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Amino Alcohol- (NPS-2143) and Quinazolinone-Derived Calcilytics (ATF936 and AXT914) Differentially Mitigate Excessive Signalling of Calcium-Sensing Receptor Mutants Causing Bartter Syndrome Type 5 and Autosomal Dominant Hypocalcemia

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Abstract

Introduction: Activating calcium sensing receptor (CaSR) mutations cause autosomal dominant hypocalcemia (ADH) characterized by low serum calcium, inappropriately low PTH and relative hypercalciuria. Four activating CaSR mutations cause additional renal wasting of sodium, chloride and other salts, a condition called Bartter syndrome (BS) type 5. Until today there is no specific medical treatment for BS type 5 and ADH. We investigated the effects of different allosteric CaSR antagonists (calcilytics) on activating CaSR mutants.

Methods: All 4 known mutations causing BS type 5 and five ADH mutations were expressed in HEK 293T cells and receptor signalling was studied by measurement of intracellular free calcium in response to extracellular calcium ($[Ca^{2+}]_o$). To investigate the effect of calcilytics, cells were stimulated with 3 mM $[Ca^{2+}]_o$ in the presence or absence of NPS-2143, ATF936 or AXT914.

Results: All BS type 5 and ADH mutants showed enhanced signalling activity to $[Ca^{2+}]_o$ with left shifted dose response curves. In contrast to the amino alcohol NPS-2143, which was only partially effective, the quinazolinone calcilytics ATF936 and AXT914 significantly mitigated excessive cytosolic calcium signalling of all BS type 5 and ADH mutants studied. When these mutants were co-expressed with

wild-type CaSR to approximate heterozygosity in patients, ATF936 and AXT914 were also effective on all mutants.

Conclusion: The calcilytics ATF936 and AXT914 are capable of attenuating enhanced cytosolic calcium signalling activity of CaSR mutations causing BS type 5 and ADH. Quinazolinone calcilytics might therefore offer a novel treatment option for patients with activating CaSR mutations.

Introduction

The calcium-sensing receptor (CaSR) is a key regulator of calcium homeostasis and is expressed in parathyroid, kidney, bone and other organs involved in calcium metabolism [1]. Binding of calcium to the extracellular domain of the receptor results in conformational changes in the transmembrane domain, which in turn activates different G-proteins [2, 3]. The best-studied signalling pathway is activation of phospholipase C by $G_{q/11}$ with a consequent rise in cytosolic free calcium ($[Ca^{2+}]_i$) [4, 5]. Activation of the receptor by rising serum calcium inhibits PTH secretion from parathyroid cells and increases calcium excretion by the kidney. This lowers serum calcium and completes the homeostatic calcium feedback loop [6].

Activating mutations of the CaSR disturb this regulatory feedback loop by lowering the calcium set-point of the CaSR and cause autosomal dominant hypocalcemia (ADH). ADH patients have mild to moderate hypocalcemia with an inappropriately normal or high urinary calcium excretion and low to normal PTH levels [7]. These patients suffer from tissue calcifications especially in the brain and kidney [8, 9] and sometimes show defects in bone mineralization [10]. Vitamin D and calcium supplementation is commonly used to raise serum calcium in ADH patients, but this does not correct the underlying molecular defect, often worsens hypercalciuria and promotes kidney stone formation or nephrocalcinosis. Treatment with PTH (1–34) increases serum calcium and reduces hypercalciuria but does not normalize urinary calcium excretion and does not prevent renal complications [10, 11].

Four activating mutations of the CaSR cause additional renal sodium, chloride and magnesium wasting which results in hyperreninemia, hyperaldosteronism, hypokalemia, and metabolic alkalosis, a condition called Bartter syndrome type 5 (BS type 5) [12–16]. The molecular basis for these different and distinct clinical phenotypes caused by activating CaSR mutations is unknown.

Since the synthesis of the first calcilytic compound NPS-2143 [17, 18] several allosteric antagonists of the CaSR from different chemical classes such as amino alcohols, diaminocyclohexanes, quinazolinones and benzimidazoles have been developed [19]. These calcilytics have the potential to directly correct the molecular cause of ADH and BS type 5. Structural and functional studies suggest common binding sites on the CaSR for amino alcohol and diaminocyclohexane

calcilytics. Quinazolinone and benzimidazole calcilytics also share a common set of binding sites on the CaSR, which are however partly different from the bindings sites of amino alcohols and diaminocyclohexanes and set these compounds apart as a distinct group of calcilytics ([19] and references therein).

In this study we tested whether amino alcohol and quinazolinone calcilytics could mitigate excessive cytosolic calcium signal transduction of CaSR mutants leading to BS type 5 or ADH thus potentially providing a novel treatment option for these patients [19].

Methods

Expression of mutant CaSR in human embryonic kidney (HEK) 293T cells

Expression vectors for wild-type (wt) and mutant CaSR were generated by site-directed mutagenesis and transfected in HEK 293T cells cultured on glass coverslips [20–22]. One μg CaSR expression vector and 0.1 μg YFP expression vector mYF-C2 or for co-transfection experiments 0.5 μg mutant and 0.5 μg YFP-tagged wt-CaSR were used for transient transfection. All 4 known *CaSR* mutations that cause BS type 5 (K29E, L125P, C131W, A843E) have been reported before [12–16]. The ADH *CaSR* mutation A835D is novel and was found in a patient referred for endocrine evaluation of incidentally detected low serum calcium. The ADH mutants T151R, P221L, G830S and A844T have been described by us before [20].

Measurement of $[Ca^{2+}]_i$ and effect of calcilytics

Transiently transfected HEK 293T cells were loaded with 5 μM Fura-2/AM (Invitrogen) and placed in superfusion buffer. Single cells of healthy appearance were selected by YFP fluorescence and used for calcium measurements by dual wavelength excitation microfluorometry. Dose response curves were carried out as described [20]. To determine the effect of calcilytics, cells were treated with 3 mM $[Ca^{2+}]_o$ for 5 min, 0.5 mM $[Ca^{2+}]_o$ for 3 min, control buffer with DMSO 0.1%, NPS-2143 (300 nM or 1 μM), ATF936 (300 nM) or AXT914 (300 nM) for 2 min, and a second stimulation with 3 mM $[Ca^{2+}]_o$ in the presence or absence of calcilytics for 5 min. Specificity of calcilytics and cell viability, was verified by a final stimulation with 10 mM $[Ca^{2+}]_o$ which can overcome inhibition by calcilytics [18]. NPS-2143 was obtained from Hangzhou Hetd Industry (Hangzhou, China), ATF936 and AXT914 were a gift from Novartis (Basel, Switzerland). All three substances were dissolved in dimethylsulfoxide (DMSO) and used at final concentration of 300 nM (NPS-2143, ATF936 and AXT914) and 1 μM (NPS-2143). The final DMSO concentration was kept constant at 0.1%.

Statistics

Nonlinear regression of dose response curves was performed with GraphPad Prism 6 (GraphPad, San Diego, CA) using $\Delta[Ca^{2+}]_i$ values. EC_{50} values and 95% confidence intervals were determined from the non-linear regression curves [20]. The regression fits of wt and mutant CaSR were tested for statistically significant differences with an F-test [23, 24] using GraphPad Prism 6 (GraphPad, San Diego, CA) by comparing two nested models. In the first model the parameters EC50 and maximum response were common for both wt and mutant CaSR and in the second model these parameters were allowed to be different. The effect of the calcilytics on the cytosolic calcium response caused by stimulation with 3 mM $[Ca^{2+}]_o$ was evaluated by Kruskal-Wallis one way ANOVA on ranks with Dunn's method for multiple comparisons versus control group with normalized $\Delta[Ca^{2+}]_i$ signals using SigmaPlot version 11.0 (Systat, Erkrath, Germany). Values shown are mean $\pm$95% confidence intervals.

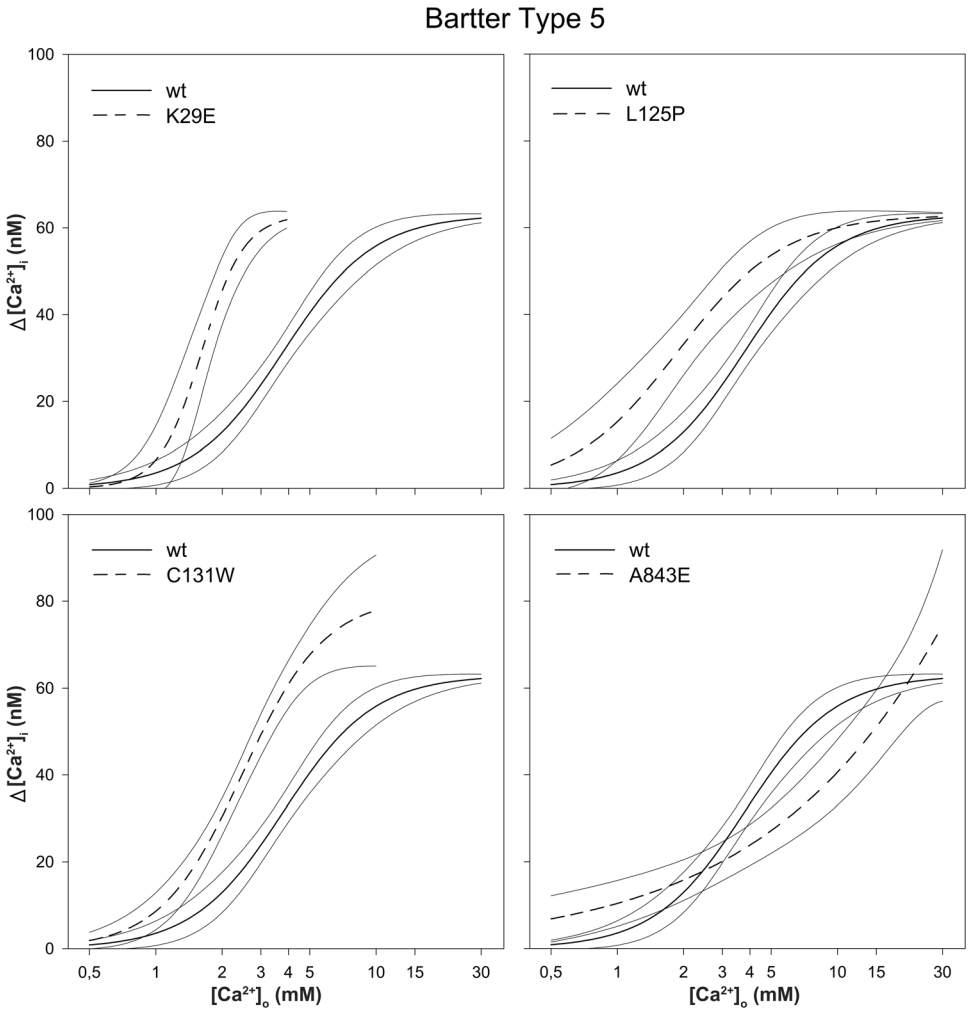
Results

$[Ca^{2+}]_i$ signalling of CaSR mutants

All four BS type 5 *CaSR* mutations and the novel ADH mutant A835D showed activation of the cytosolic calcium pathway at lower $[Ca^{2+}]_o$ concentrations than wt-CaSR (Fig. 1) with significantly left shifted dose response curves (EC_{50} 1.4 mM to 2.3 mM, $p<0.001$–0.008 vs wt-CaSR, Table 1). The A843E CaSR mutant, however, which is the only BS type 5 mutation in the transmembrane domain known to date, did not display a classical sigmoidal dose response curve, which is consistent with previously published results [14, 16, 25]. Nevertheless, an exaggerated $[Ca^{2+}]_i$ response was present at $[Ca^{2+}]_o$ below 2.5 mM when compared to wt-CaSR.

Effect of amino alcohol (NPS-2143) and quinazolinone (ATF936 and AXT914) calcilytics

To test whether the amino alcohol calcilytic NPS-2143 and the quinazolinones ATF936 and AXT914 could mitigate the exaggerated cytosolic calcium response of BS type 5 and ADH CaSR mutants cells were stimulated twice with 3 mM $[Ca^{2+}]_o$. Cells were first stimulated with 3 mM $[Ca^{2+}]_o$ for 5 min, then treated for 5 min with 0.5 mM $[Ca^{2+}]_o$ followed by a second 5 min stimulation with 3 mM $[Ca^{2+}]_o$. Two min before and throughout the second stimulation cells were perfused with medium containing NPS-2143, ATF936, AXT914, or 0.1% DMSO (control). The first stimulation was used for internal normalization to evaluate the effect of the calcilytics. In cells transfected with wt-CaSR and ADH mutants the $[Ca^{2+}]_i$ response to the second $[Ca^{2+}]_o$ stimulus was similar or lower when compared to the first $[Ca^{2+}]_i$ response. Remarkably, however, cells transfected with BS type 5 CaSR mutants displayed a significantly higher second $[Ca^{2+}]_i$ response ($p<0.05$ - $p<0.001$) (Figs. 2 and 3).


Bartter Type 5
wt
K29E
wt
L125P
wt
C131W
wt
A843E
Δ [Ca2+]i (nM)
[Ca2+]o (mM)
0
20
40
60
80
100
0,5
1
2
3
4
5
10
15
30

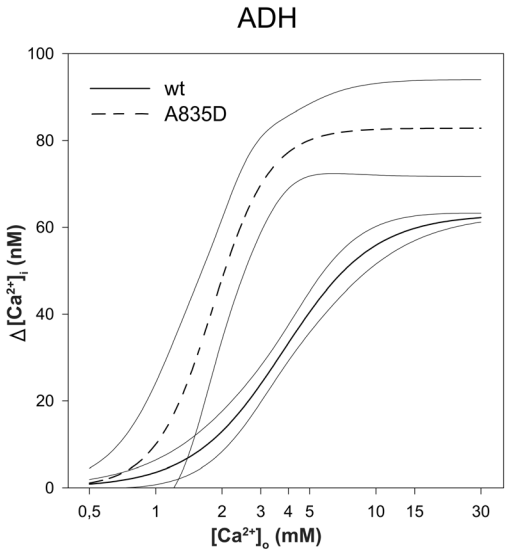

ADH
wt
A835D
Δ [Ca2+]i (nM)
[Ca2+]o (mM)
0
20
40
60
80
100
0,5
1
2
3
4
5
10
15
30

Fig. 1. Sensitivity of BS type 5 mutants, the A835D ADH mutant and wt-CaSR, to $[Ca^{2+}]_o$. Dose-response curves with 95% confidence intervals of $\Delta[Ca^{2+}]_i$ in response to a stepwise increase of $[Ca^{2+}]_o$ for BS type 5 and ADH mutants compared to wt-CaSR. Results from 11 to 27 individual cells measured in at least 4 independent experiments are shown.

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NPS-2143 (300 nM and 1 μM) significantly attenuated the $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ response of two BS type 5 mutants (K29E, L125P). Interestingly, the CaSR mutant K29E lost responsiveness to NPS-2143 when co-expressed with wt-receptor to approximate heterozygosity in patients (Fig. 2). The novel ADH CaSR mutant A835D either expressed alone or co-expressed with wt-CaSR was unresponsive to NPS-2143 up to 1 μM (Fig. 2). These data are consistent with our previous observation that NPS-2143 can reduce excessive signal transduction of some but not all ADH CaSR mutants [20].

In contrast, as depicted in Fig. 3 both ATF936 and AXT914 at 300 nM significantly mitigated excessive signalling of all BS type 5 CaSR and all ADH mutants including four ADH mutants that had been previously tested by us with NPS-2143 [20]. The quinazolinones also inhibited $[Ca^{2+}]_o$–induced $[Ca^{2+}]_i$ signalling in cells co-expressing both mutant and wt-CaSR (Fig. 3B). The $[Ca^{2+}]_i$ response to 3 mM $[Ca^{2+}]_o$ was lowered to a degree comparable to control treated cells expressing wt-CaSR only.

Discussion

Gain-of-function mutations of the CaSR either cause autosomal dominant hypercalcemia (ADH), which is characterized by hypocalcemia and hypercalciuria, or BS type 5, which in addition is associated with renal salt wasting leading to hyperreninemia, hyperaldosteronism and hypokalemia [26, 27]. The molecular background for these different phenotypes is unknown.

In the present study we investigated all four CaSR mutations that cause BS type 5 known to date (K29E, L125P, C131W in the extracellular domain and A843E in

Table 1. Results of the nonlinear regression analyses of dose-response-curves of intracellular free calcium in response to extracellular calcium.

Amino acid	Nucleotide			Regression Fit		EC_{50} $[Ca^{2+}]_o$ (mM)	
Change	Change	Location	Phenotype	p vs wt	R^2	mean	95% CI
wt				-	0.76	3.29	2.99–3.62
K29E	A85G	ECD	BS type 5	<0.001	0.96	1.42	1.33–1.51
L125P	T374C	ECD	BS type 5	<0.001	0.67	1.70	1.47–1.97
C131W	C393G	ECD	BS type 5	<0.001	0.91	1.57	1.43–1.72
A843E	C2528A	TM7	BS type 5	0.008	0.64	2.34	1.88–2.93
A835D	C2504A	ECL3	ADH	<0.001	0.69	2.02	1.79–2.28

wt, wild type CaSR; EC_{50} $[Ca^{2+}]_o$, extracellular calcium concentration giving half maximal response determined from normalized data; 95% CI, 95% confidence interval. The p-values for the regression fit was obtained by comparing nested models with EC_{50} and maximum response common between mutant and wildtype or allowed to be different. TM7, transmembrane domain 7; ECD, extracellular domain; ECL3, extracellular loop 3.

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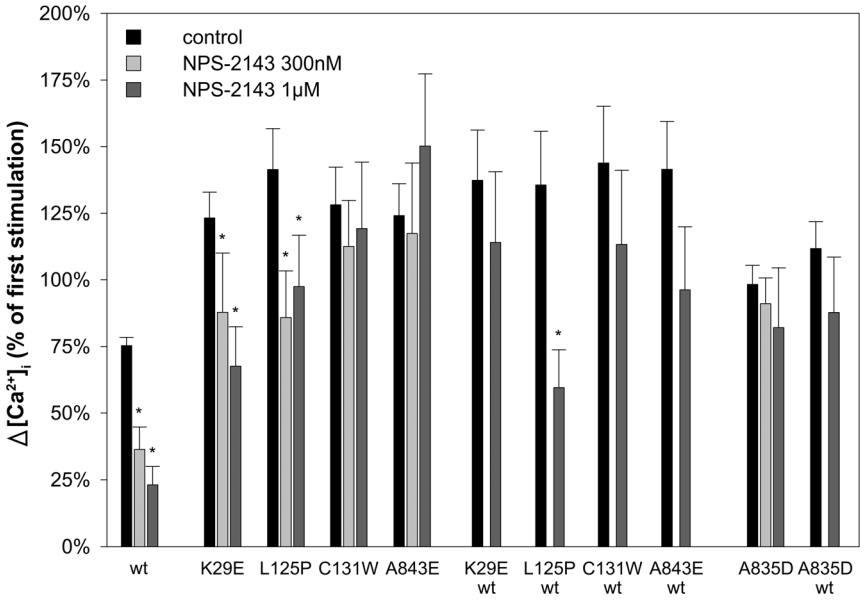

Fig. 2. Effect of the amino alcohol calcilytic NPS-2143 on CaSR mutants causing BS type 5 or ADH (A835D). $\Delta[Ca^{2+}]_i$ in response to stimulation with 3 mM $[Ca^{2+}]_o$ with and without NPS-2143 treatment normalized to the first stimulation with 3 mM $[Ca^{2+}]_o$ ($\pm$95% confidence interval) in HEK 293T cells transfected with wt-CaSR, BS type 5 or ADH CaSR mutants and co-transfected with wt and mutant CaSR as indicated. Results from 9 to 205 individual cells measured in at least 4 independent experiments are shown. *, $P<0.05$ for the effect of calcilytics vs. control (DMSO) on the $[Ca^{2+}]_o$-induced increase in $[Ca^{2+}]_i$ as determined by Kruskal-Wallis one way ANOVA on ranks with Dunn's method for multiple comparisons.

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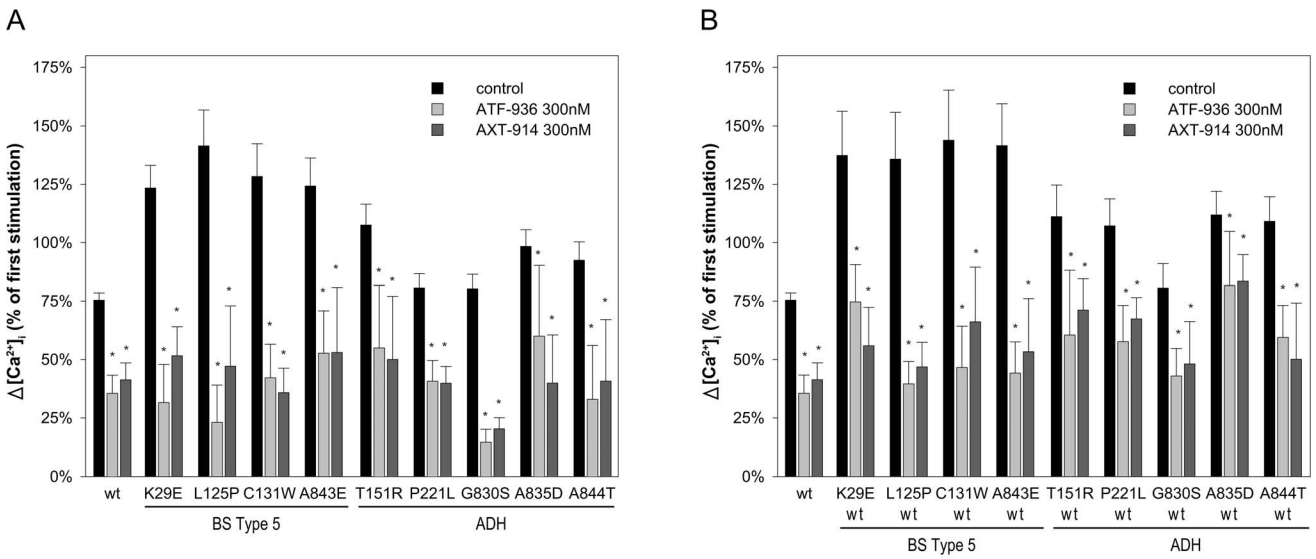

Fig. 3. Effect of quinazolinone calcilytics on BS type 5 and ADH CaSR mutants. $\Delta[Ca^{2+}]_i$ in response to stimulation with 3 mM $[Ca^{2+}]_o$ with and without ATF936 and AXT914 treatment normalized to the first stimulation with 3 mM $[Ca^{2+}]_o$ ($\pm$95% confidence interval) in HEK 293T cells transfected with wt or mutant CaSR (A) and co-transfected with wt and mutant CaSR (B). Results from 7 to 205 individual cells measured in at least 3 independent experiments are shown. *, $P<0.05$ for the effect of calcilytics vs. control (DMSO) on the $[Ca^{2+}]_o$-induced increase in $[Ca^{2+}]_i$ as determined by Kruskal-Wallis one way ANOVA on ranks with Dunn's method for multiple comparisons.

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the transmembrane region 7) and five CaSR mutations causing ADH located in the same regions. Consistent with the clinical phenotypes all *CaSR* mutations inadequately activate the cytosolic calcium signalling pathway as demonstrated by activation of this pathway even at extracellular calcium concentrations below 2 mM (Fig. 1) and [20]. CaSR K29E showed the lowest EC_{50} of all BS type 5 mutants, but causes only a rather mild phenotype in the affected patients [12, 15] suggesting that the severity of the clinical symptoms in BS type 5 may not be correlated with the degree of calcium signalling pathway activation. A further difference between BS type 5 and ADH causing CaSR mutants was observed in experiments with repeated $[Ca^{2+}]_o$ stimulation. BS type 5 CaSR mutants but not ADH mutants demonstrated an enhanced second $[Ca^{2+}]_i$ response (Figs. 2 and 3). This suggests that the calcium and possibly other signalling mechanisms may be altered differentially between BS type 5 and ADH. Elucidating these mechanisms may help to identify new therapeutic targets for BS type 5 and ADH patients.

Novel treatment strategies are indeed needed for both patient groups. Current treatment is primarily symptomatic. To treat hypocalcemia vitamin D, calcium and thiazide diuretics are used. Symptom relief is often inadequate and therapy is limited by increased urinary calcium excretion which further raises the risk of kidney stone formation or nephrocalcinosis [8, 9, 14, 28]. PTH replacement with teriparatide is another option to raise serum calcium, but inadequately high calcium excretion persists [10, 11]. In patients with BS type 5 electrolyte replacement, amiloride or spironolactone may be used to control hypokalemia. None of these therapeutic strategies, however, target the underlying molecular defect and adequately correct altered calcium and electrolyte metabolism.

Inhibitors of CaSR function called calcilytics may provide a novel therapeutic approach. They have been developed to promote PTH secretion in osteoporotic patients with the aim to increase bone formation similar to teriparatide [18, 19, 29]. Although calcilytics are not yet approved for therapeutic use, phase II trials of AXT-914 and the NPS-2143 derivative ronacaleret in healthy subjects and osteopenic postmenopausal women demonstrated an increase in serum calcium [29–31]. In studies where it was examined a reduction in urinary calcium excretion was observed [30], which would be the desired effect in ADH and BS type 5 patients. These drugs were well tolerated in the phase II trials. The most common adverse effects were mild disorders of the gastrointestinal tract and nervous system such as fatigue, headache, constipation, diarrhea, nausea and dyspepsia [29, 31], which might be related to CaSR expressed in gut [32] and brain [33]. As CaSR is also present in skin, lung, heart, mammary glands and numerous other tissues (reviewed in [27]) further short- and long-term adverse effects of calcilytics cannot be ruled out. In ADH and BS5 patients, however, calcilytics would not decrease normal CaSR function, but would restore normal function of an exaggerated CaSR activity. Nevertheless, short- and long-term adverse events may occur and should therefore be carefully monitored, when these compounds are used in clinical practice.

In this study we tested NPS-2143 for the first time on *CaSR* mutations that cause BS type 5. Only two of 4 known CaSR mutants were sensitive to NPS-2143

either expressed alone or when co-expressed with wt-CaSR to approximate heterozygosity in patients. This resembles previous data from us and others showing that some but not all ADH CaSR mutants were sensitive to NPS-2143 [20, 34, 35]. Thus, amino alcohol derived calcilytics may not be the ideal drugs to treat disorders caused by activating CaSR mutations. There are CaSR antagonists with different chemical structures such as diaminocyclohexanes, quinazolinones and benzimidazoles. Studies with mutated CaSR and molecular modelling suggest that the binding sites for amino alcohols and diaminocyclohexanes largely overlap, but that these binding sites are at least partly distinct from those for quinazolinones and benzimidazoles ([19] and references therein). This might explain the differential effects of NPS-2143 and ATF936 or AXT914 on excessive signalling of the ADH CaSR mutant A835D, as amino alcohols, but not quinazolinones form a hydrogen bond to amino acid E837 of the CaSR ([19] and references therein).

The quinazolinones ATF936 and AXT914 in contrast to NPS-2143 significantly mitigated excessive cytosolic calcium signalling of all tested BS type 5 and ADH CaSR mutants when expressed alone or co-expressed with wt-CaSR. Both compounds could suppress the calcium signalling activity of the activating CaSR mutants to levels of wt-CaSR and they were efficacious on receptor proteins with mutations in the extracellular and transmembrane region. Recently, the novel ADH CaSR mutant D410E has also been tested and found to be sensitive to AXT914 in vitro [36]. Taken together, the quinazolinone calcilytics tested here could be a promising new therapeutic approach for ADH and BS type 5 patients.

Here we studied the calcium signalling pathway, which appears to be involved in the regulation of key processes of calcium metabolism [1, 5]. However, CaSR couples via different G-proteins to a number of other intracellular signalling events [2, 3] and it is yet unknown by which signalling mechanisms CaSR regulates renal salt handling. It is therefore difficult to predict the impact of quinazolinones calcilytics on hypercalciuria and renal salt wasting in patients with activating mutations, although the amino alcohol ronacaleret reduced urinary calcium excretion in healthy probands with wt-CaSR [30]. The quinazolinone AXT914 was also well tolerated and increased serum calcium in phase II trials, but its effects on the urinary excretion of calcium and other ions have not been reported [29]. The answer will have to await the availability of calcilytic drugs for clinical treatment of patients with BS type 5 and ADH.

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Author Contributions

Conceived and designed the experiments: SL BM CS. Performed the experiments: SL. Analyzed the data: SL BH BM CS. Contributed reagents/materials/analysis tools: CH ES KFR FR. Wrote the paper: SL BH BM CS. Contributed human genetic and clinical data: CH ES KFR FR.

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