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Research Article

Preclinical development of EXT608, an investigational parathyroid hormone derivative with extended half-life for the treatment of hypoparathyroidism

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Abstract

Hypoparathyroidism, a deficiency of parathyroid hormone (PTH), results in hypocalcemia, hyperphosphatemia, and hypercalciuria. The disease is poorly controlled by calcium and vitamin D supplements or native PTH(1-84) replacement therapy. A version of PTH is being developed using D-VITylation technology, whereby vitamin D is conjugated to a therapeutic peptide, which confers a long plasma half-life by virtue of binding to the abundant vitamin D binding protein (DBP). D-VITylation of PTH caused no reduction in activity at the PTHR1 receptor, and resulted in a plasma elimination half-life of 7–15 h in rats and 24–32 h in cynomolgus monkeys. Analysis of steady-state pharmacokinetics as a function of dose showed flat profiles with smaller peak:trough ratios at low doses, indicative of slower subcutaneous absorption. In thyroparathyroidectomized (TPTx) rats, PTH(1-34)-vitamin D conjugates restored serum calcium and phosphate levels into the normal range over the 24 h dosing period, and increased bone turnover markers and reduced bone mineral density. Urinary calcium was initially elevated, but normalized by the end of treatment on day 27. In healthy monkeys, a single dose of PTH(1-34)-vitamin D conjugates elevated serum calcium levels above the normal range for a period of 24–48 h while simultaneously reducing urinary calcium. Therefore, the lead compound, EXT608, is a promising candidate as a therapeutic that can truly mimic the endogenous activity of PTH and warrants further study in patients with hypoparathyroidism.

Keywords: Bone QCT/microCT, preclinical studies, parathyroid-related disorders, hormone replacement/receptor modulators, PTH/Vit D/FGF23

Lay Summary

Parathyroid hormone (PTH) regulates calcium levels in blood. Deficiency in PTH, either from genetic causes or surgical damage to the parathyroid glands, results in low blood levels of calcium (hypocalcemia), which adversely effects the function of nerves, muscles and over time leads to loss of kidney function. Natural PTH cannot be absorbed orally from pills/capsules and if injected, only lasts a few minutes in the blood stream. Therefore, an effective hormone replacement therapy would require frequent injections of PTH. We have developed a modified version of PTH named EXT608, which remains circulating in the bloodstream for an extended time, with the goal of achieving therapeutically effective concentrations of the drug without the need for frequent injections. These studies characterize the erum concentration of EXT608 and the resulting effect on blood calcium levels as a function of time in 2 model species, rat and monkey. We demonstrate that the circulation time of EXT608 is extended far beyond that of the natural hormone, and the resulting blood calcium levels were elevated in a dose-dependent manner. The effects of EXT608 on bone structure and urinary calcium levels were also characterized. These studies provide a solid foundation for testing the effectiveness in human clinical studies.

Introduction

Hypoparathyroidism is a deficiency of parathyroid hormone (PTH), which controls calcium and phosphate homeostasis and bone remodeling.[1] PTH deficiency is classified as a rare disease with an estimated 60 000–100 000 patients suffering in the United States[2,3] and can occur when an individual is born without the parathyroid gland (typically due to a chromosomal deletion), the gland has been damaged during a surgical procedure, or when the organ is damaged due to an autoimmune response, iron accumulation, magnesium deficiency, or other idiopathic or genetic causes.[4] A lack of PTH leads to low serum calcium (hypocalcemia), high serum phosphate (hyperphosphatemia), high urinary calcium (hypercalciuria) due to decreased renal reabsorption, and reduced bone turnover leading to increased bone density and possibly impaired bone quality.[5,6] If left untreated, hypoparathyroidism affects virtually every organ system in the body. These physiological changes (hypocalcemia, hypercalciuria, hyperphosphatemia, and the near absence of PTH), alone or in combination with each other, will cause neuromuscular symptoms (persistent muscle cramps, paresthesia, tetany, seizures, cardiac arrhythmias), ischemic heart disease, kidney stones and nephrocalcinosis, and increased risk of vertebral fractures despite increased bone mineral density measurements.[7,8] Additionally, hypoparathyroidism patients suffer from emotional and cognitive disorders such as anxiety, depression, memory problems, and general "brain fog."[9] Importantly, patients with improperly controlled hypoparathyroidism report a significantly reduced quality of life, with 75% of patients reporting their disease affects their ability to work and 63% of patients reporting negative effects on their family relationships.[10,11]

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PTH plays a role in mineral homeostasis through at least three mechanisms: (1) PTH stimulates renal conversion of 25-hydroxyvitamin D (25-OH-VitD) to 1,25-dihydroxyvitamin D (1,25-OH-VitD), which, in turn, increases intestinal calcium and phosphate absorption, (2) PTH enhances calcium reabsorption at the distal convoluted tubule and collecting ducts of the kidney, and (3) PTH increases serum calcium through bone resorption mediated by osteocytes/osteoblasts.[12,13] PTH also regulates serum phosphate levels through bone resorption, vitamin D-mediated intestinal absorption, and renal phosphate excretion.[12] Conventional treatment for hypoparathyroidism is supplementation of large amounts of calcium as well as active vitamin D analogs.[12] This treatment serves to increase intestinal calcium absorption (mechanism 1) but does not increase renal calcium reabsorption (mechanism 2) or bone resorption (mechanism 3). Patients' urinary calcium concentrations must be carefully monitored as hypercalciuria can lead to nephrocalcinosis (calcium deposits in the kidney), kidney stones, and long-term renal failure.[7,14,15] Studies by Winer et al. indicate that among patients with hypoparathyroidism who were treated with conventional supplement therapy, 80% had reductions in glomerular filtration rates[16] and 40% had nephrocalcinosis.[17] However, PTH replacement therapy leads to normocalcemia in patients[16-18] and is more desirable than conventional therapy because it has the potential to increase serum calcium levels without inducing hypercalciuria.[19,20] Due to the role of PTH in bone turnover[13] and cognitive function,[21,22] a PTH replacement therapy would also be effective for treating these symptoms that are not targeted by conventional therapy.

PTH is an endogenous human polypeptide, 84 amino acids in length; however, the N-terminal 34 amino acids retain full biological activity.[23] The main obstacle to using PTH as replacement therapy is that the plasma half-life is less than 10 min when given intravenously in humans.[24,25] Given subcutaneously, the half-life is longer due to the time required for absorption, and is 1–1.3 h for PTH(1-34)[26-28] and 1.5–2.5 h for PTH(1-84).[26,29,30] Recombinant human PTH(1-84), Natpara (Takeda), was approved by the FDA in 2015 for the treatment of hypoparathyroidism as a once-daily subcutaneous injection despite the challenge of the rapid rate of elimination (Natpara is completely cleared from circulation within 10–12 h and therefore cannot maintain mineral balance throughout the dosing period). Although Natpara is able to reduce dependence on oral calcium and vitamin D supplements, it does not provide the true clinical benefits of PTH replacement, namely, reducing incidents of hypo- and hypercalcemia, and maintaining urinary calcium levels within the normal range.[31-33] Because of these limitations, the FDA has recommended Natpara only for patients that cannot be well-controlled on calcium and active vitamin D supplements. However, despite the non-ideal pharmacokinetics (PK) and pharmacodynamics (PD) of Natpara, long-term treatment has been shown to maintain serum calcium levels within the low normal range, reduce urinary calcium levels, maintain renal function, reduce cardiovascular risk, and improve bone morphology.[34-36] While improvements may be expected from increasing the frequency of dosing to twice daily,[37] in 2022, Takeda announced that Natpara will not be available after 2024.

Here, we report on the modification of PTH(1-34) with vitamin D (D-VITylation) to generate compounds with extended plasma half-life.[38] Covalent attachment of vitamin D to therapeutic molecules prevents renal clearance by virtue of association with the abundant vitamin D binding protein (DBP). Characterizations of the PK/PD in healthy rats and cynomolgus monkeys are presented, as well as the pharmacodynamic actions in thyroparathyroidectomized (TPTx) rats.

Materials and methods

Synthesis of vitamin D/PTH(1-34) conjugates

EXT601: $VitD_3$-(C3)-PEG_{2k}-maleimide was made and coupled to PTH(1-34)-Cys-OH using methods previously described.[38]

EXT607 and EXT608: The scheme for preparing EXT607 and EXT608 is shown in Supplementary Figure S1. Compound 1 (VitD-NH_2) was synthesized using a published procedure.[38,39] Mal-PEG_{36}-TFP (2) (Mal = maleimide, TFP = 2,3,5,6-tetrafluorophenyl ester) was obtained from Quanta Biodesign, Ltd. (Plain City, OH, Cat# 10555). Compound 1 (1.2 equivalents) and the PEG linker 2 (1.0 equivalents) were each dissolved in NMP (1-methyl-2-pyrrolidone) at 10 mg/mL and combined to form Mal-PEG_{36}-VitD (3). The reaction was quenched by addition of 0.1 M ammonium acetate pH = 5 buffer and purified by preparative C18 HPLC with a water/acetonitrile gradient. Fractions containing Mal-PEG_{36}-VitD (3) were combined and compound 3 was quantitated by extinction coefficient (12 800 M^{-1} cm^{-1} at 280 nm in water). PTH(1-34)-cys-R (R = OH for EXT607 and R = NH_2 for EXT608) was dissolved in 0.5 M ammonium acetate pH = 6 buffer and added directly to the pooled HPLC fractions containing Mal-PEG_{36}-VitD (3). The reaction was purified by preparative C18 HPLC with a 0.1 M ammonium acetate pH = 5.5/acetonitrile gradient and lyophilized to dryness. EXT608 had a purity of 95% as determined by UPLC, and the identity was confirmed by positive ion mode ESI mass spectrometry (monoisotopic mass: predicted 6481.6 g/mol, measured 6481.6 g/mol). EXT608 for the 21-day and 89-day repeat dosing monkey studies was synthesized by Bachem Americas, Inc. (Torrance, CA) using the above methodology.

Agonist activity at PTHR1

The agonist activity on human parathyroid receptor 1 (PTHR1, also called PTH1R) of EXT601, EXT607, and EXT608 was compared with PTH(1-34) using either a cell-based calcium flux assay or a cAMP response assay. EXT601, EXT607, and EXT608 were diluted to 100 μM in phosphate buffered saline. Cells expressing human PTHR1 were treated with 10 concentrations of test article in duplicate or triplicate at a highest concentration of 1 μM.

EXT601 was submitted to Multispan Inc. (Hayward, CA) for testing on mammalian cell lines overexpressing PTHR1 (Cat# C1301). PTH(1-34) control agonist was provided by Multispan. The calcium assay was conducted using the Screen QuestTM Fluo-8 No Wash kit (AAT Bioquest, Cat# 36315) according to the manufacturer's protocols. The calcium dye loading buffer was added to the cells and incubated for 1 h at 37°C. Calcium flux was monitored for 120 s with compound injected into the wells at the 19th second. The cAMP assay was conducted using the HTRF cAMP HiRange Kit (CisBio, Cat# 62AM6PEC) according to the manufacturer's protocols. Compounds were incubated with cells for 20 min at 37°C.

The reaction was terminated by sequentially adding D2-labeled cAMP and cryptate-labeled anti-cAMP antibody in lysis buffer. The plate was incubated at room temperature for 60 min before reading fluorescent emissions at 620 and 668 nm with excitation at 314 nm. Results are expressed as the 668 nm:620 nm ratio.

EXT607 and EXT608 were submitted to DiscoverX (Fremont, CA, United States of America, now part of Eurofins Discovery) for testing on PathHunter mammalian cell lines expressing Gq-coupled PTHR1 (Catalog Number 86-0030P-2212AG). PTH(1-34) control agonist was provided by DiscoverX. Intracellular calcium release was measured on a FLIPR Tetra instrument for 2 min following exposure using a calcium-sensitive dye loaded into the cells. Percent efficacy of activation was calculated as follows:

$$\begin{aligned}\text{Percent efficacy} = 100\% \times &(\text{mean RFU of test sample} \\ &- \text{mean RFU of vehicle control}) / \\ &(\text{mean MAX RFU of control ligand} \\ &- \text{mean RFU of vehicle control}).\end{aligned}$$

EC50 (half maximal effective concentration) values were determined from plots of percent efficacy vs concentration of agonist using the Hill equation.

Pharmacokinetic and pharmacodynamic experiments

Ethics statement

All animal procedures and activities followed ARRIVE guidelines and were performed in AAALAC-accredited facilities under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Charles River Laboratory (Stilwell, KS, formerly Xenometrics/Citoxlab, and Senneville, QC). The numbers of animals used in the studies are considered the minimum required to achieve the study objectives based on regulatory requirements, statistical power, and/or availability of historical data and are within IACUC approved guidelines.

Single-dose PK

Solutions of EXT607 were prepared in PBS for dosing at 1 mL/kg for all groups. Male Sprague–Dawley rats weighing approximately 300 g and male cynomolgus monkeys weighing 3.5–4.5 kg were used with three animals per dosing group. Intravenous (IV) doses were administered via direct tail vein injection in rats and through an indwelling catheter in the cephalic vein in monkeys. Subcutaneous (SC) doses were administered in the dorsal region between the scapulae. Rats were fasted overnight and food was returned 4 h post-dose. Blood was collected from rats via a jugular vein cannula (0.25 mL) and from monkeys by a syringe and needle from the cephalic or saphenous vein (1.0 mL), transferred into tubes containing K_2EDTA, and kept on wet ice until processed for plasma. Blood samples were collected at 0 (predose), 0.083 (IV only), 0.25, 0.5, 1, 2, 4, 6, 12, 24, 36, 48, 60, and 72 h post-dose. Plasma samples were analyzed for EXT607 concentration by ELISA (High Sensitivity Human PTH(1-34) ELISA Kit, Cat# 60-3900, Quidel Corp., San Diego, CA). Pharmacokinetic parameters were determined by a non-compartmental analysis with uniform weighting using Phoenix™ WinNonlin™ Version 6.3 [Certara L.P. (Pharsight), St. Louis, MO]. The concentration vs time data were analyzed using the IV bolus or extravascular administration models. Best fit Lambda z ranges were used.

Rat steady state PK

Multi-dose pharmacokinetics and pharmacodynamics experiments were performed in rats with daily subcutaneous dosing of EXT608. Each dosing group consisted of 9 male and 9 female Sprague Dawley rats from Charles River Laboratories [Crl:CD(SD)IGS] and were divided into 3 cohorts in order to remain within blood volume sampling limits. Male and female animals were separately assigned to dose groups by a randomizing stratification system based on body weights. Animals were group housed in solid-bottom, polycarbonate cages with Teklad 7092 corncob bedding and equipped with an automatic watering system or water bottles. The room environment was set to 20–26°C, a relative humidity of $50 \pm 20\%$, and a light dark cycle of 12 h light/12 h dark. All rats were given free and continuous access to food (Purina Mills Certified Rodent Diet 5002) and tap water. In a 21-day experiment, EXT608 was formulated in 20 mM sodium acetate pH = 5.5 and 0.8% sodium chloride, and dosed at 70 and 14 μg/kg with a dose volume of 0.5 mL/kg. At the start of dosing, male rats were approximately 11 weeks old and weighed between 310 and 400 g, and females were approximately 14 weeks old and weighed between 240 and 290 g. In a 90-day experiment, EXT608 was formulated in 10 mM sodium acetate pH = 5.5, 0.8% sodium chloride, and 0.1% polysorbate 80, and dosed at 10, 3, and 1 μg/kg with a dose volume of 0.5 mL/kg. At the start of dosing, rats were approximately 9 weeks old and weighed between 300–395 g (males) and 200–270 g (females). On the last day of dosing, blood (0.5 mL) was collected at 0 (pre-dose), 0.5, 1, 3, 8, 24, 32, 48, and 72 h post-dose and processed into plasma for PK analysis as described above.

NHP steady state PK and PD

Multi-dose pharmacokinetics and pharmacodynamics experiments were performed in cynomolgus monkeys of Chinese origin (Covance Laboratories, Alice, TX) with every-other-day interscapular subcutaneous dosing of EXT607 and EXT608. Animals were individually housed in stainless-steel cages equipped with an automatic watering system. The room environment was set to 18–29 °C, a relative humidity of $50 \pm 20\%$, and a light dark cycle of 12 h light/12 h dark. A standard certified commercial chow Envigo Teklad Global 20% Protein Primate Diet 2050C in "biscuit" form was provided to the animals *ad libitum* with additional treats or fruits/vegetables provided as part of the animal enrichment program. All animals were randomly assigned to dose groups by weight stratification. In a 5-day experiment, EXT607 was formulated in PBS and 2 males and 2 females per group were dosed at 100, 30, and 10 μg/kg with a dose volume of 1 mL/kg. At the start of dosing, monkeys were approximately 2–4 years old and weighed between 2.4–2.9 kg. On days 1 and 5, blood (1.0 mL) was collected at 0 (predose), 0.5, 1, 3, 6, 12, 24, 48, and 72 h (last day only) postdose and processed into K_2EDTA plasma for PK analysis as described above. Concurrently at the same timepoints, blood (0.5 mL) was collected and processed into serum for measurement of total calcium, phosphate, and magnesium on an Advia 1800 Clinical Chemistry System (Siemens Healthineers, Erlangen, Germany). Urine was collected on days -3 and 8, and calcium

and phosphorous were measured on a CLINITEK Advantus Analyzer (Siemens Healthineers, Erlangen, Germany). In a 21-day experiment, EXT608 was formulated in 20 mM sodium acetate pH = 5.5 and 0.8% sodium chloride, and 2 males and 2 females per group were dosed at 20 and 7 μg/kg with a dose volume of 0.05 mL/kg. At the start of dosing, monkeys were 2.6–3.1 years old and weighed between 2.3 and 2.7 kg. Blood was collected on days 1, 11, and 21 for PK and PD analysis as described above. Urine was collected on days -5 and 24, and calcium, phosphorous, and creatinine were measured as above. In a 90-day experiment, EXT608 was formulated in 10 mM sodium acetate pH = 5.5, 0.8% sodium chloride, and 0.1% polysorbate 80, and 3 males and 3 females per group were dosed at 2 and 0.7 μg/kg with a dose volume of 0.05 mL/kg. At the start of dosing, monkeys were 2.6–3.9 years old and weighed between 2.0 and 2.6 kg. Blood was collected on days 1, 45, and 89 for PK and PD analysis as described above. Urine was collected on days -9 and 90, and calcium, phosphorous, and creatinine were measured as above.

7-day TPTx study

A 7-day study with female thyroparathyroidectomized (TPTx) Crl-CD Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC) was performed by Charles River Labs, Montreal, (Senneville, QC). Surgery to remove the thyroid and parathyroid glands was performed by Charles River Labs (Raleigh, NC) with all rats receiving 1% calcium supplement in water immediately following surgery. Upon arrival at the testing facility, the 1% calcium supplement was removed and all animals were provided *ad libitum* with standard house water and PMI Nutrition International Certified Rodent Chow No. 5CR4. Upon arrival, animals were housed individually in polycarbonate bins containing Alpha Dri SS1010 bedding, equipped with an automatic watering valve. Following removal of the surgical sutures (approximately at day 10 post-surgery), the animals were pair-housed. The room environment was set to 19–25 °C, a relative humidity of 50 ± 20%, and a light dark cycle of 12 h light/12 h dark. The day after arrival, blood was collected from all animals via jugular venipuncture including the spares and baseline level of total serum calcium and phosphorus were measured. Two days later, TPTx rats had a subcutaneous implantation of thyroxine pellets under anesthesia with 5% isoflurane. TPTx rats were randomized into the treatment groups (n = 12) based on baseline total serum calcium level and body weight. Following 9 days of acclimation after the baseline bleeds, animals weighing between 250 and 300 g were subcutaneously dosed daily for 7 days with vehicle or test items formulated in PBS (days 1–7) at 1 mL/kg. EXT601 was dosed at 3.0 and 0.3 nmol/kg, and PTH(1-84) and PTH(1-34) were dosed at 3 nmol/kg.

Blood collections were performed via jugular venipuncture and processed to serum. Treatment groups were split into 2 equal cohorts (A and B) to meet overall blood sampling IACUC limits. Blood (0.4 mL) was collected for total calcium and phosphate measurements according to the following schedule: day 1: 0 h (A + B), 2 h (A), 6 h (B), 10 h (A), 18 h (B), 24 h (A); day 4: 2 h (B); day 7: 0 h (A), 2 h (B). Blood was processed to serum and analyzed using a Hitachi 7180 Clinical Analyzer (Tokyo, Japan). Blood (0.3 mL) was collected for bone biomarker analysis on days -6, 4, and 7 at t = 0 (predose) and processed to serum. Bone markers were quantitated using the following ELISA kits from Immunodiagnostic Systems (East Bolden, United Kingdom): Rat-MidTM Osteocalcin EIA (Catalog # AC-12F1), Rat/Mouse PINP EIA (Catalog # AC-33F1), and Serum Crosslaps (CTX-I) ELISA (Catalog # AC-02F1).

28-day TPTx study

TPTx rats were prepared as described above. Animals weighing between 215 and 290 g were subcutaneously dosed daily with vehicle or test items formulated in PBS for 28 days at 1 mL/kg. EXT607 was dosed at 10, 3, and 1 nmol/kg, and PTH(1-84) and PTH(1-34) were dosed at 10 nmol/kg. Blood collections were performed via jugular venipuncture and processed to serum. Treatment groups were split into 2 equal cohorts (A and B) to meet overall blood sampling limits. Blood (0.2 mL) was collected for total calcium and phosphate measurements according to the following schedule: day 1: 0 h (A + B), 2 h (A), 6 h (B), 10 h (A), 24 h (B); day 12: 0 h (B), 2 h (A), 6 h (B), 10 h (A), 24 h (B); day 27: 0 h (B), 2 h (A), 6 h (B), 10 h (A), 24 h (A + B). Blood was processed to serum and analyzed using a Hitachi 7180 Clinical Analyzer. Blood (0.5 mL) was collected for bone biomarker analysis on days 2, 6, 11, and 28 at t = 0 (predose), processed to serum, and analyzed as for the 7-day TPTx experiment. Urine samples were collected from individual animals placed in metabolic cages overnight. Samples were collected on days 1, 14, and 27, from 1 to 8 and 8 to 24 h. Peripheral QCT scans were performed using an XCT Research SA+ bone scanner. *Ex vivo* scans were obtained of the right femur for animals euthanized at the end of the treatment period. One slice was obtained in the right proximal femur metaphysis and another one in the diaphysis. Placement of the metaphysis and diaphysis slices were 19% and 50%, respectively, of the total bone length proximal to the reference line set at the distal end.

Results

Compound design and PTH receptor agonist activity

Previously we have reported the intravenous and subcutaneous pharmacokinetics in rat of PTH(1-34) with vitamin D conjugated to a cysteine added at the N-terminus (VitD-PEG_{2kDa}-cys-PTH(1-34)).[38] However, modification of the N-terminus of PTH reduces activity at the Parathyroid Hormone Receptor 1 (PTHR1) as evidenced by a 7-fold increase in the EC50 value. Here, we report on 3 related compounds where the location of the cysteine residue for conjugation on each compound is positioned at the C-terminus of PTH; the 3 compounds differ with regard to the PEG linker and the C-terminus peptide functionality (Figure 1). EXT608 was chosen as the lead compound because the discrete PEG_{36} linker (1.6 kDa) allows for ease of analytical characterization (eg, mass spectrometry and UPLC impurity analysis) as compared with the polydisperse 2 kDa PEG linker, and the C-terminal amide provides for optimization of solid phase peptide synthesis yielding fewer side products as compared with a C-terminal carboxylic acid. Modification of PTH(1-34) at the C-terminus does not significantly interfere with EXT601 activity in vitro at the parathyroid hormone receptor (PTHR1) mediated either by Gq signaling, as measured by intracellular calcium release, or Gs signaling, as measured by cAMP release (Table 1). The subtle changes in PEG-length and

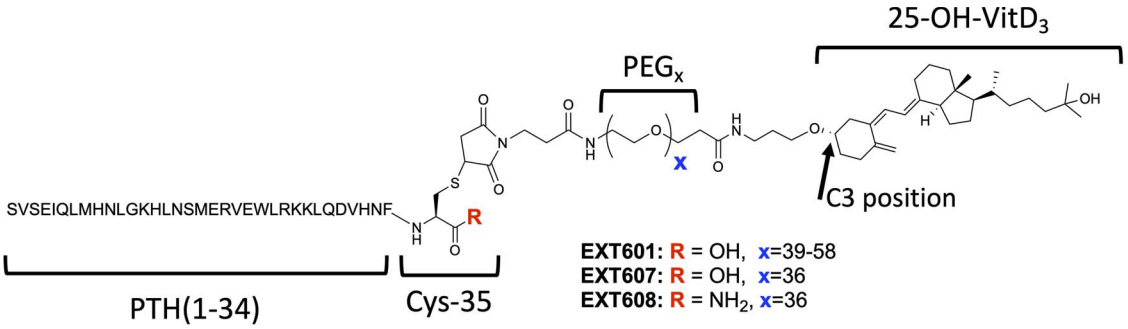

Figure 1. Structure of PTH-PEG-VitD conjugates.

Table 1. Calcium and cAMP responses of EXT601, EXT607, and EXT608 vs PTH(1-34) in mammalian cells overexpressing human PTHR1; mean (95% confidence interval).

Compound	Calcium			cAMP		
	n	EC50 nM (CI)[a]	E_{max} % (CI)[a]	*n*	EC50 pM (CI)[a]	E_{max} % (CI)[a]
PTH(1-34)	3	19.3 (15.9–22.7)	100.9 (99.3–102.6)	2	13.3 (13.0–13.6)	101.5 (101.5–101.5)
EXT601	3	13.5 (9.5–17.4)	98.0 (96.9–99.1)	2	18.8 (16.7–20.8)	100.8 (100.5–101.2)
PTH(1-34)	4	32.8 (30.3–35.3)	100.8 (98.2–103.4)			
EXT607	4	23.7 (21.1–26.3)	93.8 (89.9–97.8)			
EXT608	4	27.3 (20.7–33.8)	93.8 (89.3–98.3)			

[a]Mean (95% confidence interval)

C-terminal functionality introduced into compounds EXT607 and EXT608, as expected, did not result in substantial changes in calcium signaling. The nominal differences in measured EC50 values across the 3 compounds could be caused in part by the fact that the assays were run by different vendors, as noted by the EC50 differences of the PTH(1-34) agonist control. The compounds have not lost potency when compared with PTH(1-34) in the respective assays, which is expected because the PTH(1-34) moiety is the same amongst the 3 compounds. Emax values indicate that the modified compounds are similar to each other but might have slightly lower maximal Gq signaling activity (calcium release) as compared with PTH(1-34). Based on the similar PTHR1 activity and pharmacokinetics (see Supplementary Figure S2) of EXT601, EXT607, and EXT608, they will be considered functionally equivalent for the following results and discussion.

Rat single-dose PK

Male rats were dosed with EXT607 intravenously (IV) at 100 μg/kg or subcutaneously (SC) at 30, 100, and 300 μg/kg, and plasma EXT607 concentrations as a function of time were determined (Figure 2A). Pharmacokinetic parameters are reported in Supplementary Table S1. Subcutaneous bioavailability based on AUC ranged from 10% to 13% and the $t_{1/2}$ of elimination during the terminal phase ranged from 7 to 15 h. AUC and C_{max} increased in nearly a dose-proportional manner (1:2.8:7.5) and (1:3.1:7.8), respectively, vs (1:3.3:10) theoretical.

Monkey single-dose PK

Male cynomolgus monkeys were dosed with EXT607 intravenously (IV) at 20 μg/kg or subcutaneously (SC) at 7, 20, and 70 μg/kg, and plasma EXT607 concentrations as a function of time were determined (Figure 2B). Pharmacokinetic parameters are reported in Supplementary Table S2. Subcutaneous bioavailability based on AUC ranged from 45% to 54% and the $t_{1/2}$ of elimination during the terminal phase ranged from 24 to 32 h. While AUC increased in nearly a dose-proportional manner (1:2.9:12) vs (1:2.9:10) theoretical, C_{max} increased in a greater than dose-proportional manner (1:5.7:36).

Rat steady state PK

Male and female rats were subcutaneously administered EXT608 with daily dosing. In 2 separate experiments, rats were dosed daily either for 21 days at 14.2 and 70 μg/kg, or for 89 days at 1, 3, and 10 μg/kg. Plasma was collected beginning at $t = 0$ on the last day of dosing and EXT608 concentrations were determined (Figure 3A). No significant gender differences in systemic exposure were observed, so combined averages of male and female are reported. Pharmacokinetic parameters are reported in Table 2. Elimination $t_{1/2}$ values ranged from 10 to 14 h and are consistent with the single dose PK values (*vide infra*). While AUC dose-normalized values are consistent across the dose range, C_{max} values are greater than expected at high doses. The result is a more prominent peak of plasma EXT608 at high doses, and in contrast, a flatter PK profile at low doses. This is quantitatively expressed by a C_{max}:C_{min} (0–24 h) ratio of 30 at the high dose vs 1.8 at the lowest doses. The accumulation ratio as measured by either C_{max} or $AUC_{0-24\,h}$ was approximately 3-fold compared with day 1 values (Supplementary Table S3) except for the 70 μg/kg dose where it was 4.2 ($AUC_{0-24\,h}$) or 5.2 (C_{max}).

Monkey steady state PK

Male and female cynomolgus monkeys were subcutaneously administered EXT608 with every other day dosing. In 2 separate experiments, monkeys were dosed either for 21 days at 7 and 20 μg/kg, or for 89 days at 0.7 and 2 μg/kg. Plasma was collected beginning at $t = 0$ on the last day of dosing and EXT608 concentrations were determined using a commercial human PTH(1-34) ELISA kit (Figure 3B). No significant gender differences in systemic exposure were observed, so

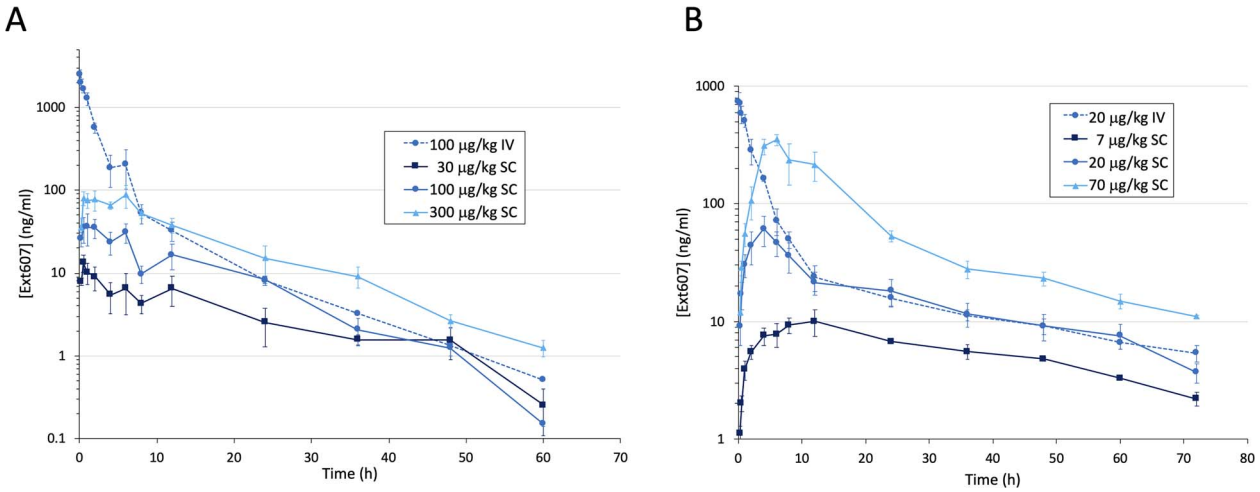

Figure 2. Rat (A) and monkey (B) single-dose PK of intravenous (IV) or subcutaneous (SC) EXT607. Plasma EXT607 concentrations were determined using a commercial human PTH(1-34) ELISA kit. Error bars indicate standard error of the mean ($n = 3$ animals/group).

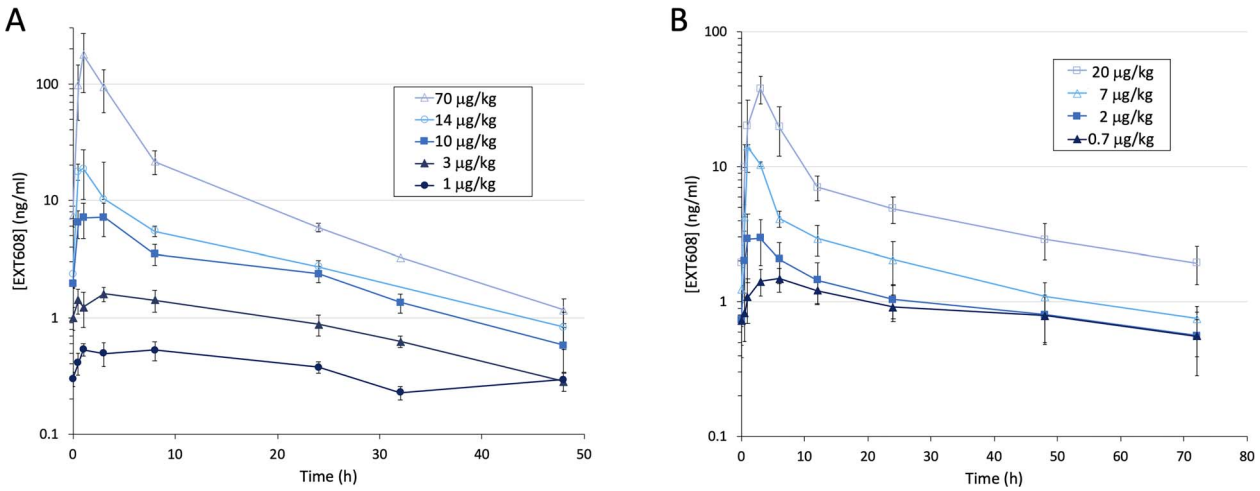

Figure 3. Rat (A) and monkey (B) steady state subcutaneous PK. Pharmacokinetics of EXT608 as measured following the last dose administration after either 21 days (open symbols) or 89 days (closed symbols). Rats were dosed daily and monkeys every other day. Plasma EXT608 concentrations were determined using a commercial human PTH(1-34) ELISA kit. Error bars indicate standard error of the mean (rats: $n = 6$ animals/group; monkeys: $n = 4$ (20 and 7 μg/kg), $n = 10$ (2 μg/kg), $n = 6$ (0.7 μg/kg)).

Table 2. Steady state PK parameters of EXT608 in rats following repeated daily SC dosing.

PK parameter	Dose				
	70 μg/kg	14.2 μg/kg	10 μg/kg	3 μg/kg	1 μg/kg
Days of dosing	21	21	89	89	89
$t_{1/2}$ (h)	11.7	13.8	10.4	12.5	11.1
T_{max} (h)	1	0.75	3	3	1
AUC_{last} (h·ng/mL)	972	204	133	42.7	14.5
Dose-normalized AUC	0.96	0.99	0.92	0.98	1.00
C_{max} (ng/mL)	177	18.8	7.16	1.60	0.534
Dose-normalized C_{max}	4.74	2.48	1.34	0.99	1.00
C_{min} (ng/mL)	5.92	2.35	1.56	0.875	0.299
C_{max}/C_{min} (0–24 h)	29.9	8.00	4.59	1.83	1.79

$t_{1/2}$, terminal half-life; T_{max}, time of maximum observed concentration; AUC_{last}, area under the concentration-time curve from time = 0 to the last measured concentration; C_{max}, maximum observed concentration; C_{min}: minimum observed concentration from time = 0 to 24 h; C_{max}/C_{min} (0–24 h): ratio of C_{max}/C_{min}.

combined averages of male and female are reported. Pharmacokinetic parameters are reported in Table 3. Elimination $t_{1/2}$ values ranged from 35 to 51 h and trended toward longer half-lives with decreasing dose. Steady-state $t_{1/2}$ values are longer than observed after a single dose (24–32 h), although the longer values were determined from fits to data points spanning only 1 to 2 half-lives and should be interpreted cautiously. Like the rat steady state PK, the monkey PK

Table 3. Steady state PK parameters of EXT608 in monkeys following repeated every-other-day SC dosing.

PK parameter	Dose			
	20 μg/kg	7 μg/kg	2 μg/kg	0.7 μg/kg
Duration of dosing (days)	21	21	89	89
$t_{1/2}$ (h)	35.9	34.7	51.0	45.0
T_{max} (h)	3.0	1.0	NE	6
AUC_{last} (h·ng/mL)	460	170	79.5	61.6
Dose-normalized AUC	0.26	0.28	0.45	1.00
C_{max} (ng/mL)	38.1	14.1	3.41	1.43
Dose-normalized C_{max}	0.93	0.99	0.84	1.00
C_{min} (ng/mL)	1.97	0.991	0.635	0.600
C_{max}/C_{min} (0–48 h)	19.3	14.2	5.37	2.38

NE, not estimated; C_{min}, minimum observed concentration from time = 0 to 48 h; C_{max}/C_{min} (0–48 h), ratio of C_{max}/C_{min}.

profiles are flatter at low doses with the C_{max}:C_{min} (0–48 h) ratios ranging from 19 at the high dose and 2.4 at the lowest dose. However, unlike the rat data, the C_{max} dose-normalized values are consistent across the dose range while AUC values are less than expected at high doses. With the exception of the 20 μg/kg dose, a small amount (1.5 to 2.0-fold) of accumulation is observed upon repeated every other day dosing of EXT608 (Supplementary Table S4).

TPTx rat 7-day efficacy study

Thyroparathyroidectomized (TPTx) rats were used as a model of hypoparathyroidism.[40] Rats received daily subcutaneous injections of EXT601 at 3 nmol/kg (19 μg/kg) or 0.3 nmol/kg (1.9 μg/kg) for a total of 7 days of treatment. As a comparison, PTH(1-34) and PTH(1-84) were also tested at 3 nmol/kg. Plasma was collected on day 1 after the first dose and tested for total calcium and phosphate levels. Animals undergoing TPTx surgery displayed significantly lower serum calcium levels which was reversed by the high dose (3 nmol/kg) of EXT601, with calcium levels reaching the normal range by 24 h (Figure 4A). In contrast, serum calcium levels were unaffected by 3 nmol/kg PTH(1-34) and PTH(1-84), or by the low dose of EXT601 (0.3 nmol/kg). Plasma collected on day 4 (2 h) and day 7 (0 and 2 h) confirmed that 3 nmol/kg EXT601 maintained calcium levels significantly higher than the TPTx: vehicle group, in contrast to all other treatment groups (Supplementary Table S5). Serum phosphate levels were significantly higher in all TPTx groups, but were not changed within the first 24 h for any treatment group. Plasma collected at all three timepoints across days 4 and 7 indicate that 3 nmol/kg EXT601 consistently and significantly lowers serum phosphate concentrations, while 3 nmol/kg PTH(1-34) and PTH(1-84) only transiently lower serum phosphate at the 2 h timepoints but not at the day 6 24 h timepoint (Figure 4B). Finally, serum samples from days 1, 4, and 7 were analyzed for biomarkers of bone formation (osteocalcin and N-terminal propeptide of type I procollagen (PINP)) and bone resorption (C-terminal telopeptides of type I collagen (CTX)).[41-43] All markers have lower levels in TPTX animals than normal animals, indicative of the lower degree of bone remodeling that occurs with hypoparathyroidism. For the markers of bone formation, osteocalcin and PINP, EXT601 at 3 nmol/kg restored biomarker levels to greater than normal, while PTH(1-84) and PTH(1-34) only induced a small increase that either reached or nearly reached statistical significance, as indicated (Figure 4C). For the CTX marker of bone resorption, the high dose of EXT601 increases levels to near wild-type, whereas no change is observed for the other treatment groups.

TPTx rat 28-day efficacy study

An extended 28-day TPTx rat study with daily dosing was undertaken to determine the long-term ability of EXT607 to reverse the effects of parathyroid removal. The doses of EXT607 were adjusted to 10, 3, and 1 nmol/kg (65, 19, and 6.5 μg/kg) and compared with 10 nmol/kg PTH(1-84) and PTH(1-34). Serum calcium and phosphate levels were determined on days 1, 12, and 27 at $t = 0$ (pre-dose), 2, 6, 10, and 24 h. EXT607 caused dose-dependent increases in serum calcium compared with TPTx controls receiving vehicle with the 10 nmol/kg dose maintaining calcium levels primarily within the normal range of healthy animals, the 3 nmol/kg dose reaching values at or just below the normal range, and the 1 nmol/kg dose causing a small increase that reached statistical significance at various timepoints (Figure 5A). 10 nmol/kg PTH(1-84) and PTH(1-34) caused no increase or only small increases in serum calcium, which reached occasional significance (Supplementary Figure S3A). EXT607 also caused a dose-dependent sustained decrease in serum phosphate on days 12 and 27 with the highest dose reaching levels observed in the sham surgery control group (Figure 5B). PTH(1-84) and PTH(1-34) caused a much smaller decrease in serum phosphate only reaching significance occasionally (Supplementary Figure S3B). Urine was collected between $t = 0$–8 h and 8–24 h post-injection on days 1, 14, and 27 and calcium, phosphate, and creatinine levels were determined. Creatine-normalized calcium was increased for the high dose of EXT607 in the 8–24 h time period on days 1 and 14, but had normalized by day 27 (Figure 5C). Urinary calcium was not significantly increased for the mid and low dose of EXT607 or for PTH(1-84) and PTH(1-34). Urinary phosphate did not show a consistent trend for any of the groups, although it tended to be higher for the 10 nmol/kg dose of EXT607 (Figure 5D).

In contrast to the 7-day TPTx experiment, TPTx animals did not have reduced levels of bone remodeling markers compared with healthy animals; the sham-vehicle group had either lower or similar levels of these markers compared with the TPTx animals (Supplementary Figure S4). Treatment with EXT607 caused a dose-dependent increase in bone remodeling markers that reached statistical significance by day 2 for CTX, day 6 for PINP, and day 11 for osteocalcin. PTH(1-84) and PTH(1-34) also increased serum concentrations of osteocalcin and CTX, but had only minimal effect on PINP.

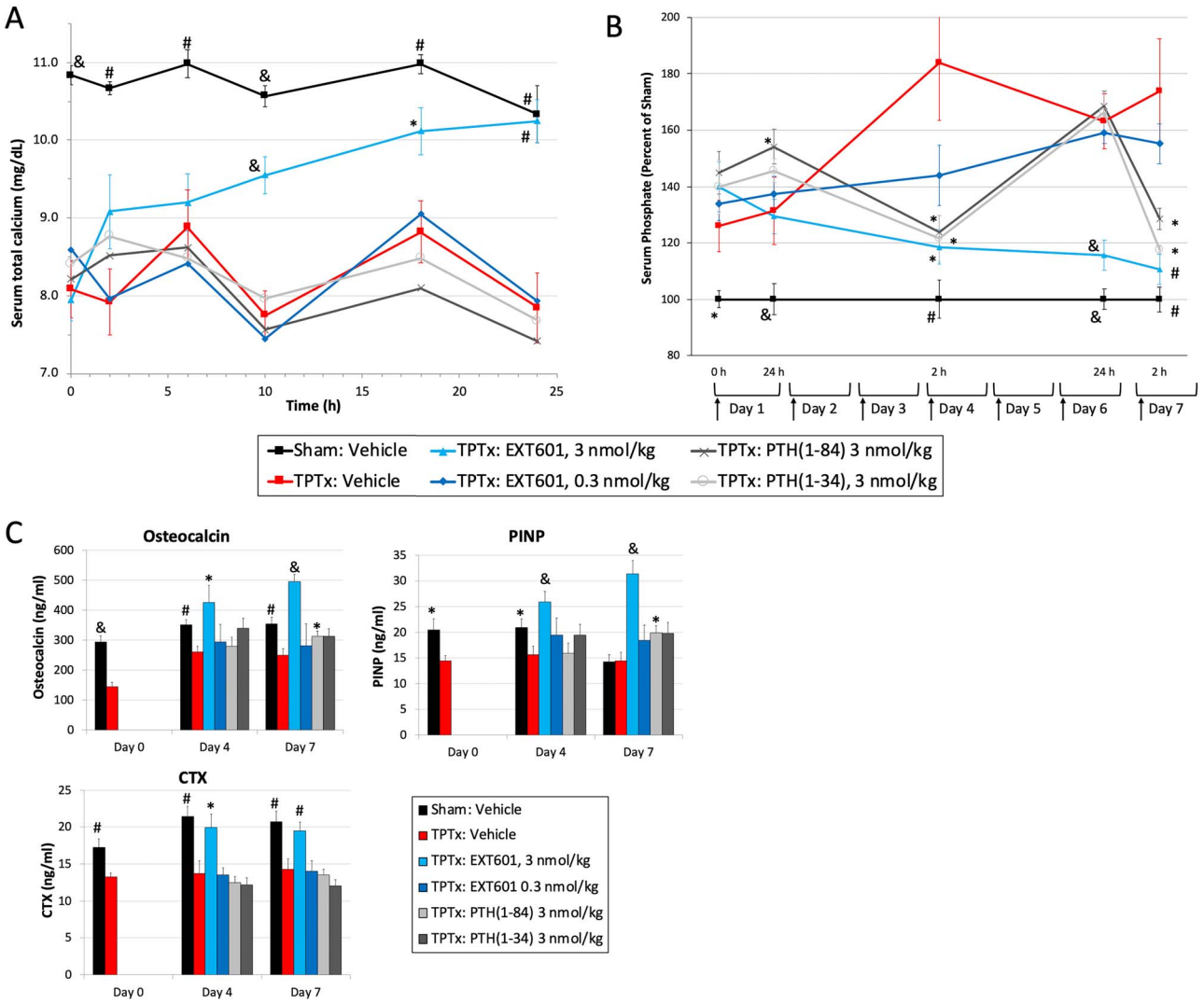

Figure 4. Effect of subcutaneous delivery of PTH compounds on serum calcium (A), phosphate (B), and bone biomarkers (C) in TPTx rats. A: Total serum calcium levels for the 24 h period after day 1 dosing. For clarity, error bars are shown only for the Sham: Vehicle, TPTx: Vehicle, and TPTx: EXT601 3 nmol/kg groups, and indicate the standard error of the mean ($n = 6$ animals/cohort). B: Serum phosphate levels throughout the 7-day dosing period. Error bars indicate the standard error of the mean ($n = 6$ animals/cohort). C: Concentration of serum bone remodeling biomarkers as measured on day 0 (pretreatment), day 4, and day 7. Error bars indicate the standard error of the mean ($n = 12$ animals/group). (A-C): *P*-values vs the TPTx: Vehicle group were calculated using a two-tailed, unpaired *t*-test (∗ < .05, # < .01, & < .001).

Bone densitometry was studied using peripheral quantitative computed tomography (pQCT) at the end of the treatment period. Measurements of bone mineral density (BMD) were made in the distal metaphysis and diaphysis regions of the femur (Figure 6). In the distal metaphysis, compared with the sham surgery group, animals in the TPTx: vehicle group had a statistically significant increase in trabecular BMD and small increases in total and subcortical/cortical BMD, which did not reach statistical significance (Figure 6A-C). Treatment with 10 nmol/kg PTH(1-84) or PTH(1-34) led to BMD values that were unchanged or slightly increased, but were not statistically different from the TPTx: vehicle group. In contrast, treatment with EXT607 led to decreases in BMD with the mid and low dose groups but not the high dose group; decreases reached statistical significance for total and trabecular BMD. In the cortical region of the diaphysis, the TPTx: vehicle group BMD was not changed from the Sham: vehicle group; treatment with 10 nmol/kg EXT607 or PTH(1-84) caused a small but statistically significant decrease in BMD (Figure 6D). Similar trends were observed for bone mineral content measurements (Supplementary Figure S5). Treatment with EXT607 caused a small, dose-dependent decrease in metaphysis area, which reached statistical significance for the high dose group (Supplementary Figure S6A). Similar trends were observed in the diaphysis for total area, cortical area, cortical thickness, endosteal circumference, and periosteal circumference (Supplementary Figure S6B-F).

5-day repeat dose monkey study

Cynomolgus monkeys (2 males and 2 females per group) were dosed subcutaneously with EXT607 at 0, 10, 30, or 100 μg/kg every other day on days 1, 3, and 5. After the day 1 and day 5 injections, serum was collected at various timepoints and analyzed for calcium levels (Figure 7A). No gender-specific differences were observed, so data are reported as a combined average of males and females. After the day 1 injection a dose-dependent increase in serum calcium was observed, which by day 5 had increased such that all EXT607

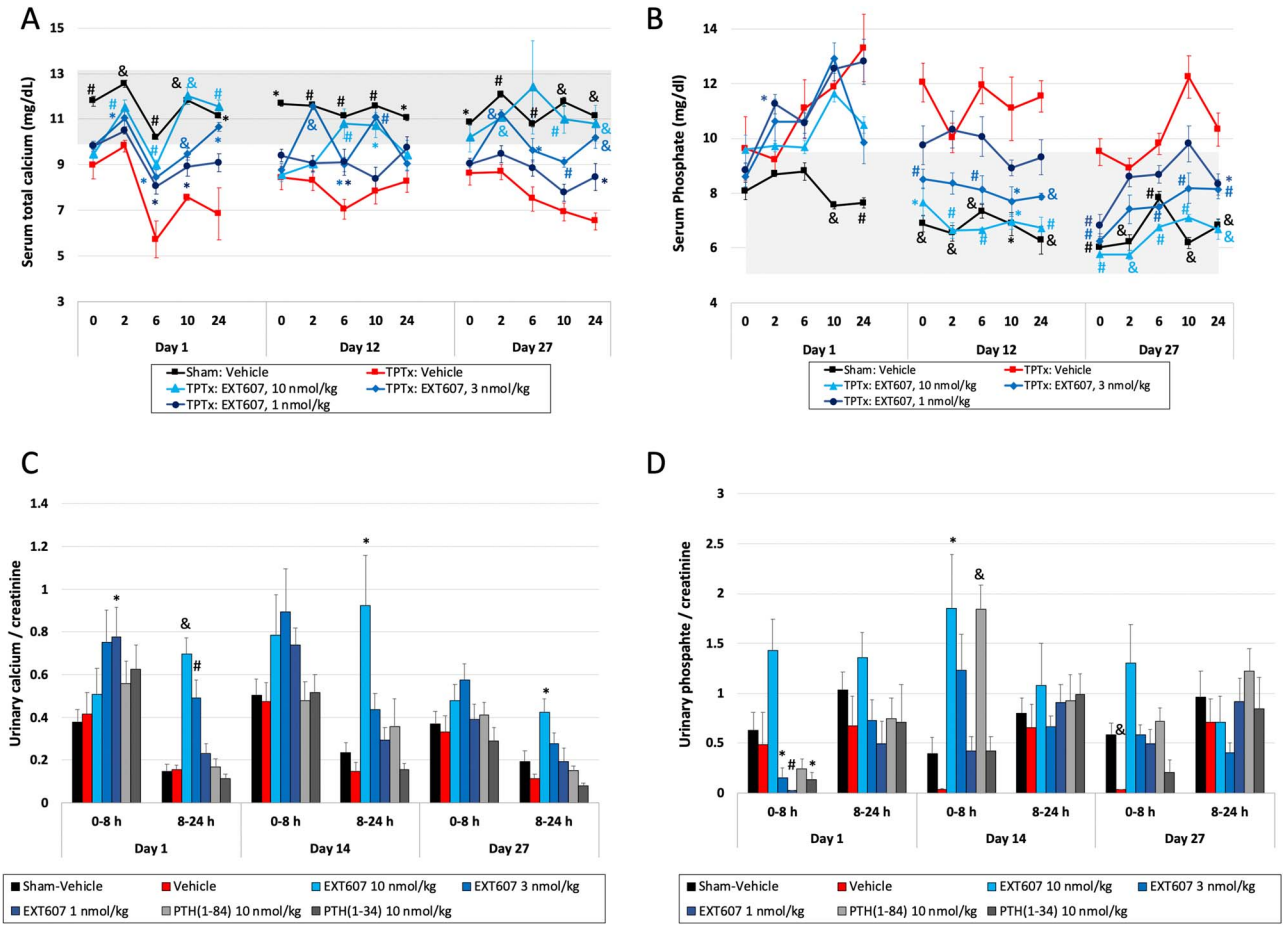

Figure 5. Effect of subcutaneous delivery of PTH compounds for 28 days in TPTx rats. Day 1, 14, and 27 measurements of serum calcium (A), serum phosphate (B), urinary calcium (C), and urinary phosphate (D). Error bars indicate the standard error of the mean ($n = 5$ animals/cohort for serum measurements and $n = 10$ animals/group for urine measurements). Normal serum calcium and phosphate levels, as determined by the observed range of measurements for the Sham: Vehicle group, are shaded gray. *P*-values vs the TPTx: vehicle group were calculated using a two-tailed, unpaired *t*-test (∗ < 0.05, # < 0.01, & < 0.001).

doses raise serum calcium levels above the normal range, although statistical significance was generally not reached due to the high variability. In contrast, no differences in urinary calcium were observed between predose day 1 samples and terminal samples collected 72 h after the day 5 injection when serum calcium levels are still elevated in the high dose group (Figure 7B and Table 4). No effect on serum phosphate was observed.

21-day repeat dose monkey study

A longer repeat dose monkey study was undertaken with EXT608 dosed every other day for a duration of 21 days at 0, 1.4, 7, and 20 μg/kg. Serum was collected at various timepoints after the day 1, 11, and 21 injections and analyzed for calcium and phosphate levels (Figure 8A and B). Calcium was significantly increased beginning on day 1, and by day 11 serum calcium remains elevated for 24 h at the 20 μg/kg dose and for 12 h at the lower doses. Serum phosphate is unaffected on day 1, but is decreased at the high EXT608 dose by day 11, and showed a dose-dependent decrease on day 21 with the 20 μg/kg dose achieving statistical significance at most timepoints throughout the 72 h monitoring period. Urinary calcium and phosphate measured at the completion of the experiment (day 21, 72 h) were unchanged from day 1 predose levels. Levels of the pre-hormonal form of vitamin D, (25-OH-VitD) are unchanged, while the active form (1,25-OH-VitD) was increased at the end of the study (day 24), reaching significance for mid and high doses (Figure 8C).

Discussion

We have synthesized and characterized PTH(1-34)-vitamin D conjugates with the aim of generating a clinical treatment for hypoparathyroidism that could maintain normal serum calcium levels for an extended period of time without increasing urinary calcium levels, thereby offering the potential to increase the efficacy, safety, and quality of life. D-VITylation prolongs the circulation time without the need to attach a large moiety such as PEG, which can significantly reduce bioactivity[44,45] and potentially illicit an immune response.[46,47] By selecting the C-terminus of PTH(1-34) as the site of vitamin D conjugation, we obtained compounds that retained full activity at the receptor (PTHR1). Because the vitamin D used is a non-active form, EXT608 has proven to be well-tolerated in animal toxicology studies, with the toxicity driven entirely by the natural pharmacological action of PTH at supraphysiological levels, resulting in pathology from elevated serum calcium levels (Figure 8).

D-VITylation of PTH(1-34) dramatically extends the plasma half-life; the half-life of PTH(1-34), which in rats is

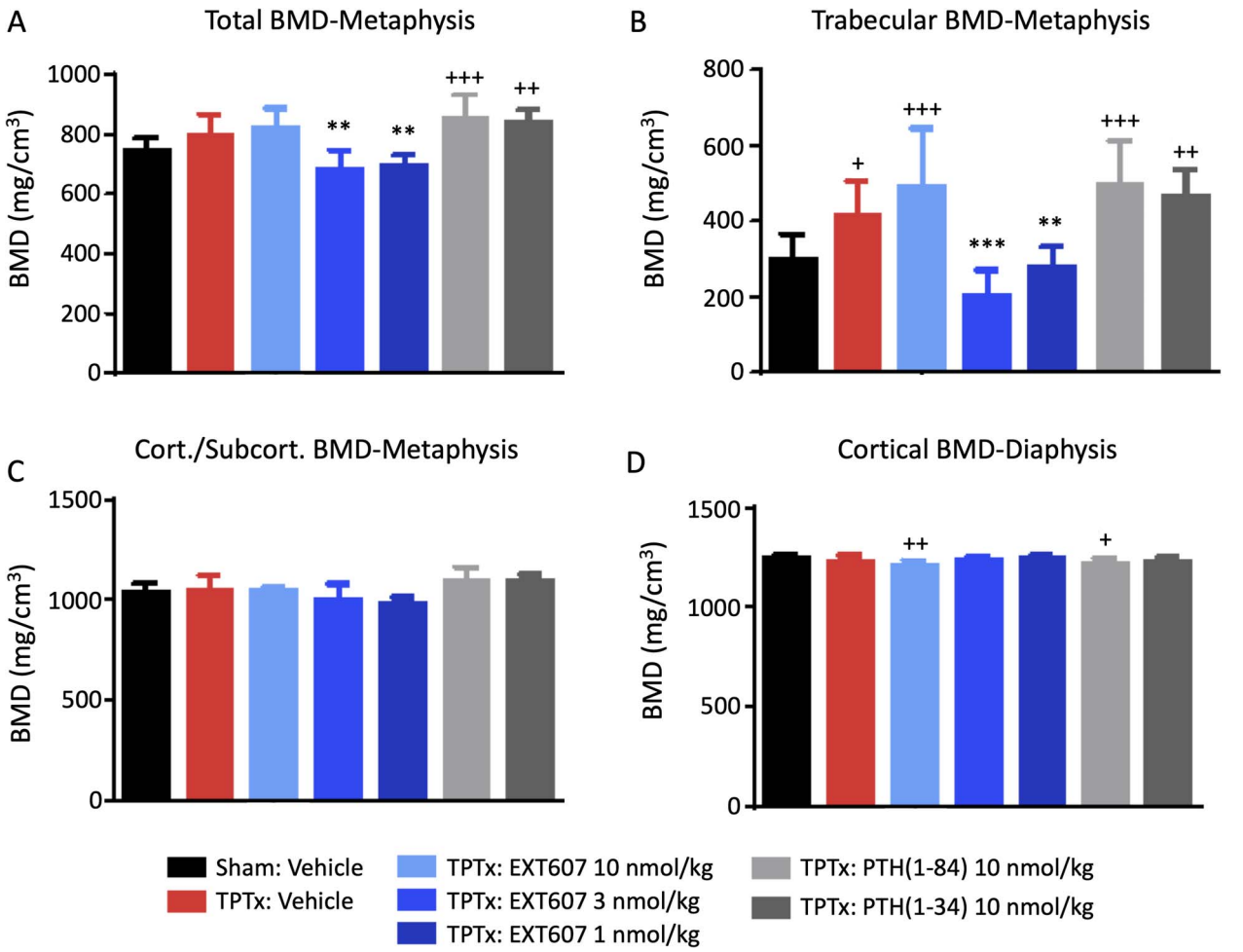

Figure 6. Bone mineral density (BMD) of TPTx rats in the distal femur metaphysis (A-C) and diaphysis (D) as measured by pQCT. Error bars indicate the standard deviation ($n = 10$ animals/group). Statistical analysis was performed using Dunnett's test (P-values: +<.05, ++<.01, +++<.001 vs Sham: Vehicle group; ∗<.05, ∗∗<.01, ∗∗∗<.001 vs TPTx: Vehicle group).

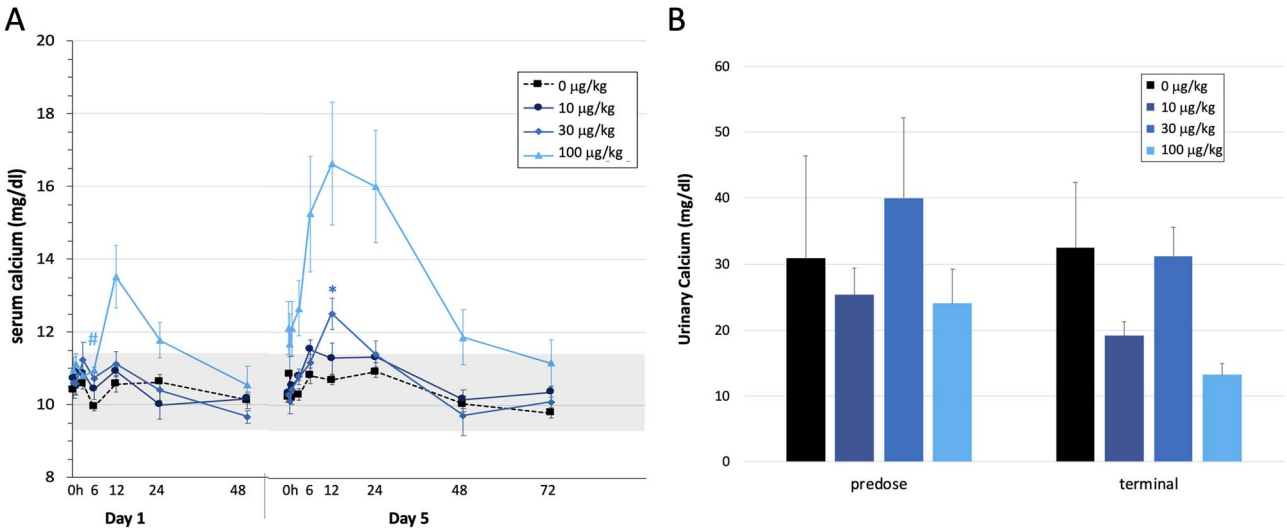

Figure 7. Effect of EXT607 on serum (A) and urinary (B) calcium in male and female cynomolgus monkeys. EXT607 was dosed subcutaneously at the indicated doses on days 1, 3, and 5 ($n = 4$, 2 males +2 females /group, mean ± SE). Normal serum calcium values are indicated by the gray area in (A) and represent the range 9.3–11.3 mg/dL as determined from the range of measured values for the 0 μg/kg group. P-values vs the 0 μg/kg group were calculated using a two-tailed, unpaired t-test (∗<.05, #<.01, and <.001).

Table 4. Relationship between serum and urinary calcium as a function of EXT607 dose.

Day	Dose	(mg/dL) Urinary Ca	(mg/dL) Serum Ca	Urinary:Serum Ca Ratio
Day -3[a]	pre-dose	30.1	9.8	3.1
Day 8[b]	0 μg/kg	32.5	9.8	3.3
Day 8	10 μg/kg	19.1	10.4	1.8
Day 8	30 μg/kg	31.2	10.1	3.1
Day 8	100 μg/kg	13.2	11.2	1.2

[a]Day -3 measurements are an average of pre-dose animals from all dosing groups. [b]Day 8 measurements were taken at the conclusion of the study, 72 h after the last dosing on day 5, but when serum calcium levels were still elevated (see Figure 7A).

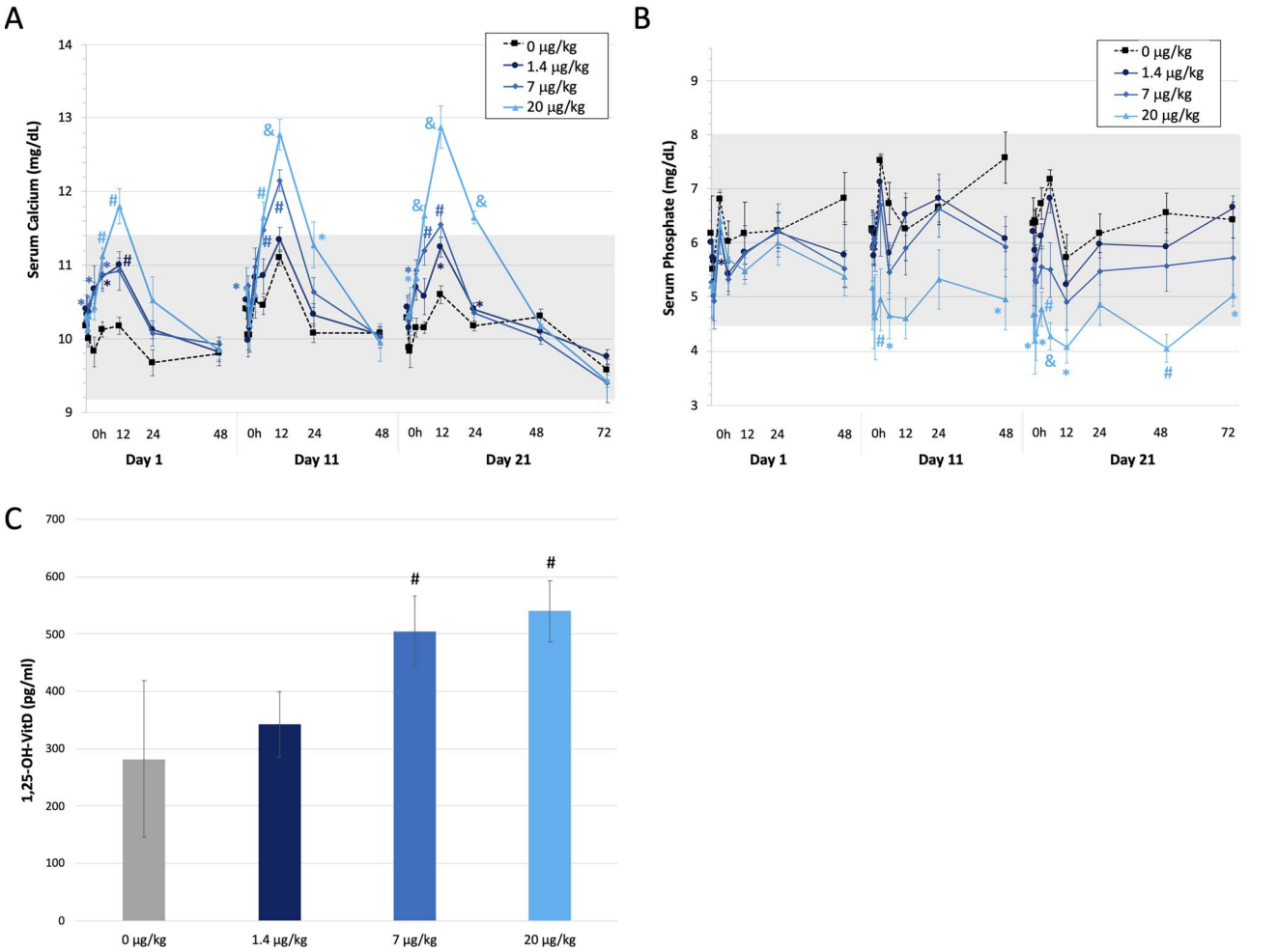

Figure 8. Effect of EXT608 on serum calcium (A), phosphate (B), and 1,25-(OH)-VitD (C) in male and female cynomolgus monkeys. EXT608 was dosed subcutaneously at the indicated doses every other day. (A-B): Serum was analyzed following dosing on days 1, 11, and 21 ($n = 4$, 2 males +2 females/group, mean ± SE). Areas shaded gray indicate the normal serum values for calcium (9.2–11.4 mg/dL) and phosphate (4.5–8.0 mg/dL) and were determined from the range of measured values for the 0 μg/kg group. (C): Serum was analyzed for 1,25-(OH)-VitD on day 24 ($n = 4$, mean ± SE). (A-C): *P*-values vs the 0 μg/kg group were calculated using a two-tailed, unpaired *t*-test (∗ < 0.05, # < 0.01, & < 0.001).

less than 15 min,[48,49] is increased by D-VITylation to 7–15 h in rats and 24–32 h in cynomolgus monkeys. Because the rate of renal clearance is allometrically related to body weight, the half-life would be expected to increase further in humans. An unexpected observation was the flatter subcutaneous PK profiles at low doses as indicated by the peak:trough (C_{max}:C_{min}) ratios (Tables 2 and 3). The change in PK profile as a function of dose is best modeled by a slower rate of EXT608 absorption at low doses. A possible explanation would be that at low doses, a higher percentage of EXT608 is bound to DBP in the subcutaneous space, which could affect the route and rate of uptake into the plasma. For example, the lymphatic system is expected to play a more important role for uptake of the larger DBP/EXT608 complex from the subcutaneous space, while free EXT608 would be expected to be transported directly into the blood.[50] We have previously observed a faster rate of absorption into plasma with VitD-peptide conjugates that have a lower affinity for DBP as compared with higher affinity conjugates.[38]

TPTx rats, in which the parathyroid glands are surgically removed, were used as a model to determine the ability of PTH-VitD conjugates to replace the functions of endogenous

PTH. First, a shorter dose range finding experiment was performed, with 7 days of daily EXT601 dosing. The highest dose of EXT601 (3 nmol/kg or 19 μg/kg) caused a rapid increase in serum calcium that persisted for 24 h. Bone turnover markers were significantly reduced by TPTx surgery, and the high dose of EXT601 restored the markers to wild-type levels or higher as assayed on days 4 and 7. Based on these findings, the daily doses were adjusted to 10, 3, and 1 nmol/kg for a 28-day TPTx study, with serum measurements performed on days 1, 12, and 27. While the serum calcium values showed some fluctuation, possibly caused by alternating measurements being performed on separate cohorts of animals, a dose-dependent increase in serum calcium was observed, with the increase fairly uniform across the 24 h measurement period on days 12 and 27. The effect on serum phosphate was delayed, with no effect on day 1 and a dose-dependent sustained decrease into the normal range on days 12 and 27. Importantly, while urinary calcium is initially increased for the high dose, by day 27 it returned to normal or below normal levels despite the higher serum levels of calcium (Figure 5A and C).

pQCT was used to study the effect of EXT607 on bone remodeling. In patients, hypoparathyroidism causes a reduction in bone remodeling leading to an increase in bone density.[13] The TPTx rat model replicates this effect as evidenced by small increases in BMD and area compared with the control healthy rats. EXT607 is largely able to reverse these effects in a dose dependent manner. The largest effect with EXT607 was observed in trabecular bone. However, the reduction in BMD did not occur in a dose-dependent manner, with the highest dose unchanged from the TPTx:vehicle group. Bone turnover marker analysis did not offer an explanation, as a dose-dependent increase was observed for all markers. However, it should be noted that for the 28-day study, TPTx surgery did not result in a decrease in markers as would be expected and was observed in the 7-day study. Both PTH(1-34) and PTH(1-84) were ineffective at reversing the effects of hypoparathyroidism in TPTx rats, a result also observed by others.[40,51] While Natpara (PTH(1-84)) is used clinically to treat hypoparathyroidism, the longer half-life observed in humans (1.5–2.5 h)[26,29,30] might explain why PTH(1-84) is able to raise calcium levels more effectively, albeit transiently, in humans. One limitation of these TPTx studies is that the short duration is not optimal for studying long-term effects on bone.

The effect of EXT607/8 on serum calcium levels in healthy cynomolgus monkeys was also investigated, where serum calcium levels were raised from normal levels to high normal or above normal values. Elevated calcium levels were maintained 24 h for mid-range doses, and 48 h for the highest dose. Despite the elevated serum calcium, urinary calcium levels remained in the normal range at the end of the dosing period (Table 4). Serum phosphate levels were not reduced immediately, but by day 11 of treatment they were persistently lowered. A limitation of studying PTH analogs in healthy animals is that serum calcium levels are increased from normal to above the normal levels, where natural negative feedback loop mechanisms exist to return to homeostasis. Thus, the kinetics of calcium elevation are expected to be different from what would be observed in patients with hypoparathyroidism, where serum calcium levels are increased from below normal into the normal range.

Several other compounds are in development for treating hypoparathyroidism that utilize different strategies to replace endogenous PTH function. TransCon PTH is a sustained-released PTH(1-34) inactive prodrug attached at the N-terminus to a large PEG scaffold (40 kDa) by a cleavable linker.[51] The amount of PTH activity is controlled by a combination of the pharmacokinetics afforded by the large PEG scaffold combined with the rate of release of free PTH. TransCon PTH demonstrated the ability to elevate and maintain constant serum calcium over a 24 h period in TPTx rats and healthy monkeys and is being developed as a once-daily injection.[19,20,52] In a Phase 3 clinical study, TransCon PTH normalized serum and urinary calcium, reduced dependence on supplements, and improved quality of life.[20] Our approach differs from TransCon PTH in that half-life extension is provided by a small molecule, vitamin D, rather than 40 kDa PEG, and EXT608 is conjugated to the C-terminus of PTH through a non-cleavable linker, and the conjugate displays full biological activity rather than utilizing sustained release of the active moiety. An alternate approach uses PTH variants that have prolonged receptor binding and activation times, as demonstrated by LA-PTH (AZP-3601), which is able to elevate serum calcium levels for 24 h in TPTx rats.[40]

The multitude of efforts to develop new therapeutics to treat hypoparathyroidism reflects the inadequacies of using native PTH(1-84) as a replacement therapy due to the short half-life and transient effect on serum calcium levels. We have developed EXT608, a PTH(1-34)-vitamin D conjugate that, due to the flat subcutaneous pharmacokinetic profile, is well-suited to mimic endogenous PTH levels without the need for frequent injections. Furthermore, in animal experiments presented here, serum calcium and phosphate were restored to normal levels in TPTx rats for the duration of the dosing period (24 h), and in healthy monkeys, serum calcium levels were elevated to above the normal range for 24–48 h with serum phosphate persistently reduced. Other benefits include the potential to raise serum calcium levels without increasing urinary calcium, and to normalize bone mineral density. For these reasons, EXT608 was selected as the lead candidate for clinical testing in hypoparathyroidism patients.

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Author contributions

Daniel Hall (Formal analysis, Funding acquisition, Investigation, Visualization, Writing—original draft, Writing—review & editing), Caroline H. Kostyla (Formal analysis, Investigation, Project administration), Laura M. Hales (Conceptualization, Funding acquisition, Writing—review & editing), and Tarik M. Soliman (Conceptualization, Funding acquisition, Supervision, Visualization, Writing—review & editing)

Supplementary material

Supplementary material is available at *JBMR Plus* online.

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Conflicts of interest

D.B.H., L.M.H., and T.M.S. are current employees of Extend Biosciences and hold stock options. C.H.K. is a former employee of Extend Biosciences and holds stock options.

Data availability

All data are either supplied within the manuscript and supplementary material or available upon request.

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