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Treatment of Autosomal Dominant Hypocalcemia Type 1 with the Calcilytic NPSP795 (SHP635)

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48 Supplemental data is included with the manuscript.

49

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58 **ABSTRACT**

59 Autosomal dominant hypocalcemia type 1 (ADH1) is a rare form of hypoparathyroidism caused
60 by heterozygous, gain-of-function mutations of the calcium-sensing receptor gene (*CAR*).
61 Individuals are hypocalcemic with inappropriately low parathyroid hormone (PTH) secretion and
62 relative hypercalciuria. Calcilytics are negative allosteric modulators of the extracellular calcium
63 receptor (CaR) and therefore may have therapeutic benefits in ADH1. Five adults with ADH1
64 due to 4 distinct *CAR* mutations received escalating doses of the calcilytic compound NPSP795
65 (SHP635) on 3 consecutive days. Pharmacokinetics, pharmacodynamics, efficacy, and safety
66 were assessed. Parallel *in vitro* testing with subject CaR mutations assessed the effects of
67 NPSP795 on cytoplasmic calcium concentrations (Ca^{2+}_i), and ERK and $p38^{MAPK}$
68 phosphorylation. These effects were correlated with clinical responses to administration of
69 NPSP795. NPSP795 increased plasma PTH levels in a concentration-dependent manner up to
70 129% above baseline (p=0.013) at the highest exposure levels. Fractional excretion of calcium
71 (FECa) trended down but not significantly so. Blood ionized calcium levels remained stable
72 during NPSP795 infusion despite fasting, no calcitriol and little calcium supplementation.
73 NPSP795 was generally safe and well-tolerated. There was significant variability in response
74 clinically across genotypes. *In vitro*, all mutant CaRs were half-maximally activated (EC_{50}) at
75 lower concentrations of extracellular calcium (Ca^{2+}_o) compared to wild type (WT) CaR;
76 NPSP795 exposure increased the EC_{50} for all CaR activity readouts. However, the *in vitro*
77 responses to NPSP795 did not correlate with any clinical parameters. NPSP795 increased
78 plasma PTH levels in subjects with ADH1 in a dose-dependent manner, and thus, serves as
79 proof-of-concept that calcilytics could be an effective treatment for ADH1. Albeit all mutations
80 appear to be activating at the CaR, *in vitro* observations were not predictive of the *in vivo*

81 phenotype, or the response to calcilytics, suggesting that other parameters impact the response to

82 the drug.

83

84 Key words: PTH, hypoparathyroidism, hypocalcemia, calcium-sensing receptor, calcilytic

85

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INTRODUCTION

Autosomal dominant hypocalcemia type 1 (ADH1) is a rare genetic disorder of mineral homeostasis (OMIM 601198) caused by gain-of-function mutations of the calcium-sensing receptor gene (*CAR)* (3q13.3-q21.1).[1,2] The extracellular calcium receptor (CaR) is a G-protein-coupled receptor for which extracellular calcium (Ca^{2+}_o) is the primary physiological ligand, and it plays the major role in regulating systemic calcium homeostasis.[3-5] Gain-of-function mutations in the G-protein subunit α11(Gα11) protein (*GNA11*) (19p13.3) downstream of CaR have also been identified as causative for autosomal dominant hypocalcemia type 2 (ADH2) (OMIM #615361).[6-8]

CaRs are prominently expressed in parathyroid glands and the kidney, specifically in the thick ascending limb of the loop of Henle, distal tubules, and collecting ducts, where they regulate parathyroid hormone (PTH) secretion and calcium reabsorption, respectively.[3,9] Increases in Ca^{2+}_o activate the receptor and trigger downstream signaling leading to suppressed PTH secretion and renal calcium reabsorption. Thus, heterozygous mutations in ADH1 decrease CaR half-maximal activation (EC_{50}) to Ca^{2+}_o such that PTH secretion is decreased and renal calcium excretion increased. Decreased PTH leads to hypocalcemia and may result in symptoms that include peripheral and oral paresthesias, muscle cramps, tetany and seizures. Increased renal calcium excretion results in development of nephrocalcinosis, nephrolithiasis, and impaired renal function in some patients.[10]

Conventional therapy for ADH1 patients who require treatment includes calcium and vitamin D analogs; however, the rise in blood calcium exacerbates the hypercalciuria, placing patients at increased risk of renal complications. [10] Thus, current practice for those on treatment is to maintain calcium levels near or slightly below the lower limit of normal. However, some

116 patients continue to experience neuromuscular and cognitive symptoms of hypocalcemia and
117 sustain renal complications. Because of the inadequacy of conventional treatment, and its
118 potential for morbidities, precision therapy that could normalize both blood calcium and urinary
119 calcium excretion in ADH1 patients who require treatment would represent a significant
120 improvement.

121 Calcilytics, CaR negative allosteric modulators, increase PTH secretion and decrease
122 renal calcium excretion. NPSP795 (SHP635), one such calcilytic, and its structurally-related
123 compounds NPSP790 and NPS2143 were shown to increase PTH in rats, dogs, and monkeys [(11)],
124 and in addition, were shown to increase blood calcium as well as decrease renal calcium
125 excretion in PTH infusion-clamped rats [(12)], suggesting a potential benefit in ADH1. Moreover,
126 the calcilytic NPS2143 has been shown to reduce signaling responses of cells with gain-of-
127 function *CAR* mutations causative of ADH1 [(13)], and increased blood calcium after a single
128 intraperitoneal injection in a mouse model of ADH1 [(14)], the Nuf mouse. [(15)] Similarly, NPS2143
129 reduced mutant Val62 Gα11 signaling responses *in vitro* and led to an increase in plasma PTH
130 and calcium concentrations in two animal models of ADH2. [(16,17)] The calcilytic JTT-305 (MK-
131 5442) resulted in increased serum calcium and decreased urinary calcium by stimulating
132 endogenous PTH secretion and prevented renal calcification in knock-in mice with human *CAR*
133 (C129S) and *CAR* (A843E) mutations. [(18)]

134 We thus hypothesized that the calcilytic NPSP795 may be effective in treating ADH1,
135 and that by assessing the effect of NPSP795 on subject mutations *in vitro* and comparing the
136 results to clinical findings we could gain insight into genotype/phenotype correlations as well as
137 potentially predict how patients with specific mutations may respond to NPSP795. The present
138 proof-of-concept study was designed to assess the pharmacodynamics (PD), pharmacokinetics

139 (PK), efficacy, as assessed by change in PTH and PTH AUC, and safety of NPSP795 in adult
140 patients with ADH1. *In vitro* studies assessed the potency of Ca^{2+}_o on CaR-induced intracellular
141 Ca^{2+} (Ca^{2+}_i) mobilization, and ERK and $p38^{MAPK}$ phosphorylation in the patient mutations, and
142 the effect of NPSP795 on these parameters and the correlation between these parameters and
143 each patients' clinical response, as assessed by PTH AUC.
144

145 **METHODS**

146 *Study participants*. Adults with a previously established clinical diagnosis of ADH1,
147 confirmed by *CAR* gene mutation, BMI ≥ 18.5 to < 39 kg/m^2, and 25-hydroxy-vitamin D > 25
148 ng/mL were enrolled in this study from June 2014 to April 2015. Key exclusion criteria were
149 treatment with PTH 1-84 or 1-34 within the previous 6 months; hypocalcemia requiring more
150 than 6 IV calcium infusions per year; hypocalcemic seizures within the past 3 months;
151 glomerular filtration rate (GFR) < 25 mL/minute/1.73 m^2; abnormal hepatic, hematologic, and/or
152 clotting function; 12-lead resting electrocardiogram (ECG) with clinically significant
153 abnormalities; concomitant medications with potential to interfere with NPSP795 metabolism;
154 and history of thyroid or parathyroid surgery.

155 *Study design*. This was an open-label, non-randomized, single-center, intra-subject dose-
156 escalating phase IIb study in adults with ADH1. The study medication, NPSP795, a calcilytic,
157 was provided by NPS Pharmaceuticals, Inc.

158 After screening and baseline assessments, subjects were admitted to the National
159 Institutes of Health (NIH) Clinical Center for a 5-day, 4-night admission. Subjects who were
160 treated with calcitriol discontinued the medication 2 days prior to the start of NPSP795. Home
161 calcium supplements were administered up until the day of the first NPSP795 infusion.

162 Thereafter, calcium supplementation was administered based on clinical symptoms of
163 hypocalcemia as deemed necessary by the investigators. Subjects fasted from midnight until 4
164 hours after each infusion start. When not fasting, they consumed approximately 1000 mg/day of
165 calcium from dietary sources throughout the study, but the diet was otherwise not standardized.
166 The ionized calcium level was measured prior to each NPSP795 infusion and had to be 0.75 to
167 1.25 mmol/L (3-5 mg/dL) to start the infusion.

168 Subjects received 5 mg/10min NPSP795 via intravenous infusion on Day 1 with a plan to
169 repeat the same dose if the ionized calcium was ≥ 1.12 mmol/L or the PTH level was > than the
170 upper limit of normal (65 pg/mL). NPSP795 was chosen as the compound for this study because
171 it was the most potent calcilytic that was available from the pharmaceutical collaborator.
172 Because this was the first study to assess the effects of this class of drugs in ADH1, NPSP795
173 was administered intravenously in order to more rigorously control blood concentrations of the
174 drug. The starting dose was selected because it was tested in previous healthy volunteer studies
175 (NPSP7975 = SB-423562***)***, was well-tolerated with no safety concerns and resulted in an
176 increase in serum PTH [(11)]. If there was no increase in ionized calcium or PTH, the dose of
177 NPSP795 was increased the following day. Every 1 hour during the testing period, an
178 investigator assessed tolerability of the infusion and monitored the ionized calcium levels and
179 blood PTH levels. Because no subjects met the ionized calcium or PTH criteria to repeat a dose
180 of NPSP795, they all received 15 mg/3.5h on Day 2 and 30 mg/3.5 h on Day 3 via intravenous
181 infusion. These doses were determined using the indirect pharmacodynamic response model
182 generated from healthy volunteer data in combination with the No Observed Adverse Effect
183 Level of the drug in pre-clinical toxicology studies in non-human primates. Subjects underwent
184 serial sampling via intravenous catheter to assess PK, PD, and safety parameters starting at

185 baseline prior to each drug infusion. Subjects resumed home medications the morning following
186 the final infusion of NPSP795 and were discharged home to the care of their local physician.
187 *Study objectives and assessments*. The primary objectives of the study were to assess the
188 safety and tolerability of NPSP795 when administered as multiple, dose-escalating *i.v.* infusions
189 and to assess the PD effects and preliminary efficacy on change from baseline ionized calcium,
190 blood and urinary calcium, and PTH. Renal ultrasounds were performed at the outpatient
191 screening visit.
192 Safety assessments included evaluation for adverse events, physical examinations, ECGs,
193 vital signs, bedside ionized calcium measurements, and standard clinical laboratory evaluations.
194 Safety and tolerability were monitored continuously during the study while subjects were
195 admitted to the NIH Clinical Center. A telephone follow-up safety assessment was performed
196 approximately 14 days following discharge.
197 *Sample collection*. To measure relevant PK/PD parameters such as NPSP795 drug
198 concentrations, ionized calcium, blood and urine calcium, PTH, blood and urine phosphate,
199 blood and urine creatinine, we collected samples from subjects at various time points depending
200 on the dose of NPSP795. For NPSP795 5 mg/10min dose, blood sampling for PK and PTH
201 determinations were performed at baseline, every 5 minutes for 15 minutes, every 15 minutes for
202 30 minutes, every 30 minutes until hour 4, and at 8 hours. Ionized calcium was measured at
203 baseline, every 15 minutes for the first 2 hours and every 30 minutes thereafter until hour 4. For
204 the 3.5h NPSP795 infusions, PK and PTH determinations were performed at baseline, every 15
205 minutes for 30 minutes, every 30 minutes until hour 4, and at hours 5 and 5.5; ionized calcium
206 was measured at baseline, every 15 minutes for the first 2 hours and every 30 minutes thereafter
207 until hour 4, and at hours 5 and 8 thereafter. Blood and spot urine sampling for determination of

208 additional PD and other parameters was performed at baseline, 1, 2, 3, 4, 8, and 12 hours for all
209 NPSP795 doses.

210 Routine blood and urine chemistries and complete blood count (CBC) were performed at
211 the NIH Clinical Center Department of Laboratory Medicine. iPTH was determined via
212 electrochemiluminescence immunoassay (Roche Cobas e601 analyzer; NIH, Bethesda, MD,
213 USA), and 25-hydroxy-vitamin D was measured by chemiluminescence immunoassay (NIH,
214 Bethesda, MD, USA). Ionized calcium measurements were performed at the subject's bedside
215 using the Radiometer ABL80 FLEX CO-OX blood gas analyzer (Radiometer America, Brea,
216 CA, USA). NPSP795 blood concentrations were determined via a liquid chromatography/
217 tandem mass spectrometry assay with a lower limit of quantitation of 10.0 ng/mL that was
218 validated in accordance with the FDA Guidance for Industry. Estimated GFR was calculated
219 using the CKD-EPI equation. FECa was determined using the following calculation: (Urine-
220 calcium*Plasma creatinine) / (Urine-creatinine*Plasma-total calcium).

221 *Study approval.* This study was conducted in accordance with the ethical principles of the
222 Declaration of Helsinki, and it received approval from the Institutional Review Board of the
223 National Institute of Diabetes and Digestive and Kidney Diseases, NIH. All participants
224 provided written informed consent before participating in any study procedures. The study was
225 registered with ClinicalTrials.gov (NCT02204579).

226 *In vitro studies: Generation of stable cell lines expressing WT or mutant CARs*. Site-
227 directed mutagenesis was performed using the Stratagene QuikChange™ II kit according to the
228 manufacturer's instructions. Briefly, a pair of complementary primers of 25–35 bases was
229 designed for each mutagenesis reaction with the mutation placed at the middle of the primers
230 (Supplemental Table 1). The template, a cassette version of human CaR in pcDNA3.1(+) was

231 amplified using Pfu II DNA polymerase with these primers for 18 cycles in a DNA thermal
232 cycler. After digestion of the template DNA with DpnI the amplified mutant DNA was
233 transformed into DH5α Escherichia coli. The incorporation of the desired mutations and absence
234 of other mutations were confirmed by automated DNA sequencing (Eurofin MWG). Successful
235 mutants and wt CaR were excised using restriction sites either side of the receptor (HindII/ApaI)
236 in the multiple cloning site, before being ligated into a pcDNA5/FRT plasmid for generation of
237 stable cell lines using the Flp-In system (ThermoFisher).

238 *Generation of cell lines*. Stably expressing cell lines were developed using the Flp-In
239 system (ThermoFisher) for generating constitutive expression cells lines. Commercially available
240 Flp-In-293 cell lines from ThermoFisher are derived from HEK293 cells and stably express the
241 pFRT/lacZeo plasmid containing an integrated Flp Recombinase Target (FRT) site. Flp-In-293
242 cells were maintained in 25-cm^2 culture flasks and transfected with both pcDNA5/FRT/CaR
243 (containing either WT or one of the ADH mutants) and pOG44 plasmid at a 9:1 ratio using
244 Lipofectamine 2000™ according to the manufacturer's instructions. Transfection of pOG44
245 leads to expression of Flp Recombinase and catalyses a homologous recombination effect
246 between the FRT sites in pcDNA5/FRT/CaR and Flp-In-293 cells. Cells were selected over 2 - 3
247 weeks through media containing 150 ng/mL hygromycin. Resistant cells were then screened for
248 activity in response to $Ca^{2+}{}_o$.

249 *Receptor expression.* As part of the initial screening process the effect of ADH mutations
250 on cell surface expression using transiently transfected FLAG-tagged WT or ADH mutant
251 constructs was examined. This version of the CaR has a FLAG-tag between residues 371 and
252 372 in a loop tolerant of in-frame insertions. [19]

253 *Cell surface expression by ELISA.* Quantification of total and cell surface expression was
254 completed using an ELISA based assay that has been previously published. [20] HEK293 cells
255 were transfected with WT or one of the 5 ADH Flag-tagged CaR constructs using
256 Lipofectamine2000™ according to the manufacturer's instructions. After ~ 24 hours transfected
257 HEK293 cells were cultured into Poly-D-Lysine (Sigma) coated 96 well plates. Once cell
258 reached an approximate density of 100% cells were washed once with TBS-T (0.05 M Tris, 0.15
259 M CaCl, 0.05% (w/v) Tween-20, pH 7.4) and fixed for 15 mins on ice with either 4%
260 paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH
261 7.4) to determine surface expression or methanol to determine total expression. After washing
262 once with TBS-T, cells were blocked for 1 h at room temperature in 1% (w/v) skim milk in TBS-
263 T, before being incubated with 1:1000 monoclonal FLAG M2 horse-radish peroxidase (HRP)-
264 conjugated antibody (Sigma-Aldrich) in blocking solution for 1 h at room temperature. After
265 incubation with the antibody, cells were washed three times with TBS-T before being incubated
266 with a HRP substrate (3,3'5,5' tetramethylbenzidence (TMB) liquid substrate solution) for 30
267 min in the dark. Enzyme reaction was stopped by adding equal volumes of 1 M HCl. Supernatant
268 samples were transferred to a new plate and A_{450} values were obtain through a plate reader.
269 *Measurements of $Ca^{2+}{}_i$ in populations of cells using a plate reader.* Cells were plated in
270 96 well plates and grown to confluence before being loaded with 2 μM Fluo-4 for 30 min. Using
271 a Fluoroskan Ascent plate reader (ThermoFisher Scientific), an initial baseline reading of ten
272 measurements (at a calcium concentration of 0.2 mM) was taken to determine the 0 sec value.
273 Calcium solutions which increased calcium concentration to 0.2 - 8 mM, in the presence and
274 absence of 1 μM NPSP795 or vehicle control (0.01% DMSO), were dispensed into a single well
275 and emission at 538 nm for each well was then followed for 30 s (1 reading every 5 seconds).

276 Dose response curves were generated from the peak fluorescence upon the addition of the
277 calcium solution and corrected for the initial baseline measurement. Peak fluorescence was then
278 normalized to a 10 mM calcium + 2 μM ionomycin response to enable comparison between
279 different plates.

280 *Measurement of ERK and p38^{MAPK} Phosphorylation.* These assays were carried out as
281 described elsewhere. [21] Briefly, cells were incubated for 10-mins in either Experimental Buffer
282 (0.5mM Ca^{2+}_o) alone or in buffer supplemented with either a) various concentrations of Ca^{2+}_o (2
283 - 10mM), or b) various concentrations of NPSP795 (1-1000nM; equal DMSO concentration in
284 all samples) in the presence of either 2.4 mM Ca^{2+}_o (ADH mutants) or 3.4 mM Ca^{2+}_o (Wild-
285 type); these Ca^{2+}_o being close to ~EC_{80} values for these receptors (with regards to ERK
286 activation). Where [$CaCl_2$] was increased, [NaCl] was reduced accordingly to normalize ionic
287 strength. Where NPSP795 was used, cells were pre-exposed to the calcilytic for 30-secs before
288 application of the high Ca^{2+}_o/NPSP795 co-treatment. The cell lysate supernatant was stored at -
289 80°C until use, whereupon an aliquot was mixed with 5X Laemmli buffer and boiled for 3-min
290 prior to immunoblotting (40-60 μl sample/lane) using the phospho-specific anti-ERK antibody or
291 anti-p38^{MAPK} antibody (1:5000; Cell Signaling). Protein equivalency of the samples was
292 demonstrated initially by Ponceau staining the blot (prior to blocking) and then by stripping and
293 reprobing with either total anti-ERK or anti-β-actin antibodies. Relative immunoreactivity was
294 determined by densitometry.

295 *Statistics.* The statistical analyses for the clinical research study were performed using
296 SAS® software version 9. A mixed model repeat measures analysis with unstructured covariance
297 matrix and Tukey adjustment was used. A p value of less than 0.05 was considered statistically
298 significant. Statistical analyses performed for the *in vitro* studies using GraphPad Prism v5

299 included one-way ANOVA and Dunnett's post-test when comparing log EC_{50} of CaR mutants to
300 that of WT. Unpaired two-tailed Student's t-tests were used to assess differences between log
301 EC_{50} of CaRs in the presence or absence of NPSP795. Statistical analyses performed to assess
302 correlations between *in vitro* and clinical findings included linear regression analysis using
303 GraphPad Prism v7.
304
305 **RESULTS**

306 *Subject characteristics*. Of the 9 patients screened, 8 were eligible, and 5 subjects with a
307 mean age of 39.8 years (range 23-55) and mean BMI of 32.8 kg/m^2 (range 29.4-37.2) completed
308 the study (Supplemental Figure 1). Baseline demographics, disease characteristics, and *CAR*
309 genotype of subjects enrolled are summarized in Table 1. All subjects had hypocalcemia,
310 inappropriately low PTH levels, and had been diagnosed in childhood. Four subjects presented
311 with hypocalcemic seizures, and 1 subject was tested for hypocalcemia after her mother was
312 diagnosed with ADH1. Subjects 1 and 4 were first-degree cousins and shared the same mutation,
313 A840V. Subject 5 was found to have a novel *CAR* mutation, E228A. Four subjects had low
314 GFR (< 90 mL/min/1.73m^2), and 3 subjects had evidence of nephrocalcinosis/nephrolithiasis on
315 renal ultrasound at baseline.

316 *Efficacy outcomes*. Subjects received 5 mg/10min NPSP795 on Day 1 with a plan to
317 repeat the same dose if the ionized calcium was ≥ 1.12 mmol/L or the PTH level was > than the
318 upper limit of normal (65 pg/mL). Because no subjects met the ionized calcium or PTH criteria
319 to repeat a dose of NPSP795, all subjects received 15 mg/3.5h on Day 2 and 30 mg/3.5 h on Day
320 3 (Figure 1). Infusions of NPSP795 resulted in significant increases in blood levels of NPSP795
321 that were sustained during the infusion period (Figure 2A-C). NPSP795 resulted in a dose- and

322 concentration-dependent increase in percent change in PTH from baseline, the primary endpoint,
323 (Figure 2 D-F), that was paralleled by changes in PTH blood levels (Figure 2G-I). The
324 maximum mean percent change (± 1SD) in PTH was 128.6% (±139%) at 15min following 5
325 mg/10min dose of NPSP795 ($p=0.048$), 49% (±47%) at 210min following 15 mg/3.5h dose
326 ($p=0.149$), and 121% (±91%) at 210 min following the 30 mg/3.5h dose ($p=0.013$) (Figure 2D-
327 F). The peak mean PTH (± 1SD) was 17.4 pg/mL (± 16) at 15min following 5 mg/10min dose
328 of NPSP795, 13.7 pg/mL (± 5.9) at 210 min following 15 mg/35h dose, and 21 pg/mL (± 14) at
329 210min following 30 mg/3.5h dose (Figure 2G-I).

330 While fractional excretion of calcium (FECa) was decreased at the higher exposures of
331 NPSP795, the changes were not statistically significant. The mean (±1SD) maximum decrease in
332 the FECa occurred during the 15 mg/3.5h (maximum -36% (± 27%) at 120min) and 30 mg/3.5h
333 (maximum of -43% (± 25%) at 120min) doses (Figure 2J-L).

334 The changes in PTH and FECa were not sufficient to result in an increase in blood
335 calcium levels (Figure 2M-O). Of note, prescribed calcitriol was discontinued 2 days prior to
336 study start, calcium supplements were discontinued at least 12 hours prior to study start, and
337 subjects were fasted for the first four hours of dosing and serial sampling. Subjects received
338 between 250-1200 mg of elemental calcium supplementation in the evening. Two subjects
339 required rescue calcium for symptoms of hypocalcemia including paresthesias and muscle
340 cramping that totaled 600 and 1,800 mg of elemental calcium (Supplemental Table 2). Thus,
341 while ionized calcium levels did not rise in response to NPSP795, they remained stable and did
342 not decline during NPSP795 administration despite fasting and withholding of calcitriol,
343 consistent with an effect of NPSP795 infusion on maintaining blood calcium levels.

344 *Safety*. NPSP795 infusions were generally well-tolerated. In total, 1 serious adverse event and 2
345 adverse events (AEs) were reported by 3 subjects during the study. Subject 1, who was obese
346 and had a family history of cholelithiasis, developed symptomatic cholelithiasis on the morning
347 of day 4 after receiving the 3 doses of NPSP795. The symptoms resolved, and he was
348 discharged home to the care of his primary physician. However, symptoms returned upon
349 discharge, and the subject underwent a laparoscopic cholecystectomy. The subject did well post-
350 operatively without additional adverse events. Considering the timing of the symptoms in
351 relation to NPSP795 dosing, the investigators concluded that the SAE was unlikely to be related
352 to the study drug. The other two AEs, facial swelling and bilateral nipple sensitivity, which
353 occurred 72 and 48 hours after the last dose of study drug, respectively, were deemed unrelated
354 to NPSP795 and resolved without incident. No deaths were reported during the study or follow-
355 up period. The study was discontinued after data from 5 subjects revealed the short-acting nature
356 of NPSP795 and lack of effect on blood ionized calcium.

357 *Response by genotype.* Despite similar blood concentrations of NPSP795 (Figure 3B),
358 there was wide variability in the response to this calcilytic across genotypes as assessed by PTH
359 AUC (Figure 3A). This is particularly evident in comparing subjects 1 and 4, who had the same
360 *CAR* mutation (A840V), and had markedly different increases in PTH AUC (Figure 3C). In
361 fact, while the NPSP795 AUC in subject 1 was generally lower than subject 4 (Figure 3D), the
362 PTH AUC in subject 1 was higher than in subject 4. In addition, subject 3 (E228K mutation) had
363 almost no response to NPSP795 in terms of PTH AUC (Figure 3E), and was considered a non-
364 responder, yet subjects 3 and 5 (E228A mutation) had similar exposure to NPSP795 (Figure 3F).
365 Baseline PTH and dose of calcium supplementation prior to study entry for each subject was

366 compared to the respective PTH AUC. There were no statistically significant correlations

367 between these clinical parameters and PTH AUC as assessed by linear regression analysis.

368 *In vitro analyses. Response by genotype.* To assess potential genotype/phenotype

369 correlations and understand variability in clinical response to NPSP795, stable cell lines

370 expressing wild-type (WT) and subject mutant CaRs were generated in HEK293-derived Flp-In-

371 293s cells, and the Ca^{2+}_o concentration at which half-maximal (EC_{50}) Ca^{2+}_i occurred was

372 measured. The same parameter was assessed for an increase in ERK and p38 phosphorylation in

373 parallel. In addition, the NPSP795 concentration at which these parameters were half-maximally

374 inhibited (IC_{50}) was determined. The EC_{50} for Ca^{2+}_i mobilization, and ERK and p38

375 phosphorylation for all mutant CaRs were significantly less than WT (Table 2). Likewise,

376 NPSP795 significantly increased all these parameters (Table 2). These changes were not

377 ascribed to differences in total or cell surface expression (Supplemental Figure 4). Also,

378 comparable results were obtained in transient and stable transfections (not shown). Together,

379 these results suggest that the enhanced signaling responses to Ca^{2+}_o, and the ability of calcilytics

380 to restore such response, were not due to differences in mutant receptor expression. All EC_{50}

381 data were compared to the clinical parameter PTH AUC. There were no statistically significant

382 correlations between any *in vitro* finding and PTH AUC as assessed by linear regression

383 analysis.

384 **DISCUSSION**

385 Hypoparathyroidism, in particular ADH1, is a disease for which there remains a need for

386 better therapeutics. [10,22,23] Given that ADH1 results from gain-of-function mutations in the *CAR*,

387 negative allosteric modulators of the CaR, calcilytics, represent a rational, precision medicine

388 approach to treating this disease. Through a public/private partnership between the NIDCR and
389 NPS Pharmaceuticals (acquired by Shire in 2015), the short-acting calcilytic NPSP795, which
390 was initially developed as a treatment for osteoporosis, , was repurposed to study the potential
391 for efficacy in patients with ADH1. In this proof-of-principle, dose-finding study the initial
392 question was if parathyroid glands in patients with ADH1, which have been essentially dormant
393 for life, would respond to a calcilytic. This was clearly shown to be the case. There were dose-
394 dependent increases in PTH that were significant at the highest drug exposure levels (Figure 3).
395 The on- and off-effect was rapid, with PTH levels rising rapidly after infusion and dropping
396 rapidly in a single phase, exponential decay pattern following cessation of drug infusion (Figure
397 2). At the highest levels of exposure, there appeared to be a decrease in FECa although it did not
398 reach statistical significance (Figure 2K-L) but was consistent with the predicted effect of a
399 calcilytic on renal calcium handling, and points to the fact that the study was underpowered to
400 see a significant effect on FECa. The short-acting nature of NPSP795 could also account for the
401 lack of sustained effect on FECa.

402 All subjects required both calcitriol and calcium prior to enrolling in the study to
403 maintain blood calcium levels. Throughout the study, all subjects were kept on a fixed,
404 relatively low dose calcium diet (1,000 mg/day), and no subjects were taking calcitriol. All
405 subjects received some supplemental calcium at bedtime to avoid symptomatic hypocalcemia
406 while fasting, and two subjects required a rescue calcium for symptoms of hypocalcemia
407 (Supplemental Table 2). The maintenance of blood calcium levels (Figure 2M-O) with
408 significantly decreased medication would be very unusual in patients with treatment-dependent
409 ADH1 such as those studied and is consistent with an effect of study drug at maintaining blood
410 calcium. However, it is likely the case that substantially higher doses of NPSP795 will be needed

411 to have significant effects on FECa and blood calcium. It was previously shown in a single-dose
412 study of PTH 1-84 in patients with hypoparathyroidism that blood levels of PTH near 200 pg/ml,
413 ten times the levels achieved in this study, were needed to illicit a clear effect on FECa and blood
414 calcium[24].

415 Of note was the marked variability in response to NPSP795 in terms of effects on PTH
416 that was not explained by differences in drug levels. The differences in PTH response to
417 NPS795 were particularly striking in the two patients with the same mutation (subjects 1 and 4,
418 first-degree cousins) (Figure 3C). Probably some of the difference in PTH response between the
419 two subjects can be explained by differences in drug levels (Figure 3D), but it is likely most of
420 the difference is explained by other factors such as metabolism, body mass, diet, sex hormones,
421 renal function, etc. In addition, there was no relationship between PTH AUC and NPSP795 drug
422 levels (Figure 3). These differences in response, especially between the first-degree cousins,
423 suggest that there are additional factors that control PTH secretion, such as disease-modifying
424 genes, that are not well understood and require further investigation.

425 This study is limited by the lack of a placebo or control group and a small number of
426 subjects that represented only 4 *CaR* mutations. Although the urinary FECa results suggested a
427 decrease with exposure to NPSP795, this did not reach statistical significance, which may have
428 been achieved with a larger number of subjects and a control group. In addition, referral bias
429 may affect our findings. As a tertiary referral center, these subjects may be more severely
430 affected which could diminish potential generalizability of the findings.

431 While this proof-of-principle study was unable to demonstrate significant effects on
432 urinary FECa and blood calcium due to the small number of subjects and limited drug exposure,

433 it clearly demonstrated that calcilytics can rapidly stimulate PTH secretion in ADH1 and that it is
434 highly likely that with adequate drug exposure calcilytics may be an effective treatment for
435 ADH1. Further, they may represent a treatment for other forms of hypoparathyroidism,
436 including ADH2, due to their independent actions on the kidney, and possibly even idiopathic
437 hypercalciuria.
438

439 **AUTHOR CONTRIBUTIONS:**

440 MSR, RIG, BB, LG, and MTC conducted the clinical trial and acquired data. MSR, RIG, DG,
441 EN, RR, and MTC contributed to designing the study and analyzing data. EK contributed to
442 performing the statistical analysis for the clinical research study. JG contributed to analyzing
443 data. SCB, DR, MS, MBK, DTW, and EN contributed to designing and analyzing the data from
444 the *in vitro* studies. SCB, MS, DR, MBK, and DTW carried out the *in vitro* studies. MSR, RIG,
445 SCB and MTC wrote the manuscript, and all remaining authors reviewed/revised the manuscript
446 prior to publication.

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528

529 **Figure 1. NPSP795 Clinical Study Design.** After an initial outpatient screening visit, subjects
530 were admitted to the National Institutes of Health (NIH) Clinical Center. An *i.v.* dose of 5
531 mg/10min NPSP795 was administered on day 1, and subjects underwent serial sampling for
532 pharmacokinetic/pharmacodynamic parameters. If subjects had an increase in ionized calcium to
533 the normal range or an increase in intact parathyroid hormone (iPTH) to greater than the upper
534 limit of normal (ULN), the same dose of NPSP795 would be administered on day 2. If subjects
535 did not meet the ionized calcium or iPTH parameters, 15 mg/3.5h NPSP795 would be
536 administered on day 2 along with serial sampling. The same criteria were used to determine the
537 dose of NPSP795 on day 3. Subjects were discharged home on day 4.

538

539 **Figure 2. Pharmacokinetic and pharmacodynamic effects of NPSP795.** NPSP795
540 concentrations significantly increased after infusion as predicted from modeling studies (A-C)
541 and resulted in a rapid and concentration-dependent increase in % change in PTH (D-F) and PTH
542 (G-I). Significant effects on PTH were seen at the highest concentrations in NPSP795 during
543 the 5 mg bolus infusion on day 1 (D) and with the 30 mg infusion on day 3 (F, I). The fractional
544 excretion of calcium (FECa) (J-L) decreased by > 30% following the 15 mg/3.5h, and >40% 30
545 mg/3.5h doses but did not reach statistical significance. The ionized calcium levels (M-O)
546 remained stable during NPSP795 infusions despite fasting and calcium and calcitriol doses being
547 withheld. Statistical analysis was performed using mixed model repeat measures analysis.

548

549 **Figure 3. Clinical response to NPSP795 by CaR genotypes.** PTH area under the curve (AUC)
550 varied widely across the different *CAR* mutations despite overall similar exposure to NPSP795
551 (B). The subject with E228K mutation in the *CAR* had little to no response to NPSP795 (A).

552 Subjects 1 and 4 were first-degree cousins with the same A840V *CAR* mutation but had widely
553 different responses to NPSP795 (C). Differences in response were not accounted for by
554 differences in NPSP795 clearance, as subject 4 had higher NPSP795 exposure (D), but less of a
555 response. Of note, subjects 3 and 5 both had E228 mutations, K and A, respectively, but
556 markedly different responses. Subject 3 had little response (E), but robust NPSP795 blood levels
557 (F).

558 **Table 1. Subject baseline demographics, biochemical profile, and disease characteristics.**

Subject #	Age	Sex	Ethnicity	CaR Mutation	Ca (8.6-10.2 mg/dL)	Phos (2.5-4.5 mg/dL)	iPTH (15-65 pg/mL)	Cr (0.67-1.17 mg/dL)	eGFR (≥90 mL/min/1.73m^2)	FECa	Renal Ultrasound
1	55	M	Caucasian	A840V$^{\Omega}$	7.5	3.6	13.4	1.17	70	0.04	Normal
2	46	M	Caucasian	Q245R	7.1	3.3	9.5	1.13	78	0.03	NC/NL
3	23	F	Caucasian	E228K	7.6	5.6	5.0	1.43	51	0.04	NC
4	51	F	Caucasian	A840V$^{\Omega}$	8	5.6	12.4	1.03	62	0.01	NC
5	24	M	Caucasian	E228A$^{\Psi}$	8.5	4.3	6.9	1.05	99	0.01	Normal

559 Non-fasting laboratory studies obtained during screening visit while on treatment with calcium and calcitriol. $^{\Omega}$First degree cousins. $^{\Psi}$Novel mutation. CaR = Calcium sensing receptor; Phos = phosphorus; iPTH = intact parathyroid hormone; Cr = creatinine; eGFR = estimated glomerular filtrate rate; FECa = fractional
560 excretion of calcium; NC = nephrocalcinosis; NL = nephrolithiasis

Table 2. Summary of *in vitro* and PTH AUC findings.

	EC_{50} Ca^{2+}_i mobilization (mM)	EC_{50} ERK phosphorylation (mM)	EC_{50} $P38^{MAPK}$ phosphorylation (mM)	IC_{50} NPSP795 Ca^{2+}_i mobilization (µM)	IC_{50} NPSP795 ERK phosphorylation (nM)	IC_{50} NPSP795 $P38^{MAPK}$ phosphorylation (nM)	Day 3 PTH AUC
WT	2.9	3.0	4.2	5.2	112	88	
Q245R	1.0	2.0	2.0	1.76	52	58	35040
E228K	0.8	1.7	1.6	0.04	91	69	1937
E228A	0.7	2.1	2.2	1.25	32	10	29280
A840V	1.3	2.0	2.3	1.15	50	70	Subject 1: 26648 Subject 4: 9141

EC_{50} = half-maximal activation; Ca^{2+}_i = intracellular calcium; IC_{50} = half-maximal inhibition; PTH AUC = parathyroid hormone area under the curve