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Title Page

TITLE: TransCon CNP, a sustained-release C-Type Natriuretic Peptide prodrug, a potentially safe and efficacious new therapeutic modality for the treatment of comorbidities associated with FGFR3-related skeletal dysplasias

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Running title page

RUNNING TITLE (max 60 characters incl. spaces): Sustained-release CNP prodrug in
Achondroplasia

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Number of Text Pages: 35

Number of Tables: 9

Number of Figures: 4

Number of References: 23

Number of words in Abstract: 250 (max 250)

Number of words in Introduction: 747 (max 750)

Number of words in Discussion: 1239 (max 1500)

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List of non-standard abbreviations:

ACH	Achondroplasia
AHV	Annualized Height Velocity
ANP	A-type natriuretic peptide
AP	Alzet pumps
BAP	Bone Specific Alkaline Phosphatase
BP	Blood Pressure
BSA	Bovine Serum Albumin
BV/TV	overall bone volume fraction
cGMP	cyclic Guanosine Monophosphate
C _{max}	Maximum Plasma Concentration
CMV promoter	Cytomegalovirus promotor
CNP	C-type natriuretic peptide
CNP-22	Endogenous CNP-22
CNP-38	CNP structurally identical to endogenous CNP (C-terminal 38 amino acids)
CNP-39	Daily CNP molecule structurally identical to Vosoritide
CTxI	C Telopeptides of Type I Collagen
DAP	Diastolic Arterial Pressure
ECG	Electrocardiogram
FA	Formic Acid
FGFR3	Fibroblast Growth Factor Receptor 3
FITC	Fluorescein Isothiocyanate
H&E	Hematoxylin and Eosin stain

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HR	Heart Rate
LLOQ	Lower limit of quantification
MAP	Mean Arterial Pressure
NEP	Neutral Endopeptidase
NPR-B	Natriuretic Peptide Receptor B
NPR-C	Natriuretic Peptide Receptor C
NTx	N-Terminal Telo peptide
OC	Osteocalcin
PBS	Phosphate-buffered Saline
PEG	Polyethylene Glycol
PINP	Procollagen Type I N Terminal Propeptide
RIA	Radio Immunoassay
PPF	Pentafluorophenol
SAP	Systolic Arterial Pressure
SPE	Solid Phase Extraction
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoro Acetic Acid
T _{max}	Time to maximum plasma concentration
vBMD	apparent density of the total region
VOI	Volume of Interest
WCX	Weak Cation Exchange

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Recommended section assignment:

Drug Discovery and Translational Medicine

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ABSTRACT

TransCon CNP is a C-type natriuretic peptide (CNP-38) conjugated via a cleavable linker to a polyethylene glycol carrier molecule, designed to provide sustained systemic CNP levels upon weekly subcutaneous administration. TransCon CNP is in clinical development for the treatment of comorbidities associated with achondroplasia. In both mice and cynomolgus monkeys sustained exposure to CNP via TransCon CNP, was more efficacious in stimulating bone growth than intermittent CNP exposure. TransCon CNP was well tolerated with no adverse cardiovascular effects observed at exposure levels exceeding the expected clinical therapeutic exposure. At equivalent dose levels, reductions in blood pressure and/or an increase in heart rate, were seen following single s.c. injections of the unconjugated CNP-38 molecule or a daily CNP-39 molecule (same amino acid sequence as Vosoritide, USAN:INN). The half-life of the daily CNP-39 molecule in cynomolgus monkey was estimated to be 20 minutes, compared to 90 h for CNP-38, released from TransCon CNP. C_{\max} for the CNP-39 molecule (20 µg/kg) was approximately 100-fold higher, compared to the peak CNP level associated with administration of 100 µg/kg CNP as TransCon CNP. Furthermore, CNP exposure for the daily CNP-39 molecule, was only evident for up to 2 h post dose (LLOQ 37 pmol/L), whereas TransCon CNP gave rise to systemic exposure to CNP-38 for at least 7 days post-dose. The prolonged CNP exposure and associated hemodynamically safe peak serum concentrations associated with TransCon CNP administration are suggested to improve efficacy, compared to short-lived CNP molecules, due to better therapeutic drug coverage and decreased risk of hypotension.

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SIGNIFICANCE STATEMENT

The hormone C-type Natriuretic Peptide (CNP) is in clinical development for the treatment of comorbidities associated with achondroplasia (ACH), the most common form of human dwarfism. The TransCon Technology was used to design TransCon CNP, a prodrug that slowly releases active CNP in the body over several days. Pre-clinical data show great promise for TransCon CNP to be an effective and well-tolerated drug that provides sustained levels of CNP in a convenient once weekly dose, while avoiding high systemic CNP bolus concentrations that can induce cardiovascular side effects.

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INTRODUCTION

Currently, no approved treatment exists for comorbidities associated with achondroplasia (ACH), the most common form of human dwarfism. Administration of C-type Natriuretic Peptide (CNP) to ACH patients and animals has been shown to stimulate endochondral bone growth and improve adverse skeletal manifestations of the disease (Yasoda et al., 2009; Lorget et al., 2012; Wendt et al., 2015; Legeai-Mallet, 2016; Biomarin_Inc, 2018). The biology of CNP has been known for many years, but the use of CNP as a pharmaceutical product has been limited by major shortcomings of the native CNP. Native CNP has a half-life of only 2-3 minutes in humans, and degradation by neutral endopeptidase (NEP) makes it difficult to maintain an efficacious therapeutic drug exposure. Because of the short half-life of native CNP, continuous intravenous administration of CNP-22 is required to restore bone growth in ACH mice (Yasoda et al., 2009). Wendt et al. (2015) recently reported the development of a daily CNP analogue, with increased NEP resistance, and an associated s.c. half-life in mice of ca 15 min, compared to <5 min for murine CNP-22 (Wendt et al., 2015). A corresponding long half-life of approximately 20 min was verified in cynomolgus monkeys (Wendt et al., 2015). The daily CNP analogue with the INN name Vosoritide, consists of the 37 C-terminal amino acids of human CNP plus the amino acids glycine and proline added to the N-terminal portion of the peptide. Proof-of-principle for this daily CNP analogue was obtained in the nonclinical setting employing ACH mice exhibiting phenotypical traits similar to that observed in human individuals with ACH (Lorget et al., 2012; Wendt et al., 2015). Daily administration of this CNP analogue improved the major phenotypical dwarfism traits by normalizing chondrocyte proliferation and differentiation facilitating bone growth in both axial and appendicular bones (Lorget et al., 2012; Wendt et al., 2015).

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The mechanism of action of CNP is believed to be via inhibition of the downstream signaling pathway of the fibroblast growth factor receptor 3 (FGFR3) at the level of *raf*, thereby restoring chondrogenesis by counteracting the overactivation of the FGFR3 pathway seen in ACH, (Laederich and Horton, 2010). In children with ACH, the daily administered CNP molecule Vosoritide, positively impacts bone growth, resulting in an increase in annualized height velocity (AHV) of approximately 1-2 cm per year (Biomarin_Inc, 2018).

As the mutated FGFR3 receptor is constitutively overactivated, we hypothesize that continuous exposure to CNP is required to optimally counterbalance the overactivated FGFR3 signaling pathway, and that short-acting CNP molecules may suffer sub-optimal efficacy due to their short half-life preventing optimal modulation of the overactive FGFR3 pathway. Another potential limitation of CNP as a therapeutic modality in ACH treatment is the vasodilatory effect of CNP (Clavell et al., 1993; Igaki et al., 1998; Chauhan et al., 2003; Scotland et al., 2005; Nakao et al., 2017). High concentrations of CNP cause vasodilation, with resulting decreased blood pressure (BP) in animals (Lopez et al., 1997; Wendt et al., 2015). Intravenous bolus administration of CNP-22 in humans induces post-dose hypotension and tachycardia, coinciding with the peak blood concentration, indicating a causal relationship (Igaki et al., 1998). Decreases in BP, occurring shortly after dose administration and coincident with high CNP exposure has also been observed in children with ACH following administration of Vosoritide (Biomarin_Inc, 2018). Thus, for CNP to be both an efficacious and safe therapy for ACH, administration should optimally provide continuous exposure to restore chondrogenesis and promote bone growth, while avoiding high systemic concentrations that can induce cardiovascular side effects. TransCon CNP, a sustained-release prodrug of CNP, is in clinical development as a potentially safe and efficacious treatment for achondroplasia. To ensure sustained delivery, CNP is

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transiently bound via the TransCon Linker to a chemically inert, branched PEG carrier that prolongs the peptide's overall circulatory half-life. This is achieved by minimizing renal clearance of the TransCon CNP prodrug and shielding of the CNP molecule from proteolytic degradation and from binding to its primary activating and clearance receptors, NPR-B and NPR-C. Following cleavage of the TransCon Linker under physiological pH and temperature, active CNP-38 peptide is slowly and continuously released and, due to the small size of the peptide, assumed to be subjected to the same distribution and pharmacodynamics mechanisms as endogenous CNP.

In this publication we describe the in vitro and in vivo screenings that led to the selection and development of TransCon CNP as a promising treatment modality for ACH and potentially other skeletal dysplasias, where CNP may impact positively on a constitutively overactivated FGFR3 pathway.

Materials and Methods

Selection of TransCon CNP

CNP-38 having the sequence LQEHPNARKYKGANKKGLSKGCFGLKLDRIQSMGSLGC (oxidized to form one intramolecular disulfide bond), fluorescein labelled CNP-38 and CNP-38 conjugates (Table 1) used for the in vivo and the in vitro studies were synthesized chemically as described in patent applications (Sprogøe et al., 2016; Rau et al., 2017).

Table 1

CNP-38 conjugates used for in vivo and in vitro studies

Deuterated CNP-38 was used as internal standard for the free CNP analysis and synthesized as described for CNP-38 with the exception that deuterated leucine (D10 Fmoc-Leu-OH, Cambridge Isotope laboratories) was incorporated at positions Leu(1), Leu(25) and Leu(36). The daily CNP-39 molecule with the following sequence PGQEHPNARKYKGANKKGLSKGCFGLKLDRIQSMGSLGC (oxidized to form one intramolecular disulfide bond) was synthesized chemically as described for CNP-38. This peptide thus consists of the amino acids proline (amino acid 1) and glycine (amino acid 2) plus the 37 C-terminal amino acids of natural human CNP. This resulting peptide is similar to Vosoritide which is in clinical development for the treatment of comorbidities associated with achondroplasia (Biomarin_Inc, 2018) and in this document referred to as the daily CNP-39 molecule.

Activity (cGMP) Assay in NIH3T3 cells.

The activity of the CNP-38 conjugates to elicit an intracellular cGMP response was determined in NIH3T3 cells. These cells express NPR-B on the cell surface (Abbey and Potter, 2003) and

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stimulation of this receptor with CNP leads to intracellular production of the secondary messenger cGMP. In brief, NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium F-12 medium with 5 % FBS and 5 mM glutamine at 37 °C and 5 % CO₂. For each assay, cells were resuspended in stimulation buffer (Dulbecco's PBS with 0.5 mM 3-Isobutyl-1-methylxanthine), seeded in a 96-well plate (5 × 10⁴/well) and incubated in duplicate with the CNP conjugate at different concentrations. After an incubation of 30 min at 37°C and 5 % CO₂, cells were lysed in the supplied lysis-buffer and the cGMP level was measured using a commercially available cGMP assay based on time-resolved fluorescence energy transfer (cGMP kit, Cat. No. 62GM2PEB, Cisbio, Codolet, France). Activity was determined using a 4-parameter logistic curve fit and by parallel line analysis of the sample in comparison to CNP-38 as a reference standard (PLA 2.0 software, Stegmann Systems GmbH, Rodgau, Germany).

NPR-C Affinity Assay

For the NPR-C affinity assay, a HEK293 cell line, stably overexpressing human NPR-C was developed. The HEK293^{NPR-C} cell line was developed as follows: The coding region of human NPR-C reference sequence was cloned into a lentiviral vector under a CMV promoter to allow for constitutive receptor expression. A bicistronic element located on the vector for puromycin resistance was used as the eukaryotic selection marker. After transduction, pools of stably growing cells were subjected to qRT-PCR for confirmation of NPR-C mRNA-expression. High receptor mRNA-expression was confirmed as compared to parental HEK293. For NPR-C affinity analysis, cells were routinely cultured in Dulbecco's Modified Eagle Medium/Glutamax medium with 10 % FBS and 0.5 µg/mL puromycin at 37°C and 5 % CO₂. Growing cells were trypsinized from the cell flask bottom, counted, and seeded in a 96-well plate (1.5 × 10⁵/well) (U-bottom wells, Greiner Bio-One GmbH, Frickenhausen, Germany) and centrifuged. The supernatants were discarded.

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CNP-38 standards and samples were serially diluted over 9 steps in PBS 0.2% BSA and transferred to duplicate microplates and mixed with the cell pellet. After 30 min incubation at room temperature, Fluorescein-labelled CNP-38 was added to each well at a fixed concentration and cells were incubated for additional 45 min at room temperature. Subsequently, cells were analyzed by flow cytometry using the mean fluorescence intensity of the FITC channel as the read out. The CNP-38 standard and sample curves were generated in parallel line analysis software (PLA 2.0, Stegmann Systems GmbH, Rodgau, Germany) using a 4-parameter logistic fit for calculation of the relative NPR-C affinity of sample versus the CNP-38 standard (Ratio IC_{50} CNP-38 / IC_{50} CNP-38 conjugate).

NEP Resistance

The degradation half-life of various CNP conjugates was measured in a NEP digestion assay. Recombinant human NEP (2.5 μ g/mL final concentration, Enzo Life Science, no. BML-SE532-0010) and the internal standard pentafluorophenol (PFP; 40 μ g/mL final concentration) were added to a solution of the CNP conjugate (100 μ g CNP equivalents/mL) contained in digest buffer (50 mM Tris-HCl, 10 mM NaCl, pH 7.4). The solution was incubated at 37 °C and 500 rpm for up to 4 days in a thermomixer. Samples were removed at several time points during the 4-day incubation period. The reaction was stopped by adding TCEP (tris(2-carboxyethyl)phosphine; 25 mM final concentration) and incubating the mixture at 95 °C, at 500 rpm for 5 minutes. The half-life of each CNP variant was calculated using the loss of the HPLC-UV peak area over time relative to PFP.

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IN VIVO STUDIES

Animals and husbandry: All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by and performed in AAALAC-accredited facilities (Charles River Laboratories, Senneville, Canada and Envigo, Cambridgeshire, UK). The total number of animals used in the current studies was considered the minimum required to properly accomplish the objectives of the studies. All studies and procedures were conducted in accordance with the highest standards of animal care, and in compliance with federal, state, and local laws and institutional regulations.

Cardiovascular studies:

The hemodynamic tolerability of s.c. administered TransCon CNP and the daily CNP-39 molecule, was investigated in both mice and cynomolgus monkeys, whereas unconjugated CNP-38 was only evaluated in mice.

Mice

In male telemetered adult Crl:CD1(ICR) mice, the cardiovascular evaluation of unconjugated CNP-38 and TransCon CNP included potential effects on blood pressure (BP) and heart rate (HR). Animals (n=4) were dosed s.c. with single doses of vehicle, CNP-38 or TransCon CNP at 800 µg CNP/kg. Animals were dosed with TransCon CNP and CNP-38 on different days but always together with time-matched vehicle controls. Systemic BP (systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressure) and HR (derived from blood pressure monitoring), were recorded from an implanted DSI transmitter (DSI TA11PA-C10) using a digital data capture system linked with a DSITM Ponemah data acquisition (DSI PONEMAH 5.2) and analysis system (OpenART version 4.3). The data capture system allowed recording of the

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cardiovascular parameters whilst the mice were in individual cages. On each of the dosing occasions, a pre-dose recording of a duration of approximately 60 min was performed.

Immediately after each dose administration, the mice were returned to their cages, and a 48-h cardiovascular monitoring initiated. Systemic BP and HR were reported at the following time points: -0.5, -0.33, -0.17, 0.08, 0.25, 0.5, 1, 2, 6, 12, 18, 24, 30, 36, 42 and 48 h post-dose, with each time point being the mean of 5 min of data. MAP measurements were tested for statistical difference to vehicle group (using Williams' test; ANCOVA adjusted for differences between the treatment groups at baseline).

Monkeys

In adult male telemetered cynomolgus monkeys, the cardiovascular evaluation encompassed potential effects of TransCon CNP on BP, HR, electrocardiogram (ECG) and body temperature. TransCon CNP and the daily CNP-39 molecule was administered as single s.c. injections in an escalating dose design (n=4/dose level) including 6 test sessions. On test session 1, all animals received vehicle. On test session 2 and 3 all animals received dose levels of the daily CNP-39 molecule (positive control) at 50 and 100 µg CNP/kg, respectively. On test sessions 4-6, all animals received TransCon CNP at 10, 40, or 100 µg CNP/kg, respectively. A washout period of 3 days was allowed between test session 1 and 2, 7 days between test session 2, 3 and 4, and at least 14 days between test sessions 4-6. Body temperature and the following cardiovascular parameters were recorded via a DSI transmitter implant (DSI TL11M2-D70-PCT) and using the DSI digital data capture system Ponemah Physiology Platform P3 software system (DSI PONEMAH 5.1 and 5.2, ADaPT 2.0): systemic BP (SAP, DAP, MAP and pulse pressures (PP)), HR (derived from blood pressure), and ECG duration/intervals (PR, RR, QRS, QT, and QTca) up to 24 h after administration of the daily CNP-39 molecule and up to 48 h after administration

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of vehicle or TransCon CNP. Blood samples were collected at 48 and 168 h after administration of TransCon CNP, to verify that animals received the correct dose.

Mouse efficacy study: Pharmacological effects of daily bolus versus continuous subcutaneous infusion of CNP-38 in mice

Groups (n=9) of 3-week old (± 1 day) male FVB mice (Charles River Laboratories, Sennneville, Canada) were administered 203 $\mu\text{g/kg/d}$ CNP-38 for 5 weeks either by continuous s.c. infusion or by single daily s.c. injections. Continuous infusion was applied by osmotic Alzet pumps (AP) (Model 1002, 0.25 $\mu\text{l/h}$ for study day 1-15; Model 1004, 0.09 $\mu\text{l/h}$ for study day 15-34). CNP-38 was formulated either at 577 $\mu\text{g/mL}$ (Model 1002, day 1), 2254 $\mu\text{g/mL}$ (Model 1004, day 15) or 40.6 $\mu\text{g/mL}$ (daily subcutaneous injection) in vehicle (30 mM acetate buffer pH 4 containing 5 % sucrose and 1 % benzylic alcohol). The vehicle was used as control in both the daily injection and in the continuous infusion groups. The pumps were implanted subcutaneously in the scapular area at day 1 (Model 1002) and were exchanged on day 15 (Model 1004) to adjust for the increased body weight and to ensure adequate dose coverage over the 5-week infusion period. Doses for continuous infusions were calculated using an average body weight of 17 g at study day 7 (Model 1002) and 24 g at study day 25 (Model 1004) according to FVB mouse growth charts. Daily subcutaneous injections were given at the same time of the day (± 2 h) and based on the most recent body weight. The body length was measured weekly. At day 35 the mice were anesthetized and the length of the spine (lateral view, distance from the most cranial end of L1 vertebral body to the most caudal end of the L6 vertebral body and accounting for curvature manually), the right femur (distance from the most proximal femoral head ossification center to the most distal ossification center in dorso-ventral view) and the right tibia (distance from the most proximal ossification center to the most distal ossification center in dorso-ventral view) was determined by

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digital X-Ray (Faxitron, MX20). Treated groups were compared with corresponding vehicle control groups. Statistical analysis was performed using a one-factor ANOVA model for the logarithm of length. The 4-level factor comprised all combinations of treatment (vehicle, CNP38) and administration route (AP, s.c.). The relative change from control was derived from the estimated treatment differences in the model. Data were analyzed using SAS 9.4 software (SAS institute, Cary, United States).

Design of Cynomolgus monkey PK and efficacy study. Groups of 4 male cynomolgus monkeys were administered 0, 40 or 100 μ g/kg TransCon CNP once weekly for 26 weeks by the s.c. route. An additional group of 4 animals received the daily CNP-39 molecule (positive control), once daily for 26 weeks. The monkeys were of Chinese origin and received from RMS Houston, Houston, TX, USA. The animals were acclimatized for 5 weeks prior to study start. The animals were 25-29 months old and weighed between 2.0 kg and 3.1 kg at start of dosing and hence skeletally immature. Animals were socially housed (up to 2 animals of same sex and same dosing group together). PMI Nutrition International Certified Primate Chow No. 5048 was provided twice daily, except during designated procedures. The chow was provided in amounts appropriate for the size and age of the animals. Target temperatures of 20°C to 26°C with a relative target humidity of 30% to 70% were maintained. A 12-h light/12-h dark cycle was maintained, except when interrupted for designated procedures. Animals were sacrificed 7 days after the last dose administration.

Pharmacokinetics in cynomolgus monkey. The pharmacokinetic (PK) profile of CNP released from the TransCon CNP prodrug was investigated and compared to that of daily administered CNP-39 molecule, during the time course of a 24-week juvenile monkey efficacy study (study design described above). Blood samples for CNP-38 analysis were collected and analyzed

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following the first and the 26th dose at the following timepoints: 0 (prior to dosing), 6, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h post-dose. Samples from animals treated with the daily CNP-39 molecule were also obtained after the first and the 26th dose; at pre-dose, 0.08, 0.25, 0.5, 1, 2, and 4 h post-dose.

For analysis of free CNP-38, blood was collected into pre-cooled lithium heparin tubes, mixed, maintained chilled on wet ice. Within 60 seconds of collection, 250 μ L heparinized blood was transferred into a polypropylene tube kept on ice containing 50 μ L acidic citrate buffer (0.5 M sodium citrate pH 4.0). After mixing, acidified plasma was generated via centrifugation at 4 °C. Free CNP-38 was quantified after plasma precipitation, weak cation exchange (WCX) solid phase extraction (SPE), and subsequent HPLC-MS/MS analysis with deuterated CNP-38 as internal standard. The standards (150 μ L) (calibration range 2 to 250 pmol/L in blank Li-Heparin cynomolgus monkey plasma, LPT GmbH & Co. KG, Hamburg, Germany, acidified with 0.5 M sodium citrate pH 4.0 (5/2, v/v)), the QCs, and the acidified monkey plasma samples (150 μ L) were precipitated with 150 μ L 0.2 % formic acid in methanol containing the internal standard. After centrifugation at ambient temperature the supernatant was transferred to a 96 well plate. The transferred supernatant was diluted with 100 μ L 0.01 M methionine in water. The so far processed standards, QCs, and samples were applied to pre-conditioned cavities of a WCX SPE well plate (SOLA μ WCX 2mg/1mL, Thermo Scientific 60209-004) and centrifuged. Washing was performed by centrifugation using 30 μ L 0.01 M methionine in water followed by two times 30 μ L 0.01 M methionine in water/acetonitrile (80/20, v/v). Elution was performed using three times 30 μ L 11.4 % trifluoro acetic acid (TFA) in water/acetonitrile (~1/4/4 (v/v/v)). The solutions were then evaporated until dryness in a stream of nitrogen. The residues were reconstituted with 50 μ L water/methanol/Formic acid (11/8/1 v/v/v) and subsequently analyzed

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by HPLC-MS/MS. Chromatography was carried out on an Agilent 1290 Infinity (Agilent Technologies) with water plus 0.2 % Formic Acid (FA) and acetonitrile plus 0.2 % FA as eluents with post-column infusion of 0.25 % DMSO in water as supercharger. MS/MS analysis was done with selected MRM transitions for CNP-38 (ESI, positive mode, m/z 581.1. to 656.2) and the deuterated internal standard (m/z 585.4 to 659.4) on an AbSciex 6500+ QTrap (AbSciex, Darmstadt, Germany). The concentration of free CNP in study samples was calculated from a calibration curve (linear regression, $1/x^2$ weighted). The lower limit of quantification (LLOQ) of the assay was 2 pmol/L.

The daily CNP-39 molecule was measured using a commercially available competitive radioimmunoassay (RKU-012-03, Phoenix Pharmaceuticals, Germany). The protocol provided by the manufacturer was used to qualify the assay using Li-Heparin plasma from cynomolgus monkey. All samples (20 μ L) were diluted in 180 μ L radio-immunoassay (RIA) buffer supplied and incubated with the supplied rabbit anti-CNP antibody (100 μ L) for 5 h at room temperature. All samples were subsequently incubated with the supplied iodine tracer (100 μ L) and immunoprecipitation was carried out for 16-24 h at 5°C. On day 2, 100 μ L Goat Anti-Rabbit IgG Serum and 100 μ L Normal Rabbit Serum was added and samples were further incubated for 90 minutes at room temperature. Upon incubation, 500 μ L RIA buffer was added and the sample was centrifuged for 20 minutes at 3000 rpm (4°C). The supernatant was discarded by tube inversion. All tubes were immediately placed in a gamma-counter and measured for 5 minutes. Quantitation of study samples was carried out over an analytical range of 150-1080 pg/mL, using calibration standards of the daily CNP-39 molecule (75; 150; 300; 450; 600; 750; 900; 1080 pg/mL) and quality controls (250; 400; 800 pg/mL). Samples were analyzed across 10 analytical runs. Assay coefficient of variation (at each QC level) was below 20%. The quantification was

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performed using the radioactivity of the sample expressed in counts per minute. The calibration curve was fitted by non-linear regression using the 4- parameter logistic function (Watson/LIMS: logistic auto-estimate, no weighting).

The PK evaluation was performed by non-compartmental analysis (NCA) using the Phoenix® WinNonlin® Software.

Bone Markers. A panel of musculoskeletal endpoints was assessed during the 26 week treatment period, including biochemical markers of bone turnover (Bone formation markers: Bone Specific Alkaline Phosphatase (BAP; MicroVue™ BAP EIA Kit (Quidel cat # 8012)), Procollagen Type I N Terminal Propeptide (PINP; UniQ PINP RIA Kit (Orion Diagnostica, cat # 67034)), Osteocalcin (OC; MicroVue Osteocalcin EIA Kit (Quidel cat # 8001)) in serum, as well as the bone resorption markers C Telopeptides of Type I Collagen (CTxI; Serum CTx (CrossLaps ®)) ELISA Kit (Immunodiagnostic Systems (IDS), Cat #: AC-02F1) in serum, and N-Terminal Telopeptide (NTx; Osteomark ® NTx Urine ELISA Kit (Abbott, cat. no. X9006 or 9006)). The latter was measured in urine.

For the biochemical markers of bone turnover, animals were fasted overnight before blood sampling. Blood samples were collected between 08:00 and 10:00 a.m. prior to dosing and processed to serum. Animals were deprived of food during the urine collection procedure.

Musculoskeletal End Points. Aspects of bone quality and growth were assessed using a high resolution pQCT bone scanner (XtremeCT, Scanco Medical XtremeCT system II), digital radiography (tibia and femur lengths; Dragon Digital-X-ray System using VXvue, Viewworks), and morphometric measurements (body and tail length). XtremeCT, radiography and morphometry were performed on all animals under anesthesia once prior to initiation of dosing and at Week 4, 8, 13 and 26. Animals were given an intramuscular injection of Glycopyrrolate,

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Ketamine HCl Injection, U.S.P., and Dexmedetomidine prior to scanning, which was performed under isoflurane anesthesia. Atipamezole was administered (intramuscularly) following scanning to aid in recovery from anesthesia.

Bone quality measured by XtremeCT. XtremeCT was used to measure the volume of the right distal ulna growth plate in vivo and to measure overall bone quality, using the distal metaphysis and diaphysis of the right radius as a representative bone and bone location. Scans were performed using an XtremeCT system II (Scanco Medical). A volume of interest (VOI) was defined at the physis/metaphysis and at the diaphysis, contoured and analyzed. The reference line was placed at the distal aspect of the ulna, proximally to the styloid process, and included the widest part of the distal radius. For the radius metaphysis, the VOI was defined as all 125 slices. The first slice to contour on the radius was placed at a distance equal to 1% of the ulna length from the widest part of the distal radius and proximal to the growth plate. For the radius diaphysis, the VOI was defined as all 186 slices and was delimited using manual contouring of the first 2D slice, then followed by automatic contouring using the “Iterate forward” function on all slices. For the ulna growth plate, the VOI was defined as all contoured slices. Contouring was performed as follows: For the total region, with an outer threshold of 74 and an inner threshold of 200, manual contouring was done, with auto fit, on the larger part of the distal ulna, proximal to the growth plate. This contour was then applied to the larger part of the ulna, distal to the growth plate. Then, automatic contouring using the “morph” function was done between the two parts. For the distal region: automatic contouring on the distal ulna, distal to the growth plate, and for the proximal region: automatic contouring on the distal ulna, proximal to the growth plate, was applied. The evaluation method used is presented in Table 2, employing a three-dimensional constrained Gaussian filter with finite filter support (1 voxel) and filter width

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($\sigma = 0.8$). The images were subsequently binarized to separate the object from background using a global thresholding procedure.

Table 2

Evaluation Analysis Parameters for XtremeCT Data

Bone Length Measurements. For the physical measurements of body length, the animal was placed on its back with the head straight and hindlimbs extended (with toes up) and measurements were done using a measuring tape from the top of the head to the heels. The tail length was measured from the base of the tail ventrally to the tip of the tail. Measurements were recorded in cm with one decimal place.

Digital radiographs of the right femur/tibia and the right radius/ulna were obtained in ventro-dorsal view (one view for both sides, including the pelvis). Furthermore medio-lateral views were taken of the right femur/tibia and a cranio-caudal view was taken for the right radius/ulna. Radiographs were evaluated qualitatively and consisted of an evaluation of the bone including assessment of the growth plate. Physeal closure (right proximal tibia and distal femur) was scored as follows: 0: widely open physis, 1: open physis, 2: physis partially closed, 3: physeal scar, 4: physis closed. Tibia and ulna lengths were derived from the digital radiographs using the following landmarks: Ulna: olecranon to styloid process and tibia: most proximal ossification center to the most distal ossification center.

Growth parameters were compared across groups using an ANCOVA model of the relative change from baseline including treatment as a fixed effect and the baseline value as a covariate. Group estimates and contrasts from this model are presented with 95%-confidence intervals.

Histomorphometry. Growth plate histology was evaluated in the proximal tibial growth plate obtained from control and treated animals at terminal sacrifice. The epiphyseal plate width of the proliferative and hypertrophic zones was derived from tibial H&E (Hematoxylin and Eosin stain) sections from controls and treated animals sacrificed following 26-weeks of treatment. One coronal H&E section of 4 μ m in thickness and approximately 2 cm in length was taken from the proximal tibia at the level of the medial collateral ligament to include both the articular cartilage, epiphysis, growth plate, metaphysis and diaphysis. The proliferative zone was defined as the region of chondrocytes displaying a flattened-disc morphology and extending to the level where the cell assumed a more rounded cellular morphology. The hypertrophic zone was defined as the region beginning with the consistent cylindrical cellular profile and extending to the metaphyseal chondro-osseous junction. The width of the hypertrophic zone was calculated using an Image-Pro Plus image analysis system (MediaCybernetics).

Results

In vitro screening of CNP prodrugs: NEP resistance, activity and NPR-C affinity

It was proposed by Oefner et al. (2000) that substrates of the NEP protease (like CNP) have a size limit of < 3 kDa due to the restricted volume of the active site of the enzyme. Wendt et al. (2015) confirmed short-term in vitro NEP resistance (140 min exposure) of several CNP variants exceeding the 3 kDa range, like e.g. CNP-38 (4.1 kDa) or N-terminally PEGylated 2 kDa PEG CNP-22 (4.2 kDa). In our in vitro NEP resistance studies conducted over several days, CNP-38 was degraded slowly with a half-life of approximately 13 h (Table 3). In order to further improve NEP resistance, a 5 kDa PEG was attached to the N-terminus of CNP-38 which resulted in almost complete resistance against NEP proteolysis (Table 3). In contrast, a 5 kDa PEG

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moiety to the Lys26 side chain of the ring part of CNP rendered the CNP molecule less resistant to proteolysis, resulting in a half-life of 65.4 h. Initial NEP proteolysis was reported at the Cys6-Phe7 bond of CNP-22 (Watanabe et al., 1997). This is not supported by our findings, as the corresponding Cys22-Phe23 bond of CNP-38 should be protected more efficiently by the PEG attachment at the nearby Lys26 side chain, compared to the N-terminus (Leu1). Increasing the PEG size from a 5 kDa moiety to a more bulky 40 kDa branched PEG (4x10 kDa PEG) led to full NEP resistance of the Lys26 side chain conjugated CNP-38.

The biological activity of the various CNP conjugates were further evaluated in vitro in a cell-based assay. Conjugation of the 5 kDa PEG at the N-terminus of CNP-38 did not fully prevent NPR-B binding and resulted in 14% residual potency. In contrast, conjugation of the 5 kDa PEG to the Lys26 side chain of CNP-38 effectively reduced potency to below 1 % (Table 3). This is not surprising as Lys26 is part of the Leu-Lys-Leu motif of the CNP intramolecular ring structure that was reported by Furuya et al. (1992) as essential for CNP binding to NPR-B and subsequent activation of the cGMP signaling pathway. For the 4x10 kDa PEG Lys26 side chain conjugate (TransCon CNP) a low activity of < 1 % was also evident.

The affinity of the CNP-38 PEG conjugates to the NPR-C receptor was screened for in a competitive ligand binding cell assay. PEG 5 kDa conjugation to the N-terminus or Lys26 side chain of CNP-38 did not lead to reduced affinity to NPR-C compared to CNP-38. He et al. (2005) have shown that the NPR-C receptor interacts with the intramolecular ring structure of CNP-22. The four N-terminal amino acids of CNP-22 are largely disordered and are pointing away from the binding cleft and hence do not impact binding to NPR-C. Consequently, further screening focused on PEG conjugates of the Lys26 side chain of CNP-38. Using larger, e.g. 40 kDa PEG conjugates reduced affinities of the CNP-38 PEG conjugates were obtained and were

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furthermore strongly influenced by the architecture of the PEG molecule. An affinity of <1% was obtained for a branched 40 kDa PEG conjugate with the CNP attachment site close to the branching site of the four 10 kDa arms (4x10 kDa PEG) (Table 3). In contrast, a 4x10 kDa PEG conjugate with CNP-38 attached away from the branching site, at the distal ends of each the 10 kDa arms (C(10 kDa PEG CNP-38)₄), did not significantly reduce affinity to NPR-C. Keeping the attachment site close to the branching point but decreasing the number of arms from four to two (2x20 kDa PEG) had less effect on the NPR-C receptor affinity compared to the 4x10 kDa PEG conjugate.

Table 3

In vitro NEP resistance, activity and NPR-C affinity of CNP-38 conjugates

Pharmacokinetics of TransCon CNP and a daily CNP-39 molecule in Cynomolgus Monkey.

The daily CNP-39 molecule was rapidly absorbed into the systemic circulation following s.c. administration to cynomolgus monkeys with a T_{max} ranging between 5 min and 1 h postdose. C_{max} was followed by a rapid decline with an associated half-life of approximately 21 minutes. The last measurable time point was 2 h post dose (LLOQ 37 pmol/L). Based on the half-life, only approximately 1.5% of C_{max} was estimated to be still in the systemic circulation at 4 h post-dose. Following repeated daily administration of the daily CNP-39 molecule, an apparent increase in both C_{max} and half-life, was observed during the time course of the study; from the first dose administration till the last administration in week 26. Following administration of TransCon CNP, released CNP-38 was measured in the systemic circulation following the first and 26th dose. On both dosing occasions CNP-38 was quantifiable for up to 168 h post-dose at

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both 40 and 100 μg CNP/kg. The main PK parameters from the first dose administration are presented in Table 4.

Table 4

Mean Pharmacokinetic Parameters of CNP (after first dose) released from TransCon CNP following administration of TransCon CNP compared to the daily CNP-39 molecule in Monkeys.

As indicated above, CNP-38 released from TransCon CNP has a half-life of ca. 90 h, indicating that sustained increased systemic CNP levels are achieved in cynomolgus monkey following weekly dosing with TransCon CNP. The systemic peak levels of CNP-38 following administration of TransCon CNP (100 $\mu\text{g}/\text{kg}$) was estimated to be almost 100-fold lower than the C_{max} for the daily CNP-39 molecule (20 $\mu\text{g}/\text{kg}$). In contrast to the daily CNP-39 molecule which was cleared rapidly from the systemic circulation, resulting in a dose holiday of close to 20 h, the systemic CNP-38 level following s.c. TransCon CNP administration was above the LLOQ of the assay (2 pmol/L) throughout the 168-h sampling period.

Cardiovascular Assessment in Mice. Dose-dependent reductions in BP were observed in mice administered a s.c. dose of 800 $\mu\text{g}/\text{kg}$ unconjugated CNP-38. The lowest MAP was recorded at 2 h post-dose and subsided around 12 h post-dose and no effect on HR was observed (Figure 1). No TransCon CNP-related effects on BP or HR were recorded at a dose level of 800 μg CNP/kg administered as the TransCon CNP prodrug (Figure 1).

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Figure 1

Mean Arterial Blood Pressure (MAP) Following a Single Subcutaneous Administration of Vehicle, CNP-38 (800 µg CNP/kg) or TransCon CNP (800 µg CNP/kg) to Mice

Cardiovascular Assessment in Cynomolgus Monkeys. In cynomolgus monkeys, a single s.c administration of TransCon CNP at 10, 40, and 100 µg CNP/kg or the daily CNP-39 molecule at 50 µg CNP/kg resulted in no changes in systemic BP, HR, ECG parameters or body temperature up to 48 or 24 h post-dose, respectively. Three of the four male monkeys were successfully administered the daily CNP-39 molecule at 100 µg CNP/kg, whereas one animal received only 60 µg CNP/kg. The remaining 3 animals, that received the full dose of CNP-39, had decreased SAP from 19 to 28% between 0.25 and 1.25 h post-dose (from 19 to 28% compare to pre-dose). Exposure measured as total prodrug (data not shown) was confirmed in all animals administered TransCon CNP at 48 and 168 h post-dose, with the exception of 2 animals administered 40 µg CNP/kg for which the exposure, for unknown reasons, could not be confirmed. In the remaining animals, the plasma concentrations of the TransCon CNP prodrug were similar across animals with respect to the dose levels and time points. The effect on SAP is presented below (Figure 2).

Figure 2

Systolic Arterial Blood Pressure (SAP) Following a Single Subcutaneous Administration of TransCon CNP and Daily CNP-39 molecule to Cynomolgus Monkeys compared to vehicle control

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Stimulation of bone growth by daily s.c. injection or continuous infusion of CNP-38 in mice. Three-week old male FVB mice (n=9) received CNP-38 at a dose of 203 $\mu\text{g/kg/d}$ either as a daily subcutaneous bolus injection or as continuous s.c. infusion by osmotic Alzet pump. CNP-38 administered via both routes were generally well tolerated and animal body weights developed according to historical FVB mouse growth charts (data not shown). Weekly measurements showed a sustained growth measured as animal body length during the entire study period, following both daily s.c. bolus injection and by continuous s.c. infusion (data not shown). CNP-38 given as a daily bolus elicited significant growth in the appendicular and axial skeleton (Table 5).

Table 5

Bone length (mm [95% CI] (% change from control)) determined by X-ray after 5 weeks of daily bolus injection or continuous infusion of CNP-38 or vehicle control in FVB mice

Applying the same amount of CNP-38 per day as a continuous subcutaneous infusion lead to an even more pronounced growth effect, and significant increases in responses on several growth parameters were observed as compared to the daily s.c. bolus injection (Table 5).

Efficacy in monkey

Biomarkers of Bone Turnover. Over the 26-week study period, control animals generally presented slight increases in bone formation markers associated with stable values or slight decreases in bone resorption markers; indicating bone turnover in favor of bone formation (data not shown). During the treatment period, TransCon CNP at 40 $\mu\text{g CNP/kg/week}$ did not show any meaningful changes in serum BAP, PINP or OC levels. Animals administered TransCon CNP at 100 $\mu\text{g CNP/kg/week}$ or the daily CNP-39 molecule at 20 $\mu\text{g CNP/kg/day}$ on the other

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hand presented increases in serum BAP and PINP levels at week 26 as compared to controls (Table 6). No meaningful impact of TransCon CNP or treatment with the daily CNP-39 molecule was observed on serum OC and CTxI levels or urine NTx levels (data not shown).

Table 6

Percent increase in bone formation markers BAP, PINP and OC and bone resorption markers CTx and NTx compared to controls following weekly TransCon CNP or daily CNP-39 molecule administration for 26 weeks to cynomolgus monkeys

Bone growth. During the treatment period, there were no meaningful treatment-related changes in growth plate closure at the right femur, tibia and radius/ulna (proximal and distal physis). Compared to the observed growth in control animals, body length was increased by 5% and 3%, and tail length by 9% and 3%, at the end of treatment, for TransCon CNP and the daily CNP molecule, respectively (Table 7).

Table 7

Estimated relative effects of CNP treatment on growth of body, tail, tibia and ulna (relative increase in growth compared to vehicle treatment [% (95% CI)] in monkeys)

A linear growth over the 26-week treatment period was evident for both tibia and ulna (Figure 3A) for all dose groups, as expected for growing juvenile monkeys. Weekly dosing of TransCon CNP to the skeletally immature cynomolgus monkey at a dose of 40 µg CNP/kg/week generally resulted in higher values for tibia length (most notably at week 26), as well as in body and tail lengths compared to controls. At 100 µg CNP/kg/week, significant increases in tibia length were

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observed (Figure 3B) amounting to a 6% increased length of tibia at week 26 compared to control animals (Table 7). Administration of the daily CNP-39 molecule resulted in an increase in tibia length of 3% at week 26 compared to control.

Animals treated at 100 µg CNP/kg/week also exhibited an increase in right ulna length of 3%, whereas no meaningful changes in ulna length were observed in animals administered the daily CNP-39 molecule or 40 µg CNP/kg/week group as compared to control animals.

Figure 3

Growth from baseline of body, tail, tibia and ulna following weekly administration of TransCon CNP to monkeys (n=4/group) compared to the daily CNP-39 molecule (positive control) and vehicle treatment (negative control); A) for duration of study (26 weeks), and B) comparing relative change from baseline ($\pm 95\%$ CI) at week 26

Bone Quality (XtremeCT). In TransCon CNP and daily CNP-39 molecule treated animals, no meaningful changes in the bone quality parameters were observed. Likewise, the distal ulna growth plate volume was not impacted by weekly TransCon CNP or daily CNP-39 molecule treatment (data not shown). At the distal radius metaphysis, controls presented signs of bone consolidation as expected for a growing animal evidenced by slight increases in the apparent density of the total region (vBMD) as assessed by XtremeCT (data not shown). In the trabecular bone compartment there were slight increases in the overall bone volume fraction (BV/TV) and in vBMD, characterized by slight increases in trabecular number, thickness and separation (Table 8).

Table 8

Summary of XtremeCT Values (Mean±SD) as Percentage Change from Acclimation Period (n=4) after weekly s.c. administration of TransCon CNP or daily CNP-39 molecule for 26 weeks

In the cortical compartment of the distal metaphysis of the radius slight increases in cortical thickness were associated with slight decreases in tissue bone mineral density and vBMD (Table 8).

In the distal radius diaphysis (Table 8), controls presented signs of bone growth as evidenced by slight increases in cortical thickness associated with marginal decreases in BMD and slight decreases in vBMD. Other parameters investigated remained stable over time. Controls presented slight increases in the distal ulna growth plate volume over time (data not shown).

Histomorphometry. TransCon CNP at 100 µg CNP/kg/week and the daily administered CNP analogue at 20 µg CNP/kg/day induced statistically significant higher mean proliferative or hypertrophic zone widths of the proximal tibial physis compared to control animals (Table 9). In comparison to control animals, the width of the proliferative zone for TransCon CNP was increased by 37% compared to 16% for the daily CNP-39 molecule. The width of the hypertrophic zones was similar for the weekly TransCon CNP and the daily CNP-39 molecule and amounted to an increase in width of 38-39% compared to control animals. These higher mean proliferative and hypertrophic zone widths correlated microscopically with an increased cellularity of the proliferative and hypertrophic zones, respectively and a statistically significant increase of 14% in the thickness of the growth plate for TransCon CNP treated animals (Figure

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4). There were no statistically significant histomorphometry changes in the proximal tibial physis of monkeys administered TransCon CNP at 40 µg CNP/kg/week.

Table 9

Histomorphometry data of the proximal tibial growth plate following weekly administration of TransCon CNP compared to the daily CNP-39 molecule for 26 weeks to monkeys [% change from vehicle control]

Figure 4

Histomorphometry imaging of the proximal tibial physis following weekly administration of TransCon CNP compared to vehicle control for 26 weeks to monkeys

Safety

During the 24-week treatment period no abnormal clinical signs were observed for any of the treatment regimens, including observations before and after C_{max} for both the weekly and the daily CNP-39 molecule. No changes in standard clinical chemistry and hematology were observed and no impact on serum ANP levels were observed. Furthermore, histopathological assessment by H&E (ICH recommended organ list) did not suggest any adverse effects of CNP.

Discussion

Based on the presented in vitro screenings, the CNP-38 PEG 4x10 kDa conjugate TransCon CNP was selected for clinical development. This prodrug of CNP-38 exhibited an almost complete

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resistance against NEP proteolysis and very low NPR-C affinity and low ability to activate NPR-B. The interaction with NPR-B and NPR-C was markedly reduced by attaching the PEG carrier to the Lys26 side chain of CNP-38, which is part of the intramolecular ring structure that interacts with the active binding site for both receptors (Igaki et al., 1998). A 5 kDa PEG attached to CNP-38 was found sufficient to reduce binding to and activation of NPR-B, whereas a four arm PEG 4x10 kDa moiety with the CNP attachment close to the PEG branching site was required to additionally minimize NPR-C binding. This is in agreement with the crystal structure of the NPR-C CNP complex, that shows only weak interaction between CNP's Lys26 side chain, but stronger interactions between other hydrophobic motifs of the peptide and the receptor (He et al., 2005) which also binds A- and B-type natriuretic peptides. NEP resistance could be achieved through attachment of a 40 kDa PEG carrier to the Lys26 side chain (and may also be beneficial for the protection against potential other proteolytic pathways.)

The extent of the growth of tibia, femur and spine in the FVB mice after bolus injection of CNP-38 at 203 $\mu\text{g/kg/day}$ (50 nmol/kg/d) is comparable to the results reported for 287 $\mu\text{g/kg/day}$ (70 nmol/kg/day) bolus injection of vosoritide, employing a similar experimental design (Wendt et al., 2015). Overall growth effects on femur, tibia and spine were much stronger when CNP-38 was applied as a continuous subcutaneous infusion compared to a daily s.c. bolus injection indicating improved efficacy as a result of continuous exposure. For tibia and spine the continuous infusion of CNP more than doubled the effect, compared to daily bolus administration and hence intermittent exposure of CNP-38, suggesting that bone growth, even in a healthy animal with normal endogenous CNP levels, could be greatly accelerated by sustained systemic or local CNP exposure. Yasoda et al. (2009) tested the impact on bone growth of continuous infusion of CNP-22 in 3-week old mice. Only intravenous infusion of high doses of

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CNP-22 (1 $\mu\text{g/kg/min}$ = 655 nmol/kg/d) led to significant effects on bone growth, whereas subcutaneous injections of similar doses of CNP-22 did not produce significant effects. This discrepancy between the effect of s.c. dosing of CNP-38 and CNP-22 can be explained by the relatively fast inactivation of the CNP-22 by NEP in mice compared to the increased stability of CNP-38 towards NEP shown in vitro.

Also, in healthy juvenile monkeys TransCon CNP dosed s.c. once weekly gave rise to significant increases in growth based on several morphometric outcomes (body, tail and long bones (tibia and ulna)). The effect on ulna was only observed for TransCon CNP and varied markedly between animals, which may explain why no significant change in ulna growth plate volume was observed in the current study by XtremeCT. Wendt et al. (2015) likewise reported lack of a significantly increased volume of the right proximal tibial growth plate, following treatment with vosoritide in the same dose range as applied in the current study, whereas tibial growth plate expansion was evident when measured at the histologic level. This further suggests that the different methodologies employed in assessing growth plate dynamics vary greatly in their ability to pick up growth-promoting effects at the growth plate level.

At 100 $\mu\text{g/kg/week}$ TransCon CNP the observed relative growths were at least comparable to that observed for 20 $\mu\text{g/kg/day}$ CNP-39 (observed ratios between relative growth outcomes were 1.4-3.3), and this despite the lower overall weekly dose of TransCon CNP (100 μg) relative to the accumulated CNP dose following daily administration of 20 μg CNP-39. Based on the histomorphometric analysis of the tibial growth plate, a more marked effect of TransCon CNP on the proliferating zone giving rise to an overall greater and significant expansion of the growth plate thickness as such, do suggest that continuous exposure to CNP may afford a better effect on the growth plate and thereby on bone growth, relative to intermittent CNP exposure; hereby

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confirming the observations from the murine continuous s.c. infusion study described above. The associated increases in the bone formation markers BAP and/or PINP, most likely due to osteoblast mediated increase in the subphyseal bone observed in the proximal tibial bone following both TransCon CNP (100 µg CNP/kg/week) and daily administration of the daily CNP-39 molecule (20 µg CNP kg/day), suggest that BAP and/or PINP could be used as noninvasive markers for bone growth mediated by a CNP molecule.

In comparison to endogenous CNP-22 which has a half-life of 2-3 min, CNP-38 released from TransCon CNP exhibits a half-life in monkeys and in humans (Ascendis_Pharma, 2018) of approximately 90 h. This pronounced extension in half-life of this otherwise extremely labile peptide underlines the excellent features of the TransCon platform. The TransCon technology platform has been clinically validated across 3 different products; TransCon hGH, for treatment of growth hormone deficiency (Phase 3); TransCon PTH for hypoparathyroidism (Phase 1) and TransCon CNP for treatment of comorbidities associated with ACH (Phase 1).

As indicated above, TransCon CNP gives rise to close to 100-fold lower C_{\max} compared to the CNP-39 molecule. High concentrations of CNP cause vasodilation, and decreased blood pressure in animals (Lopez et al., 1997; Wendt et al., 2015) and IV bolus administration of CNP-22 in humans induces post-dose hypotension coinciding with the maximum blood concentrations (C_{\max}), indicating a causal relationship (Igaki et al. 1998). The hypotensive effect of high systemic CNP concentrations is speculated to be mediated either via the normal paracrine effect of CNP on the vascular smooth muscle cells via natriuretic peptide receptor C (NPR-C) interaction (Lopez et al., 1997; Chauhan et al., 2003) or directly via natriuretic peptide receptor B (NPR-B) activation in endothelial cells but could also result from competitive inhibition of e.g.

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ANP clearance via NPR-C (Igaki et al., 1998), or be a chronotropic effect on the sinoatrial node suggested to be mediated by NPR-B (Beaulieu et al., 1996; Beaulieu et al., 1997).

By avoiding high peak CNP levels, a sustained-release CNP construct such as TransCon CNP holds great promise for reduced cardiovascular side effects compared to daily CNP bolus administration. Studies in mice confirmed that hemodynamic tolerability is greater when CNP-38 is conjugated to TransCon compared to unconjugated CNP-38. Furthermore, the hemodynamic effect caused by unconjugated CNP-38 and the daily CNP-39 molecule generally occurred within the first h after dosing and did not occur in all animals. In agreement with previously reported data for CNP variants and the expected vasodilatory response of CNP, administration of unconjugated CNP-38 and the daily CNP-39 molecule resulted in decreased BP (Clavell et al., 1993; Barr et al., 1996; Igaki et al., 1998; Scotland et al., 2005; Wendt et al., 2015). In contrast no adverse cardiovascular effects were observed following single or repeated weekly doses of TransCon CNP in cynomolgus monkeys at 100 µg CNP/kg. The good cardiovascular safety profile was confirmed in the first human clinical trial with TransCon CNP, where single doses up to 150 µg CNP/kg were administered to adult healthy volunteers without associated significant adverse cardiovascular effects (Ascendis_Pharma, 2018).

Overall TransCon CNP shows great promise in providing an efficacious and safe therapeutic modality in ACH, by providing continuous exposure to promote bone growth, while avoiding high systemic CNP concentrations that can induce cardiovascular side effects.

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ACKNOWLEDGMENTS

This work was supported by Ascendis Pharma A/S and Ascendis Pharma GmbH. The authors wish to thank Bettina Lust, Franziska Gille, Nicole Hassepaß, Nora Kaluza, Steffen Schröder and Tom Woods for the preparation of the CNP compounds, Bioassay GmbH, Heidelberg, for development and conduct of the activity and NPR-C affinity studies and staff at Charles River Laboratories, Montreal and Edinburgh, and at ENVIGO, Huntingdon for conduct of the in vivo studies.

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FOOTNOTES

This research was sponsored by Ascendis Pharma, where VMB, CER, PMH and MKH are employees of Ascendis Pharma A/S and UH, JZ, FF and AB are employees of Ascendis Pharma GmbH, and have received cash and equity compensation from Ascendis Pharma during their employment.

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LEGENDS FOR FIGURES

Figure 1. Mean Arterial Blood Pressure (MAP) Following a Single Subcutaneous Administration of either Vehicle, CNP-38 (800 µg CNP/kg) or TransCon CNP (800 µg CNP/kg) to Mice.

Footnote: MAP recorded up to 48 h post dose. Means (\pm SEM). *Significant difference from vehicle $P < 0.05$.

**Significant difference from vehicle $P < 0.01$. William's test comparing treatment groups with vehicle control at each time point.

Figure 2. Systolic Arterial Blood Pressure (SAP) Following a Single Subcutaneous Administration of TransCon CNP and Daily CNP-39 molecule to Cynomolgus Monkeys compared to vehicle control.

Footnote: SAP recorded up to 48 h post dose. A: Means. B: Baseline adjusted means (\pm SEM).

Figure 3. Growth from baseline of body, tail, tibia and ulna following weekly administration of TransCon CNP to monkeys ($n=4$ /group) compared to the daily CNP-39 molecule (positive control) and vehicle treatment (negative control); A) for duration of study (26 weeks), and B) comparing relative change from baseline ($\pm 95\%$ CI) at week 26.

Footnote: Estimates and test are derived from an ANCOVA model including treatment as a fixed effect and baseline value as a covariate. *Significant difference in absolute growth from negative control $P < 0.05$. Neg.Ctrl: Negative control; Pos.Ctrl: Positive control.

Figure 4. Histomorphometry imaging of the proximal tibial physis following weekly administration of A) vehicle control compared to B) TransCon CNP (100µg CNP/kg/week) for 26 weeks to monkeys.

Footnote: PF: Proliferative zone; HZ: Hypertrophic zone; PFi: Increased cellularity and width of proliferative zone

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HZi: Increased cellularity and width of hypertrophic zone

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TABLES

Table 1. CNP-38 conjugates used for in vivo and in vitro studies

CNP-38 conjugate (linker type, linker attachment site)	Description
5 kDa PEG-linker-CNP-38 (permanent, Lys26)	linear 5 kDa PEG carrier conjugated to the Lys26 side chain of CNP-38 via a permanent linker
5 kDa PEG-linker-CNP-38 (permanent, N-terminus)	linear 5 kDa PEG carrier conjugated to the N-terminus of CNP-38 via a permanent linker
4x10 kDa PEG-linker-CNP-38 (permanent, Lys-26)	4-arm branched 4x10 kDa PEG carrier conjugated to the Lys26 side chain of CNP-38 via a permanent linker ^a
2x20 kDa PEG-linker-CNP-38 (transient, Lys-26)	2-arm branched 2x20 kDa PEG carrier conjugated to the Lys26 side chain of CNP-38 via a TransCon Linker ^a
4x10 kDa PEG-linker-CNP-38 (transient, Lys26), TransCon CNP	4-arm branched 4x10 kDa PEG carrier conjugated to the Lys26 side chain of CNP-38 via a TransCon Linker ^a
C ^b (10 kDa PEG-linker-CNP-38) ₄ (permanent, Lys26)	Four CNP-38 molecules conjugated to a 4-arm branched 4x10 kDa PEG. One CNP-38 is attached via its Lys26 side chain and via a permanent linker to the distal end of each of the four PEG arms

^a The linker attachment site to the PEG carrier is close to the branching site of the PEG arms

^b C represents the central carbon atom which is the branching point of the four PEG arms.

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Table 2. Evaluation Analysis Parameters for XtremeCT Data

	Evaluation Ulna Growth Plate	Evaluation Radius Metaphysis	Evaluation Radius Diaphysis
Sigma	0.8	0.8	0.8
Support	1	1	1
Lower Threshold	95	150 (Total/cortical) and 110 (trabecular)	150
Upper Threshold	1000	1000	1000

Table 3. In vitro NEP resistance, activity and NPR-C affinity of CNP-38 conjugates

CNP-38 Conjugate	NEP Resistance	Activity^a	NPR-C Affinity^{b, c}
(linker type, attachment site)	Half-life [h]	[%]	[%]
CNP-38	12.6 ± 3.9 ^d	100	100
5 kDa PEG-linker-CNP-38 (permanent, N-Terminus)	Stable ^e	14	199 ± 12
5 kDa PEG-linker-CNP-38 (permanent, Lys26)	65.4	< 1 ^f	83 ± 7
2x20 kDa PEG-linker-CNP-38 ^g (transient, Lys26)	ND ^h	< 1 ^f	11 ± 4
4x10 kDa PEG-linker-CNP-38 ⁱ (permanent, Lys26)	Stable ^e	< 1 ^f	< 2 ^f
4x10 kDa PEG-linker-CNP-38 ⁱ (transient, Lys26) TransCon CNP	ND	< 1 ^f	< 1 ^{f, j}
C(10 kDa PEG-linker-CNP-38) ₄ ^k (permanent, Lys26)	ND	ND	68 ± 11

^a Activity to elicit intracellular cGMP response in a NIH3T3 cell assay, relative to CNP-38. ^b n = 2-3. ^c Ratio IC50

CNP-38 / IC50 CNP-38 conjugate. ^d n = 4. ^e Almost no degradation after 96 h. ^f Limitation due to assay range. ^g

Branched 40 kDa PEG carrier having the CNP attachment site near the branching site of two 20 kDa arms. ^h ND, not

determined. ⁱ Branched 40 kDa PEG carrier having the CNP attachment site near the branching site of four 10 kDa

arms. ^j Calculated from a 25x concentrated sample. ^k Branched 4x10 kDa PEG carrier having a total of four CNPs

attached. The CNP attachment site is not close to the branching site of the four 10 kDa arms, but at the distal end of each of the arms.

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Table 4. Mean Pharmacokinetic Parameters of CNP (after first dose) released from TransCon CNP following administration of TransCon CNP compared to the daily CNP-39 molecule in Monkeys

Group	Dose (µg CNP/kg)	T_{max}^a (h)	C_{max} (pmol/L)	AUC_(0-t) (h* pmol/L)	T_{1/2} (h)
Daily CNP-39 molecule	20/day	0.25	3850	2560	0.35
TransCon CNP	40/week	48	16.5	1748	92.6
TransCon CNP	100/week	24	39.4	4186	86.7

AUC: Area under the curve; ^aMedian T_{max} is presented

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Table 5. Bone length (mm [95% CI] (% change from control)) determined by X-ray after 5 weeks of daily bolus injection or continuous infusion of CNP-38 or vehicle control in FVB mice

	s.c. bolus injection		s.c. continuous infusion	
Measurement	Vehicle control	CNP-38 203 µg/kg/d	Vehicle control	CNP-38 203 µg/kg/d
Femur length ^a	14.2 [13.7;14.8]	15.0 [14.5;15.6] (5.5 %)*	13.6 [13.0;14.1]	14.6 [14.1;15.2] (7.1 %)*
Tibia Length ^b	17.9 [17.7;18.1]	18.7 [18.5;18.9] (4.0 %)*	17.6 [17.4;17.8]	19.8 [19.6;20.0] (12.2 %)*,#
Spine length ^c	53.8 [52.4;55.2]	59.8 [58.5;61.2] (11.3 %)*	53.6 [52.2;55.0]	67.0 [65.6;68.4] (25.0 %)*,#

95% CI; 95% Confidence interval of mean. *Different from vehicle control, #Different from growth under s.c. bolus injection (one-factor ANOVA model on logarithmic transformed data) P<0.05.

^a: right femur

^b: right tibia

^c: lateral view

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Table 6. Percent increase in bone formation markers BAP and PINP following weekly TransCon
CNP or daily CNP-39 molecule administration for 26 weeks to cynomolgus monkeys

	TransCon CNP 40 µg CNP /kg/week	TransCon CNP 100 µg CNP/kg/week	Daily CNP-39 molecule 20 µg/kg/day
No. Animals per Group	4	4	4
BAP	-	14 ^a	51 ^a
PINP	-	53 ^a	144 ^a

BAP; Bone Specific Alkaline Phosphatase. PINP; Procollagen Type I N Terminal Propeptide. ^aSignificantly
different from control group - P < 0.05, based on statistical analysis of group means

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Table 7. Estimated relative effects of CNP treatment on growth of body, tail, tibia and ulna
(relative increase in growth compared to vehicle treatment [% (95% CI)]) in monkeys

	TransCon CNP 40 µg CNP /kg/week	TransCon CNP 100 µg CNP/kg/week	Daily CNP-39 molecule 20 µg CNP/kg/day
Body	1.0 (-2.3; 4.4)	4.8 (1.1; 8.5)*	3.3 (0.0; 6.6)*
Tail	1.4 (-2.7; 5.5)	8.6 (3.7;13.4)*	2.8 (-1.3; 6.9)
Tibia	1.7 (-2.0; 5.4)	6.0 (2.1; 9.9)*	2.9 (-1.0; 6.8)
Ulna	0.9 (-2.8; 4.7)	2.7 (-1.1; 6.5)	0.8 (-2.9; 4.5)

95% CI; 95% confidence interval of the relative increased growth compared to growth in vehicle group.

*P<0.05

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Table 8. Summary of XtremeCT Values (Mean±SD) as Percentage Change from Acclimation Period (n=4) after weekly s.c. administration of TransCon CNP or daily CNP-39 molecule for 26 weeks

Treatment		Control	Daily CNP-39 molecule	TransCon CNP	
CNP dose		Vehicle	20µg/kg/day	40µg/kg/week	100µg/kg/week
Right Radius, distal Metaphysis - trabecular Region					
Trabecular Region vBMD	mg HA/cm ³	19.8±10.3	18.2±14.9	27.0±17.9	16.5±10.7
Trabecular Tissue BMD	mg HA/cm ³	-0.27±2.1	0.45±1.8	-0.12±1.1	1.7±0.83
Trabecular BV/TV	%	18.2±8.2	18.7±15.0	29.9±19.2	18.2±12.4
Trabecular Number	1/mm	13.2±11.6	5.8±6.0	12.9±8.8	8.9±5.3
Trabecular Thickness	mm	4.6±3.7	8.9±6.7	16.2±14.5	14.0±11.8
Trabecular Separation	mm	-14.6±8.4	-5.9±7.0	-12.0±9.0	-10.5±5.0
Right Radius, distal Metaphysis - cortical Region					

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Cortical Region vBMD	mg HA/cm ³	-5.2±1.2	-7.0±2.2	-8.7±2.9	-2.7±2.3
Cortical Tissue BMD	mg HA/cm ³	-3.8±1.4	-5.9±1.6	-5.9±1.2	-2.4±1.8
Cortical Thickness	mm	-3.6±16.0	-9.4±11.2	-16.7±11.5	4.5±10.7
Right Radius, distal diaphysis - cortical Region					
Cortical Region vBMD	mg HA/cm ³	-1.8±1.6	-3.3±1.6	-7.8±9.8	-2.4±2.3
Cortical Tissue BMD	mg HA/cm ³	-4.8±1.9	-6.7±1.4	-6.3±3.6	-3.7±1.6
Cortical Thickness	mm	7.0±3.0	8.2±5.0	1.1±8.4	8.9±4.7

BV: bone volume; TV: tissue volume; vBMD: apparent density of the total region

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Table 9. Histomorphometry data of the proximal tibial growth plate following weekly administration of TransCon CNP compared to the daily CNP-39 molecule for 26 weeks to monkeys [% change from vehicle control]

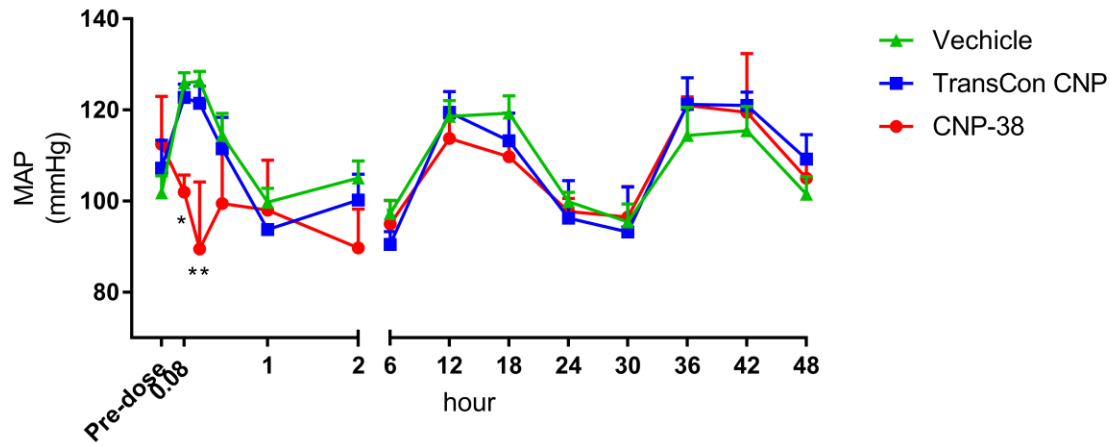
		Treatment	
		TransCon CNP	Daily CNP-39
		100 µg	molecule
		CNP/kg/week	20 µg/kg/day
No. Animals per Group	4	4	4
Bone, Tibia (No. measured)	4	4	4
Proliferative zone width	-2	37 ^a	16 ^a
Hypertrophic zone width	7	38 ^a	39 ^a
Epiphyseal plate thickness	-4	16 ^a	7

^a Significantly different from control group - $P \leq 0.05$, based on statistical analysis of group means

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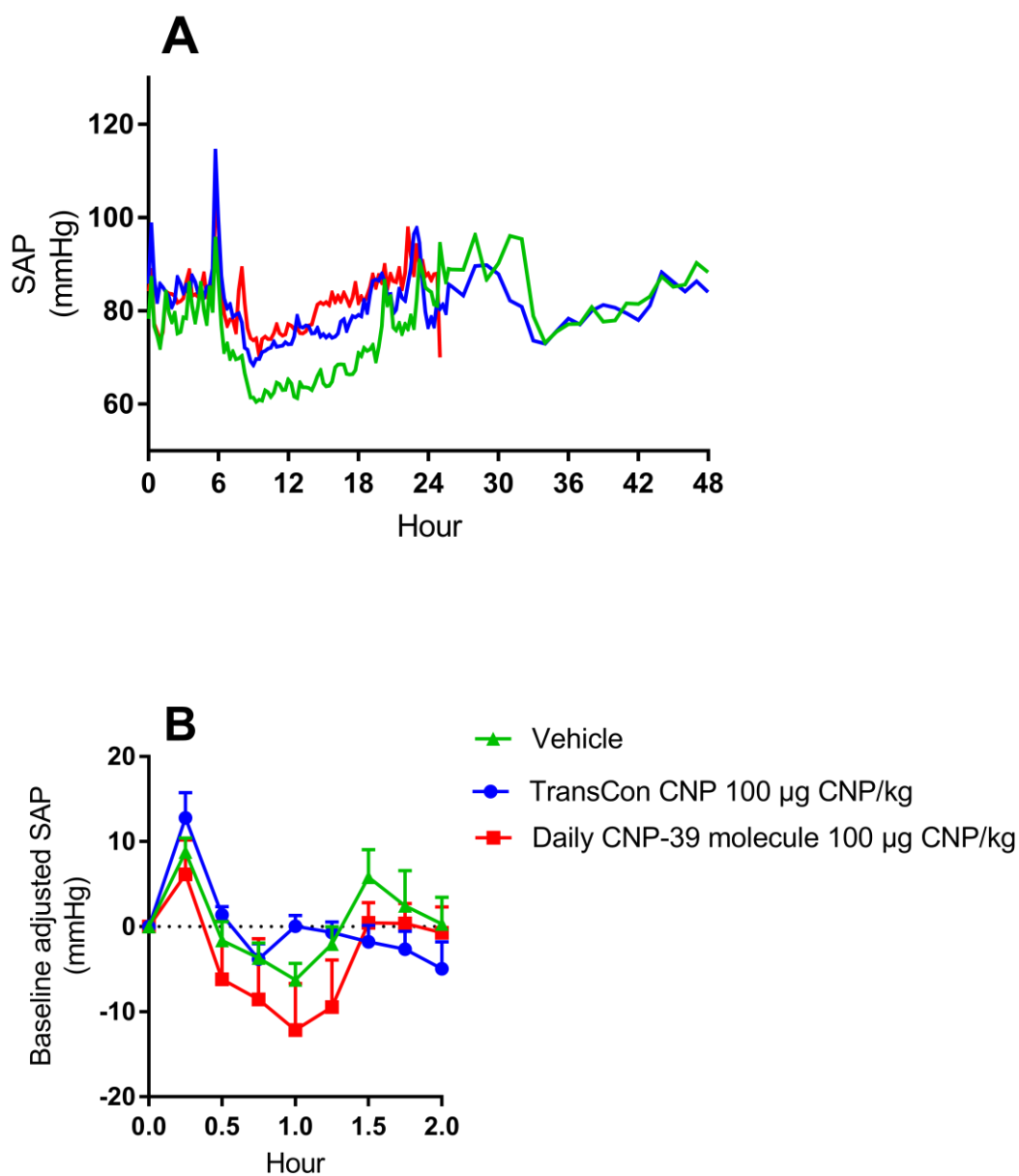
FIGURES

Figure 1



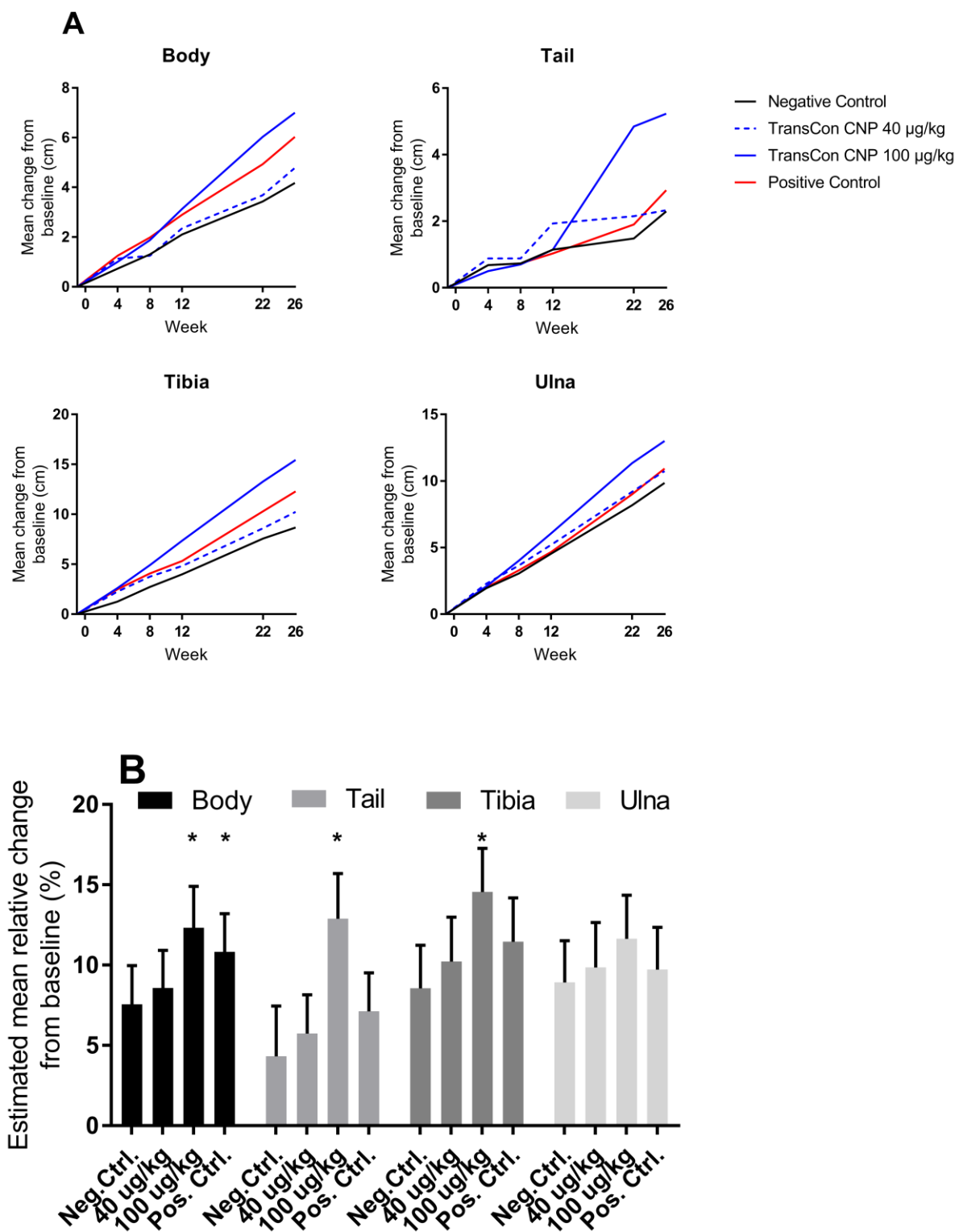
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Figure 2



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Figure 3



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Figure 4

