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Development of an hypoparathyroid male rodent model for testing delayed clearance PTH molecules

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Abstract

Background: Parathyroid hormone (PTH) replacement is a promising approach in the management of hypoparathyroidism but long-acting analogues need to be developed. To date, animal models for testing PTH required parathyroidectomy by surgery. We have developed a non-surgical rodent hypoparathyroid model and tested a delayed clearance PTH molecule (DC-PTH).

Methods: Male Wistar rats were gavaged with either 30 mg/kg Cinacalcet-HCl (Cinacalcet) or vehicle only. Animals were then dosed with either single or repeated subcutaneous doses of PTH 1-34 or a DC-PTH at 20 nmol/kg. Control animals received vehicle only. Serum samples were analysed for ionised calcium (iCa), PO_4, PTH and DC-PTH. A Pharmacokinetic-Pharmacodynamic (PK-PD) model was built for Cinacalcet, PTH 1-34 and DC-PTH using Phoneix64®.

Results: Cinacalcet reduced iCa levels between 2-24 hrs returning to baseline by 72 hrs post dose with nadir at 8 hours (ANOVA $P < 0.0001$), associated with a fall in rat PTH. For phosphate there was a variable biphasic response. Single dose PTH abrogated the Cinacalcet induced fall in iCa for up to 2 hrs. DC-PTH prevented the fall in iCa from 4hrs post dose and gave a prolonged response, with iCa levels quicker to return to baseline than controls. DC-PTH has a half-life of 11.5h, approximately 44 times longer than human PTH 1-34. The PK-PD models defined the reproducible impact of Cinacalcet on iCa and that DC-PTH had prolonged biological activity.

Conclusions: The administration of Cinacalcet provides a robust and reproducible non-surgical animal model of hypoparathyroidism. DC-PTH holds promise for treatment of hypoparathyroidism in the future.

Introduction

Hypoparathyroidism is a rare condition that results from a failure of PTH secretion from the parathyroid glands; it may be either congenital, as in DiGeorge syndrome, or acquired such as autoimmune polyendocrine syndrome type 1 or following neck surgery which is the commonest cause (1). The goal of treatment is to maintain a normal calcium level and prevent the long-term complications of hypoparathyroidism. Conventional treatment is with supplemental calcium and vitamin D which in most patients will prevent hypocalcaemia but does not prevent, and may even exacerbate, the long-term complications of hypoparathyroidism including renal failure and extra-skeletal calcification. The most appropriate treatment would be to replace PTH.

Parathyroid hormone (PTH) is synthesized as a 115-amino acid polypeptide called pre-pro-PTH, which is cleaved within parathyroid cells at the amino-terminal portion, first to pro-PTH (90 amino acids) and then to PTH (84 amino acids) (2,3). The latter is the major storage, secreted, and biologically active form of the hormone. PTH 1-84 is secreted by exocytosis within seconds after induction of hypocalcemia. Intact PTH has a plasma half-life of two to four minutes (3). The classical PTH/PTH-related protein (PTHrP) receptor, otherwise known as PTH1R, binds intact PTH and the biologically active amino-terminal fragments of PTH, such as PTH 1-34. PTH maintains calcium homeostasis through binding to its PTH1R, in bone and kidney. In bone PTH mobilizes skeletal calcium, at the kidney PTH enhances renal tubular calcium reabsorption and stimulates the synthesis of 1-alpha hydroxylase in the proximal tubules and, thus, conversion of calcidiol to calcitriol and thereby increases vitamin D-mediated absorption of calcium from the gut. PTH, along with fibroblast growth factor 23 (FGF23), is a key hormonal determinant of serum phosphate concentration and inhibits mostly proximal but also distal tubular reabsorption of phosphorus.

The treatment goal in endocrinology is to replace physiology; however, this has been a challenge in hypoparathyroidism because of the short half-life of PTH. Infusions of PTH 1-34 have been shown to normalise calcium levels and urine calcium excretion but are generally not practical (4,5). Treatment with daily recombinant human PTH 1-84 (rhPTH 1-84) reduces the requirement for calcium and vitamin D supplementation and in the long-term urinary calcium excretion (1). rhPTH 1-84 is approved for patients with HypoPT who cannot be well-controlled on conventional therapy (6). A number of approaches to generating a long acting PTH are currently in development including; Transcon PTH (7), pegylated PTH (8), and Long-acting PTH (9). We have developed a delayed clearance PTH fusion protein (DC-PTH) by linking PTH 1-34 to a modified growth hormone binding protein (GHBP). The rational for this is based on our previous experimental work that demonstrated by linking growth hormone to GHBP resulted in a very delayed clearance molecule (10). We hypothesised that the linking of GHBP reduced proteolysis and renal clearance and, as a native protein, GHBP would not raise an immune response (11).

To date, animal models used for testing PTH molecules are complex, single use, and expensive. The rodent model requires surgical parathyroidectomy or thyro-parathyroidectomy (9,12,13). More recently a mouse model has been developed in which PTH glands can be highlighted using GFP for easy removal by surgery or for the targeted ablation of the PTH glands using diptheria toxin (14).

To test our DC-PTH molecule we developed a simple non-surgical rodent model of hypoparathyroidism using Cinacalcet. Cinacalcet is a calcimimetic that binds and activates the calcium sensing receptor (CaSR) suppressing PTH secretion and synthesis (15). The aim of this study was to use Cinacalcet to suppress calcium levels in normal rats and to reverse these effects with the administration of PTH or PTH analogues. Thus, creating a non-surgical animal model in which both PK and PD parameters for a DC-PTH can be quantified.

Materials & Methods

Animals: 7-8 month old male Wistar rats, weighing ~400 to 450 g, were used in these studies (Purchased from Royan Institute, Isfahan, Iran). The rationale for choosing male rats was to reduce the number of variables being tested as male rats have the advantage of constant testosterone and oestradiol, whereas female mice have fluctuating oestradiol levels and there is likely an interaction between PTH and oestrogen on tissues such as the skeleton. Animals were kept individually in cages in standard animal housing conditions at room temperature (25±2°C) in natural light with access to food and water ad libitum for at least two days prior to study. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 2011; National Academies Press; Washington, D.C.). All animal work was conducted by the Department of Cell and Molecular Biology and Microbiology, University of Isfahan, Iran. Ethical permissions were obtained from the University of Isfahan. (Approval date: 27th April 2019. Animal ethics committee reference number: IR.UI.REC.1398.188).

Test compounds: Cinacalcet-HCl (Cinacalcet, Sigma, Cat No: SML2012) was dissolved in 20% Captisol (Sigma, Cat No: M4837) to 18 mg/ml. DC-PTH (Delayed clearance PTH) is a fusion between human PTH 1-34 and GHBP 1-238 and expressed and purified from a stable CHO cell line (Flp-In-CHO (RRID:CVCL_U424) and stored in 20 mM citrate, 0.15M NaCl, 10% glycerol, pH 6.0 (Citrate buffer). Human PTH 1-34 (Anaspec, Cat No: AS-20708) was dissolved at 1 mg/mL in Citrate buffer immediately before use.

Route of administration: Anaesthetised rats were gavaged with Cinacalcet at 30 mg/kg using a low dead space insulin syringe and a specialized, commercially available gavage needle (18-

25 gauge, 3.7-inch length). Control animals received vehicle only. PTH 1-34 and DC-PTH were administered by subcutaneous injection at 20 nmol/kg.

Sampling: Blood samples (1 mL) were taken from the orbital sinus into microfuge tubes and allowed to clot. Samples for PTH measurements were transferred into microfuge tubes and stored at -20°C. Remaining serum was placed at 4°C in sealed tubes ready for calcium and phosphate measurements.

Assays: Ionized calcium (iCa) was determined using an EasyLyte fully automated electrolyte analyser (Medica Corporation). Phosphorus was determined using a Hitachi 917 Clinical Chemistry Analyser (Roche). For PK analysis PTH 1-34 and DC-PTH serum levels were measured using a High Sensitivity Human PTH (1-34) Elisa kit (Immutopics, Quidel, Cat# 60-3900, RRID:AB_2893017). Endogenous rat PTH was measured using a rat intact PTH Elisa (Immutopics, Quidel, Cat# 60-2500, RRID:AB_2827505).

Cinacalcet Model for Hypoparathyroidism: To test for the effect of Cinacalcet, male Wistar rats were gavaged with 30 mg/kg Cinacalcet (n =10). For PK/PD experiments, animals initially gavaged with Cinacalcet were immediately dosed with 20 nmol/kg PTH 1-34 either as a single injection (n = 10) or a repeated dose, given every hour for 6 hrs for a total of 7 doses (n = 2). Controls received vehicle only (n = 9). Serum samples were taken at pre-dose and 1, 2, 4, 8, 12, 24, 48 & 72 hrs post Cinacalcet and analysed for iCa, phosphate, endogenous rat PTH and PTH 1-34.

In vitro Biological Activity for DC-PTH: was measured using the rat osteosarcoma cell line, UMR-106 (ATCC Cat# CRL-1661, RRID:CVCL_3617). Briefly, 40,000 cells per well of a 48 well plate were transfected with 0.2 µg reporter plasmid pGL4.29/CRE/Luc2/Hygro

(Promega Corp, Cat No: E8471) and 20 ng transfection control plasmid pRL-TK (Promega Corp, Cat No: E2241) using TransIT-LT1 (Mirus). Transfected cells were left for 4 hrs before changing media to DMEM (Gibco) supplemented with L-Glutamine and 0.1% (w/v) bovine serum albumin (BSA) followed by a further incubation o/n. Next day cells were challenged with either DC-PTH or human PTH 1-34 for 5 hours at 37ºC/5% CO_2. Cells were then lysed and Luciferase activity measured using a Berthold Autolumat Plus Luminometer with the Promega Dual Luciferase Assay kit. Data were analysed using GraphPad Prism Software (Version 9.1.1) and EC_{50} values (nM ±standard deviation) obtained from a Four Parameter Logistic (4PL) curve fit.

Testing of DC-PTH in Model for Hypoparathyroidism: Animals initially gavaged with Cinacalcet were immediately administered with a single subcutaneous dose of DC-PTH at 20 nmol/kg (n = 4). Control animals received vehicle only (n = 9). Serum samples were taken at pre-dose and 1, 2, 4, 8, 12, 24, 48 & 72 hours post Cinacalcet and analysed for iCa, phosphate and DC-PTH.

Pharmacokinetic (PK)-Pharmacodynamic (PD) Model: PK and PK-PD evaluations were performed by non-compartmental analysis and compartmental modelling using Phoenix64® by XenoGesis (Nottingham, UK). PK and PK-PD models were generated using the Maximum Likelihood method using multiplicative (PK) and additive (PK-PD) weighting and the naïve pooled engine.

Statistics: Statistical analyses on samples were performed using GraphPad Prism Software (Version 9.1.1). Data are expressed as mean±SEM. Comparisons between experimental

groups were performed by a one-way ANOVA followed by Tukey multiple comparisons test between treatments and controls. A P value <0.05 was considered significant.

RESULTS

Cinacalcet model of hypoparathyroidism: Cinacalcet produced a significant reduction in iCa levels between 2-24 hours returning to baseline at 48-72 hours post dose with the iCa nadir at 8 hours (ANOVA $P < 0.0001$, Figure 1a). iCa (mmol/L±SEM) at predose, 1.2 ± 0.029 and at 8 hours, 0.9 ± 0.016 ($P < 0.0001$). For phosphate an initial lowering up to 2 hours in all groups was followed by a rise above control from 8-24 hours (ANOVA, *ns*), returning to baseline at 48 hours (Figure 1b). Rat PTH fell in line with the fall in serum iCa induced by Cinacalcet (Figure 1c). A single dose of PTH 1-34 showed a peak serum concentration at 10 minutes post dose and abrogated the Cinacalcet induced fall in iCa for up to 2 hours (Figure 2a & b): $AUC_{0\text{-}2hrs}$±SEM (iCa mmol/L.hr), 0.076 ±0.018 versus 0.163±0.024, $P = 0.0181$). Repeated hourly injections of PTH 1-34 over 6 hours maintained iCa levels in Cinacalcet treated animals similar to those in vehicle only treated animals for up to 8 hrs post dosing (Figure 2c & d).

DC-PTH *In vitro* Biological Activity: EC_{50}'s of 63.52±7.6 nM (n = 3) and 1.11±0.49 nM (n = 4) were obtained for DC-PTH and PTH 1-34 respectively.

Testing DC-PTH in Cincalcet model of hypoparathyroidism: DC-PTH abrogated the effect of Cinacalcet from 4 hours post dose restoring iCa to baseline levels at 48 hours and giving a prolonged response (Figure 3a). $AUC_{0\text{-}72hrs}$ (iCa mmol/L.hr) for reduction in iCa was significantly smaller than treatment with Cincacalcet only: Mean±SEM $AUC_{0\text{-}72hrs}$ for DC-

PTH 3.9±0.7 vs 10.5±0.97 for Cinacalcet (P = 0.0003). The PK profile of DC-PTH closely matched the in vivo effect on iCa (Figure 3b).

Non-compartmental analysis of exposure of DC-PTH and PTH 1-34: Non-compartmental analysis of individual animal data following administration of DC-PTH or PTH 1-34 was performed in Phoenix64® and indicates that DC-PTH has a half-life of 11.5 hours which is 44 times longer than that for PTH 1-34 at 0.26 hours.

Cinacalcet PK-PD model: To build the pharmacokinetic model of Cinacalcet the oral exposure of Cinacalcet in rats was extracted from a previous publication (Nemeth *et al.,* JPET 308: 627-635, 2004. DOI: 10.1124/jpet.103.057273) and fit to a single compartment extravascular PK model which reasonably captured the observed Cinacalcet levels (Figure 4). While Cinacalcet decreased PTH levels resulting in a lowering of iCa, PTH acts in response to low iCa to increase its release. A PK-PD model is therefore proposed where an initial dose of Cinacalcet results in the inhibition of iCa formation with a dose of PTH acting to increase levels of iCa. Both are assumed to occur as a function of Michaelis-Menten kinetics with inhibition of formation of iCa related to the concentration of Cinacalcet (Conc_C) with maximal rate Imax_C and 50% inhibition at concentration IC50_C. Stimulation of formation of iCa is related to the concentration of PTH (Conc_PTH) with maximal rate Imax_PTH and 50% inhibition at IC50_PTH. These act in addition to zero order synthesis of iCa at rate Kin and first order degradation at rate Kout. The resulting rate of change of iCa, (d/dt(iCa)) is therefore described by the differential equation (Eqn1) with steady state iCa levels defined as Kin/Kout.

Eqn 1: d/dt(iCa) = Kin* (1 – Imax_C * Conc_C / (Conc_C+IC50_C)+
ImaxPTH*Conc_PTH/ (Conc_PTH+IC50_PTH)) - Kout * iCa)

As pre-dose iCa levels vary somewhat between animals, the levels relative to T=0 (or the first time-point if this data isn't available) expressed as a % have been modelled throughout. The parameters Imax_C and IC50_C were fit to this model using the observed data on the effect of Cinacalcet alone on iCa. As exposure of Cinacalcet was not measured, the Cinacalcet model PK parameters described in Table 1 were fixed. The model fit to the observed data (Figure 5) and fitted model parameters are shown in Table 2. Overall, the model is able to reasonably capture the observed decrease in iCa following dosing with Cinacalcet. The %CV is relatively high for all parameters but this could reflect variability in the exposure of Cinacalcet which was not measured in this study and is consistent with the observed variability in response at each time-point. The Cinacalcet-iCa model was fixed in future models to assess the impact of PTH 1-34 or DC-PTH on iCa.

Human PTH 1-34 and DC-PTH PK-PD: The derived PK models for PTH 1-34 and DC-PTH were used in the combined model of iCa as a function of Cinacalcet and PTH 1-34 (Figure 6a & b). In rats treated with 30 mg/kg Cinacalcet, a single 20 nmol/kg dose of PTH 1-34 had a modest impact on iCa levels over 8 hours with levels at later time-points similar to untreated animals (Figure 6a). In contrast, iCa levels return to within 90% of baseline from 10 hours onwards following a single 20 nmol/kg dose of DC-PTH (Figure 6b). Modelling suggests that DC-PTH is less potent than PTH 1-34 at restoring iCa levels. However, due to a combination of higher plasma exposure at 20 nmol/kg and a longer half-life, DC-PTH is able to exert a more profound effect on iCa levels versus time in the Cinacalcet induced rat model of hypoparathyroidism from a single 20 nmol/kg dose than PTH 1-34 either from a single dose or repeat dose at 20 nmol/kg.

DISCUSSION

We have developed and tested a rodent model of hyoparathyroidism using Cincacalcet that can be used in the development of new therapies for hypoparathyroidism. Based on our experimental data we have built a PKPD model that predicts the impact of Cinacalcet on iCa and can be used to examine the impact of PTH analogues on iCa. We have then tested a delayed clearance PTH, DC-PTH and demonstrated that it has a long plasma half-life and a prolonged biological activity restoring iCa levels to baseline in the rodent model of hypoparathyroidism.

It is well known that Cinacalcet lowers PTH and therefore iCa and this has been shown in rodents and humans and Cinacalcet is a licensed therapy to lower PTH in humans (16). However, no-one has investigated the impact of replacing PTH after the administration of Cinacalcet. The administration of PTH analogues to healthy rodents has minimal impact on iCa with previous studies showing that both single or multi-dose administration of PTH results in a rapid insignificant lowering of calcium accompanied by a rapid return to baseline levels (17,18), and so a model of hypoparathyroidism is required to test potential PTH analogues. To date, testing of PTH analogues has required a surgical model which has limitations as parathyroidectomy in the rodent usually involves removal of the thyroid, the surgical model can only be used for short term administration and the animals can only be used once. The Cinacalcet model we have developed has a number of advantages over the surgical model in that no surgery is required, and the animal has normal thyroid function. The administration of Cinacalcet resulted in suppression of iCa for up to 72 hours allowing the testing of long acting or delayed clearance PTH analogues. The dose of 30 mg/kg Cinacalcet was chosen based on the preclinical studies during the development of Cinacalcet (15). The change in iCa after Cinacalcet paralleled the change in rodent PTH levels but phosphate showed an initial fall then the expected rise in levels following Cinacalcet. This

biphasic response of phosphate is seen in previous publications after administration of Cinacalcet (15), although we don't have a physiological explanation for why phosphate levels initially fall. Cinacalcet not only works on the CaSR in the parathyroid glands but also has effects on the CaSR in the kidneys, and therefore may change the characteristics of PTH analogs on renal calcium handling and this needs to considered when developing PTH analogs.

The DC-PTH molecule we tested was a fusion of PTH 1-34 fused to GHBP and expressed in mammalian cells as a single molecule. This was based on our previous work with a fusion of GH to GHBP that showed delayed clearance (19). As far as we are aware this is the first fusion molecule of the peptide PTH that has been expressed and purified from mammalian culture. Previous analogues of PTH have either been generated as conjugates, by peptide synthesis, or extracted from E.coli. The DC-PTH was less potent *in vitro* than PTH 1-34, and this was also shown in the PKPD modelling. The DC-PTH had a half-life in rats of 11.5 hours which is 44 times longer than that for PTH 1-34 at 0.26 hours and with allosteric modelling DC-PTH would be expected to have a half-life in humans of >40 hours compared to that published for PTH 1-84 of 2-3 hours (20). DC-PTH had a considerably longer biological action than PTH 1-34 in the rodent model of hypoparathyroidism. The reduced potency of DC-PTH coupled with prolonged biological action may be an advantage for a PTH replacement therapy in hypoparathyroidism avoiding the fluctuations in iCa seen with PTH 1-84 (6). The protocol used in our study was to administer DC-PTH at the same time as Cinacalcet and therefore there was an initial fall in iCa as DC-PTH was absorbed. It would be of interest to see if prior administration of DC-PTH prevented the initial fall in iCa as would be expected from the data with PTH 1-34 which maintained iCa when co-administered with Cinacalcet. The dose of DC-PTH used, 20 nmol/kg, was that used in previous investigations

of PTH analogues in rodent models (9,18) and future development will need to look for a dose-response.

The PKPD model presented is able to describe the impact of PTH 1-34 and DC-PTH on iCa in the Cinacalcet rodent model of hypoparathyroidism in a time-dependent manner. A limitation was that the Cinacalcet PK was based on published literature; however, the observed versus predicted plasma concentration of Cinacalcet from the derived data showed a very good fit. The PKPD model can be developed and used to plan repeat dosing studies and potentially adapted for use in man if phase 1 studies were considered in healthy individuals. The current model is based on a single dose of Cinacalcet which allowed the investigation of PTH analogues without the need for parathyroidectomy. Potentially the animal model could be developed with repeated Cinacalcet administration to examine prolonged effects of PTH suppression on the skeleton.

In conclusion, we have developed a rodent model of hypoparathyroidism and demonstrated the potential of DC-PTH as a therapeutic for hypoparathyroidism.

Author contribution statement:

The first and last authors vouch for the accuracy, completeness of the data and analyses. All authors critically reviewed the manuscript, participated in the design and analysis of the trial.

Data Availability Statement

No supplemental data is supplied in the manuscript. Data will be made available upon reasonable request.

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Figure Legends

Figure 1: **(a)** Serum levels of iCa, **(b)** Phosphorus, and **(c)** Rat PTH 1-84 in normal Wistar rats after oral administration of 30 mg/kg Cinacalcet. Values represent mean±SEM. Cinacalcet results in a prolonged suppression of PTH associated with a fall in iCa.

Figure 2: (a) Effect of a subcutaneous single dose injection of human PTH 1-34 on Cinacalcet induced reduction of iCa and **(b)** PTH 1-34 levels during PTH 1-34 treatment arm. **(c)** Effect of a subcutaneous multi dose injection of human PTH 1-34, given every hour for 6 hours on Cinacalcet induced reduction of iCa and **(d)** PTH 1-34 levels during PTH 1-34 treatment arm. Values represent mean±SEM. Single injection of PTH 1-34 maintained iCa for 2 hours and repeated injections for 8 hours compatible with the PK profile.

Figure 3: (a) Effect of a subcutaneous single dose of DC-PTH on Cinacaclet induced reduction of iCa and **(b)** DC-PTH levels during DC-PTH treatment arm. Values represent mean±SEM. DC-PTH restored iCa to baseline levels from 8 hours.

Figure 4: Observed versus Predicted plasma concentration of Cinacalcet (solid line = line of unity). Shows good model fit for derived Cinacalcet data.

Figure 5: Percentage of iCa (mmol/L) relative to first time point for Predicted (solid line) and Observed (circle) versus time following a 30 mg/kg dose of Cinacalcet.

Figure 6: Percentage of iCa (mmol/L) relative to first time point for Average Observed (±SD) and Cinacalcet model (±SD) levels of relative iCa versus time following **(a)** a single 20 nmol/kg dose of PTH 1-34 + Cinacalcet and **(b)** a single 20 nmol/kg dose of DC-PTH + Cinacalcet.

Table 1: Model PK (Pharmacokinetic) parameters derived from Observed versus Predicted plasma concentration of Cinacalcet. Ka: rate of absorption, V/F: volume of distribution, and CL/F: clearance.

Parameter	Units	Estimate	CV%
Ka	1/h	4.07	29.7
V/F	mL/kg	393000	7.88
CL/F	mL/h/kg	193000	6.52

Table 2: Fitted model parameters for effect of Cinacalcet on iCa. tvKin: zero order rate of synthesis of iCa, tvKout: first order rate of degradation of iCa, tvImax: maximal rate of simulation of formation of iCa due to PTH and tvIC50: concentration of PTH that results in 50% of the maximal rate of stimulation of formation of iCa.

Parameter	Value	Units	CV%
tvKin	30.3	1/h	138
tvKout	0.303	1/h	133
tvImax	0.415	1/h	139
tvIC50	31.7	ng/mL	306