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Development of a calcium-sensing receptor molecular imaging agent

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Abstract

Background—Calcium-sensing receptor (CaSR) is expressed by parathyroid cells and thyroid C-cells (from which medullary thyroid carcinoma [MTC] is derived). A molecular imaging agent localizing to the CaSR could improve the detection of parathyroids and MTC preoperatively or intraoperatively. We synthesized a novel compound containing a fluorine residue for potential future labeling and demonstrated that the compound inhibited CaSR function in vitro.

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DISCUSSION

Dr John A. Olson, Jr. (Baltimore, MD): You acknowledged that parathyroid adenomas can often but variably down-regulate the CaSR, which would impact this strategy.

The other thing that is sometimes abnormal is mislocalization of the CaSR from the cell surface to intracellular locations on the processing pathway. Do your compounds penetrate the cell membrane, and would they target CaSR in the intracellular space?

Dr John Phay: We do not know if the compounds were actually getting into the cells. The interaction between the compounds and the receptor is typically an allosteric interaction. I think the compounds probably do not penetrate the cell membrane. Data on other similar compounds show that the interaction is quite a long-acting association. That is obviously an excellent question, and something we plan to investigate in the future.

Dr James Lee (New York, NY): So you had mentioned that you might be able to use this to target medullary thyroid cancer. Do you see this as a way of targeting specifically for ablative therapy, or are you able to somehow selectively pick the calcium-sensing receptors on medullary cells versus parathyroid cells?

Dr John Phay: As far as we know, there's no difference in the CaSR receptor in medullary thyroid cancer versus parathyroids versus kidney. Using the CaSR as a target to decrease cancer cell growth is a possibility. Unfortunately, one would worry about injury to the kidneys and parathyroids. So our initial focus is going to be localization, but that's something we will consider, in the future.

Dr Jason D. Prescott (Baltimore, MD): Along the lines of Dr Olson's question, looking at your transfection experiments, your GFP-fused protein, looks like it's pretty well localized to the cytoplasm, suggesting that maybe you're having an effect that's cytoplasmic. Have you looked for localization of your fusion protein at the cell membrane? Is it actually getting there as well? Because it's clearly in the nucleus and the cytoplasm. Maybe you're getting an effect where your drug is penetrating.

Dr John Phay: That is a possibility. We are actively working on binding assays with the receptor. We have had some trouble with the solubility of the compounds. With better binding studies, for example, comparing the amount of compound in the plasma membrane fraction versus the cytosolic fraction, we should be able to determine the intracellular penetration.

Dr Mark S. Cohen (Ann Arbor, MI): My question is related to the compound itself. Do you know anything about the toxicity of the compound in other cells? And what's your planned route of delivery?

Dr John Phay: We do not know about the toxicity of the compounds in other cells. In our initial experiments, we did not observe any apparent toxicity. We do know that the compounds can be given intravenously because it's something that we have already been able to do.

Methods—We synthesized compound **M**, a derivative of a known calcilytic compound, Calhex-231. Human embryonic kidney cells transfected with green-fluorescent protein-tagged CaSR or control vector were preincubated with compound **M** before the addition of calcium. Immunoblotting for total mitogen-activated protein kinase (MAPK: ERK1/2), activated MAPK (phosphorylated ERK1/2), and glyceraldehyde 3-phosphate dehydrogenase was performed.

Results—Synthesis of compound **M** was confirmed by mass spectrometry. Inhibition of the MAPK signaling pathway by compound **M** was demonstrated in a dose-dependent manner by a decrease in phosphorylated ERK1/2 with no change in total ERK1/2 levels. Compound **M** inhibited MAPK signaling slightly better than the parent compound.

Conclusion—We have developed a novel molecule which demonstrates functional inhibition of CaSR and has a favorable structure for labeling. This compound appears to be appropriate for further development as a molecular imaging tool to enhance the surgical treatment of parathyroid disease and MTC.

MEDULLARY THYROID CANCER (MTC) originates from parafollicular thyroid cells, also called C-cells, which produce the hormone calcitonin. MTC is the third most common thyroid cancer, comprising approximately 3% of all cases of thyroid cancer.¹ Because these cells do not express the sodium-iodine symporter, radioactive iodine is not used in the treatment of MTC. Therefore, surgery has been the main treatment for patients with localized MTC, which includes thyroidectomy and complete central neck dissection.

Similar to MTC, hyperparathyroidism (HPT) is a disease primarily treated by surgery. Intraoperative identification of both normal and abnormal parathyroids can be difficult because of their small size and variable anatomic location. For patients with HPT, although preoperative imaging using technetium-99m sestamibi scintigraphy or ultrasonography is usually performed before parathyroid operation, there are limitations attributed to equipment sensitivity and user experience.^{2,3} Consequently, a molecular imaging agent with high sensitivity and specificity to MTC and parathyroid glands would not only decrease operative time, but more importantly, likely improve the outcomes of operative treatment.

Both C-cells and parathyroid glands express high levels of calcium-sensing receptor (CaSR), a cell-surface G protein-coupled receptor that plays a key role in sensing changes in the serum calcium level.^{4,5} Originally cloned from bovine parathyroid, the CaSR has also been found in many other species, including humans, where it is highly expressed in parathyroid tissue and kidneys, organs primarily involved in blood calcium regulation. In thyroid C-cells, CaSR controls the release of calcitonin in response to increasing levels of circulating calcium. Stimulation of CaSR by extracellular calcium or several other ions generates the production of inositol trisphosphate resulting in a rapid increase of intracellular calcium and subsequent activation of several downstream signaling pathways, including the mitogen-activated protein kinase (MAPK).⁶ This signaling cascade has been studied extensively in human embryonic kidney (HEK)-293 cells, which do not express CaSR, by using cells stably expressing CaSR or transient transfection of the CaSR gene.^{7,8}

Previous studies on negative, allosteric modulators of CaSR (referred to as antagonists or calcilytics) have focused on developing treatments for osteoporosis or other bone-related

diseases. NPS-2143 (IC₅₀ 43 nM) and compound 1 (IC₅₀ 64 nM) both inhibit recombinant-CaSR activation expressed in HEK-293 cells by extracellular calcium.^{9,10} Calhex 231 is another small allosteric antagonist to CaSR, which was designed as a potential modulator of parathyroid hormone for the treatment of osteoporosis.¹¹ The structure of this CaSR antagonist is favorable for further modification and labeling. To date, none of these CaSR antagonists have been translated into clinical use.¹²

We have synthesized two isomers of Calhex 231 and multiple analogues. We are initially interested in using a small label, such as ¹²³I, ¹²⁵I, or ¹⁸F, to minimize their influence on the binding to the receptor. Using iodine as an imaging agent is a problem in the neck because of the extremely high affinity of normal thyroid follicular cells for iodine. This results in a high background due to the normal thyroid cells taking up even trace amounts of unincorporated radioiodine. Therefore, to further develop one of these analogues as an imaging agent, we present the synthesis of a novel compound containing fluorine. A fluorine molecule is conducive to labeling with ¹⁸F, which can be imaged on a positron emission tomography (PET) or a hand-held probe. After confirmation of synthesis, we demonstrated the in vitro function of the new analogue through its ability to inhibit CaSR-specific phosphorylation of ERK1/2, a component of the MAP kinase signaling pathway, in a dose-dependent manner.

METHODS

Synthetic chemistry

An approach leading to the rationale of synthesizing compound **M** from the known N-Boc azirane (**B**, prepared from **A**) is described in Fig 1. Lithium perchlorate-catalyzed nucleophilic ring opening (**B**) with (R)-1-(1-naphthyl) ethylamine (**C**) was a simple variation of the known *N*-sulfonyl aziridination protocol reported in the literature for this reaction sequence, to produce a mixture of diastereomers **D** and **E**, which were separated easily by silica column.^{13,14} The N-Boc protection on individually separated **D** and **E** were then removed under 4 M HCl/dioxane conditions to give the amine hydrochloride salts **F** and **G**, respectively. The amines **F** and **G** were then coupled with 4-chloro benzoic acid under *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride conditions to give the amides (**H** and **I**) in their respective series. **H** and **I** are isomers of the parent compound, Calhex-231. We then extended these studies to generate compound **M** by coupling **F** to the fluoro acid counterpart **L** under *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride conditions. The samples were characterized by high-resolution mass spectrometry using a Waters Micromass LCT Spectrometer (Core Instrumentation Facilities at The Ohio State University, Columbus, OH).

Cell culture and transfections

HEK-293 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in high glucose Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and nonessential amino acids (all from Life Technologies, Carlsbad, CA). The cells were grown at 37°C in a humidified incubator containing 5% CO₂.

The method to assess the antagonistic ability of compounds **I** and **M** to inhibit the activation of CaSR was modified from Loretz et al.¹⁵ Transient transfections with either plasmid pEGFP-N1 (Clontech Laboratories, Mountain View, CA) or green fluorescent protein (GFP)-CaSR (a gift from Michael Levine of Children's Hospital of Philadelphia, PA) were performed using Lipofectamine Plus reagents (Life Technologies) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were switched to calcium-free DMEM supplemented with 0.2% (w/v) bovine serum albumin (BSA) and 0.5 mM CaCl₂ for 18 hours. The medium was renewed 2 hours before any experiments. Compounds **I** and **M** were dissolved in dimethyl sulfoxide (DMSO) and added to the cells at various concentrations for 30 minutes and incubated with 4 mM CaCl₂ for 10 minutes before collecting the cells. DMSO was used as a solvent control in all experiments.

Immunoblotting

Cell pellets were lysed in Mammalian Protein Extraction Reagent (Thermo Scientific, Pittsburgh, PA) supplemented with 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 20 µM 4-aminophenyl-methane-sulfonyl fluoride, and 0.3 mM okadaic acid. Total protein was quantified using the Pierce BCA Protein Assay kit (Thermo Scientific). Protein samples were then size fractionated using SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking with either 5% nonfat milk (for detection of total protein) or 5% BSA (for detection of phosphorylated protein), the membranes were incubated with primary antibodies at 4°C overnight. The next day the membranes were washed with TBS-T (25 mM Tris-HCl [pH 7.4], 140 mM NaCl, 3 mM KCl and 0.1% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibody for 1–2 hours at room temperature, and detected by the use of chemiluminescence (GE Healthcare, Piscataway, NJ).

Antibodies against total p44/42 MAPK (ERK1/2), activated p44/42 MAPK phosphorylated at Thr202 and Tyr204 (phosphorylated ERK1/2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (catalog numbers 4695, 9101, 2118; Danvers, MA). Other antibodies used were against GFP (catalog number 632380; Clontech Laboratories) and CaSR (catalog number ab19347; Abcam, Cambridge, MA). Densitometry was performed on the immunoblots using the Image Gauge version 3.3 software (Fuji Photo Film Co., Ltd, Tokyo, Japan).

Statistical analysis

The ratios of pERK1/2/ERK1/2 from the immunoblots were compared between compounds **I** and **M** averaged across the different doses. A linear mixed effect model was used for analysis to take account of the correlation among observations from the same experiment.

RESULTS

Synthesis of compounds **I** and **M**

From Fig 1 we have successfully synthesized Calhex 231 isomers, compounds **H** and **I**, and analogue **M**. Because our previous studies have shown the Calhex 231 isomers to have

similar potency in inhibiting ERK1/2 phosphorylation (H. Ding and AM. Yusof, unpublished data), subsequent in vitro experiments were performed with compound **I**.

Within the context of the current studies, Fig 2 shows the chemical structure of compounds **I** and **M**, and their corresponding mass spectrometry analysis verifying their synthesis. Comparing the structure of both analogues, compound **M** has a fluorine atom in its appendage, which could incorporate an ^{18}F atom for imaging. The mass spectrometry data confirms the appropriate mass (M^+) for these compounds.

In vitro characterization of the antagonistic activity of compounds **I** and **M**

The antagonistic activities of compounds **I** and **M** were then evaluated in HEK-293 cells transfected with either GFP-tagged CaSR or GFP alone by measuring their ability to inhibit CaSR-mediated ERK1/2 phosphorylation by extracellular calcium activation. Calcium-mediated activation of CaSR causes rapid dose-dependent phosphorylation of ERK1/2.^{16,17} CaSR was stimulated with 4 mM Ca^{2+} , which we had determined in dose and time course experiments to be optimal for CaSR-mediated ERK1/2 phosphorylation (H. Ding and AM. Yusof, unpublished data).

As shown in Fig 3, in cells transfected with GFP-CaSR, both compounds **I** and **M** inhibited Ca^{2+} -induced phosphorylation of ERK1/2 in a concentration-dependent manner but did not affect total ERK1/2 levels. Comparing the ratio of intensity of the pERK1/2/ERK1/2 bands, from the immunoblots of repeated independent experiments, and averaging across the different concentrations of the compounds, we determined that analogue **M** had a decreased pERK1/2/ERK1/2 ratio than compound **I** (mean pERK1/2/ERK1/2 ratio: **M** = 0.91 ± 0.37 compared with **I** = 1.19 ± 0.37 , $P = .01$). This indicates that compound **M** is a slightly more potent inhibitor of ERK1/2 phosphorylation than compound **I**. Importantly, no inhibition was observed in cells transfected with the GFP control vector as seen by the absence of changes in the levels of ERK1/2 phosphorylation indicating that the effect is CaSR-specific. There was also no effect in the levels of ERK1/2 phosphorylation in the GFP-only control (concentration 0) in the transfected cells.

The GFP-CaSR and GFP cell lysates from the dose-dependent experiments were then examined to confirm CaSR and GFP overexpression. Figure 4 is a representative figure showing clear overexpression of the CaSR and GFP protein in the GFP-CaSR transfected cells incubated with various concentrations of compound **M**. Only GFP overexpression was detected on the immunoblots of the GFP control experiments.

DISCUSSION

Understanding and regulating the activity of CaSR has important medical ramifications because the receptor has been implicated in numerous diseases, including HPT and osteoporosis. This has resulted in the development of several synthetic, allosteric modulators of the receptor which are either positive modulators (referred to as calcimimetics) or negative modulators for the potential treatment of these diseases. Currently, only one calcimimetic, cinacalcet (IC_{50} 28 nM), is used clinically for the treatment of secondary HPT at doses of 60–360 mg/day.^{18,19} Nevertheless, parathyroidectomy is the next treatment

alternative when this option fails. Thus far, none of the published CaSR antagonists have been translated for clinical use.

Our interest in the CaSR is because of its expression in MTC and parathyroids. To date, the main treatment for both MTC and HPT is surgery, where improved preoperative and intraoperative imaging to better guide the procedure would be beneficial.

Calhex 231, a negative allosteric modulator of CaSR, was developed as a potential treatment and/or prevention of osteoporosis.¹¹ Using the inhibition of calcium-induced tritiated inositol phosphate accumulation, the IC₅₀ was 0.33 ± 0.02 μM. We have synthesized analogues of Calhex 231 and demonstrated their specific CaSR-inhibitory function in vitro using HEK-293 cells transfected with GFP-tagged CaSR. These compounds were not optimal for labeling for in vivo use. Therefore, we synthesized the analogue compound **M**, which has a fluorine sidechain making it amenable for labeling with ¹⁸F for imaging.

We compared the inhibitory function of a Calhex 231 isomer, compound **I**, and the novel analogue, compound **M**. Our in vitro assay involving overexpression of GFP-tagged CaSR in HEK-293 cells showed that both compounds inhibit calcium-induced phosphorylation of ERK1/2 in a dose-dependent manner, indicating specific inhibition of CaSR, with compound **M** being slightly more potent.

We have successfully developed and synthesized a novel molecule which showed functional inhibition of CaSR and has a structure favorable for labeling. Going forward, we anticipate that the effort towards a PET-active compound **M** could be easily accomplished in a few steps starting from its corresponding hydroxyl analogue. An effective imaging agent has the potential to dramatically improve the operative treatment of MTC and parathyroid disease.

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Fig 1.

Scheme of synthesis of Calhex 231 isomers and analogue **M**. Synthesis of calcium- sensing antagonist analogue **M** by nucleophile ring opening of cyclohexene oxide **A** by 1(R)-Naphthylethylamine (**C**) to produce **D** and **E** that after clean separation and deprotection is followed by selective coupling of diamine **F** with acid **L**.

Fig 2.
Chemical structure and mass spectrometry analysis of compounds **I** and **M**. Mass in dalton.

Fig 3.
Inhibition of CaSR-dependent activation of ERK1/2 phosphorylation by compounds **I** and **M**. HEK-293 cells transfected with GFP-CaSR or GFP DNA were incubated with increasing concentrations of each compound before the addition of 4 mM Ca^{2+} for another 10 min. The cell lysates were analyzed for phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (ERK1/2). GAPDH is the loading control.

Fig 4. Overexpression of GFP-CaSR and GFP in transiently transfected HEK-293 cells incubated with increasing concentrations of compound **M** before the addition of 4 mM Ca^{2+} for another 10 min. The immunoblots (IB) were analyzed for CaSR and GFP. c is the GFP-CaSR control, and GAPDH is the loading control.