437 and (R)-AC-265347 were affected to a much smaller degree at I841A7.43 (Fig. 4; Tables 1 and 2). In agreement with previous results, all four of these CaSR polymorphic variants displayed impaired responses to MgCl2 or CaCl2 compared with those for wild-type CaSR, with reduced potency, reduced maximum response, or both. AC-265347 and (S)-AC-265347 each reduced serum PTH (Fig. 6). The approximate ED50 values for PTH suppression were 0.01 mg/kg for AC-265347 and (S)-AC-265347 and 0.1 mg/kg for (R)-AC-265347, a rank order potency correlated with their in vitro potencies. Cinacalcet also suppressed serum PTH, with a similar maximal effect and an ED50 of approximately 0.1 mg/kg. (S)-AC-265347 also suppressed serum ionizable calcium [Ca2+]o, in a dose-dependent manner, although it required much higher doses than it needed to suppress serum PTH (Fig. 7A). A similar difference between the potency required to suppress serum PTH and that required to suppress [Ca2+]o, has been observed for phenylalkylamine calcimimetics such as cinacalcet (Fox et al., 1999; Nemeth et al., 2004). AC-265347 also produced a significant suppression of serum [Ca2+]o (data not shown); however, although rats treated with (R)-AC-265347 did have lowered serum [Ca2+]o, the trend was not statistically significant (Fig. 7B).

Oral administration of AC-265347 rapidly suppressed serum PTH levels in rats, with the maximal suppression of plasma PTH occurring 30 min after drug administration (Fig. 8A). Lower levels of plasma PTH were maintained for 6 h at 30 mg/kg AC-265347. Plasma concentrations of AC-265347 after oral administration were determined over a 24-h time course (Fig. 8B). Peak plasma concentrations of AC-265347 were 67 and 311 ng/ml for the 10 and 30 mg/kg groups, respectively, and

![Fig. 6. AC-265347 lowers plasma PTH in rats. The indicated doses (milligrams per kilogram, subcutaneous) of (S)-AC-265347 (A), (R)-AC-265347 (B), AC-265347 (C), or cinacalcet (D) were administered to male Sprague-Dawley rats (n = 6/drug group; n = 12 vehicle), blood samples were taken at 1 h postdose, and plasma levels of PTH (reported as nanograms per milliliter) were measured as described under Materials and Methods. *, p < 0.05; **, p < 0.01, compared with vehicle (Veh) analyzed by analysis of variance with Dunnett post-test analysis.

![Fig. 7. AC-265347 lowers plasma Ca2+ in rats. The indicated doses (milligrams per kilogram, subcutaneous) of (S)-AC-265347 (A) or (R)-AC-265347 (B) were administered to male Sprague-Dawley rats (n = 6/drug group; n = 12 vehicle), blood samples were taken at 1 h postdose, and plasma levels of ionizable calcium ([Ca2+]o, reported as millimolar concentrations) were measured as described under Materials and Methods. *, p < 0.05; **, p < 0.01, compared with vehicle (Veh) analyzed by analysis of variance with Dunnett post-test analysis.](image-url)
occurred at 1-h postdose. These levels compare favorably with those reported previously for cinacalcet (73 and 124 ng/ml at 10 and 36 mg/kg p.o., respectively) (Nemeth et al., 2004). The plasma concentration-time curves of AC-265347 correlated very well with its observed effects on serum PTH levels.

Discussion

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

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

The benzothiazol CaSR allosteric agonists appear to interact with CaSRs differently from the phenylalkylamines calcimimetics. Although studies using CaSRs lacking the N-terminal extracellular domain indicated that both classes of compounds interact with the transmembrane domain spanning regions of the CaSR (Fig. 3), studies using point mutations within transmembrane domain 7 clearly indicate that they use different amino acid residues to bind to CaSRs. We observed that mutations E837A \(^{397}\) and I841F \(^{443}\) strongly reduced phenylalkylamine-induced signaling, in agreement with previous studies on cinacalcet and other structurally related calcimimetics and calcilytics (Petrel et al., 2004). In contrast, these mutations had little effect on AC-265347- or (S)-AC-265347-induced signaling (Fig. 4).

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

Several studies reported that the calcimimetic 3-(2-chlorophenyl)-N-\((1R,1\prime)-1-(3-methoxyphenyl)ethyl\)propan-1-amine (NPS R-568) improves the signal transduction characteristics of loss-of-function polymorphic variants of the human CaSR associated with human disease (Rus et al., 2008; Lu et al., 2009; White et al., 2009). NPS R-568 is structurally very similar to cinacalcet, and therefore one would expect cinacalcet to have similar effects on those polymorphic receptors, and, vice versa, one would expect NPS R-568 to have effects similar to those of cinacalcet and calindol on the polymorphic receptors studied here. The polymorphisms studied in those previous reports were different from the ones reported in this study, and thus direct comparisons of this study to those studies are not possible. In addition, there are a large number of other polymorphic variants of the CaSR known to exist that were not tested in this study or any of the studies cited above (Pidasheva et al., 2004). It seems reasonable to speculate that benzothiazol CaSR allosteric agonists such as AC-265347 may stimulate greater responses than cinacalcet at these other variant receptors too.

Cinacalcet is a potent inhibitor of the CYP2D6 cytochrome P450 isofrom with a \(K_i\) of 87 nM (Nahashima et al., 2007). A wide variety of drugs are metabolized by CYP2D6, and the potential for cinacalcet to cause significant drug-drug inter-

![Graph](image-url)

**Fig. 8.** AC-265347 is orally active. A, the indicated doses of AC-265347 were orally administered to catheterized male Sprague-Dawley rats \(n = 5\)/drug group, \(n = 8\) vehicle), blood samples were taken at the indicated time points, and plasma PTH levels were measured as described under Materials and Methods. The PTH levels in the 10 mg/kg group were significantly \(p < 0.01\) lower than those for vehicle at 0.5 and 1 h postdose and significantly \(p < 0.05\) lower than that for vehicle at 2 h postdose. The PTH levels in the 30 mg/kg group were significantly \(p < 0.01\) lower than those for vehicle at 0.5, 1 and 2 h postdose. Significance was assessed by analysis of variance with Dunnett post-test analysis.

vehicle: □, 10 mg/kg; ■, 30 mg/kg.

Cinacalcet is a potent inhibitor of the CYP2D6 cytochrome P450 isofrom with a \(K_i\) of 87 nM (Nahashima et al., 2007). A wide variety of drugs are metabolized by CYP2D6, and the potential for cinacalcet to cause significant drug-drug inter-
actions has been documented (Harris et al., 2007). In contrast, we observed no significant interaction of AC-265347 with CYP2D6 at concentrations up to 100 μM. Thus, drug-drug interactions should be less of a concern with AC-265347 or its analogs.

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

Authorship Contributions

Participated in research design: Ma, Tabatabaei, Olsson, and Burstein.
Conducted experiments: Ma, Owens, and Schmeltzer.
Contributed new reagents or analytic tools: Gustafsson and Jensen.
Performed data analysis: Owens, Ma, and Burstein.
Wrote or contributed to the writing of the manuscript: Burstein.

References


Address correspondence to: Dr. Ethan S. Burstein, ACADIA Pharmaceuticals, Inc., 3911 Sorrento Valley Blvd., San Diego, CA 92121. E-mail: eburstein@acadia-pharm.com