Neutral Endopeptidase-Resistant C-Type Natriuretic Peptide Variant Represents a New Therapeutic Approach for Treatment of Fibroblast Growth Factor Receptor 3–Related Dwarfism

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ABSTRACT
Achondroplasia (ACH), the most common form of human dwarfism, is caused by an activating autosomal dominant mutation in the fibroblast growth factor receptor-3 gene. Genetic overexpression of C-type natriuretic peptide (CNP), a positive regulator of endochondral bone growth, prevents dwarfism in mouse models of ACH. However, administration of exogenous CNP is compromised by its rapid clearance in vivo through receptor-mediated and proteolytic pathways. Using in vitro approaches, we developed modified variants of human CNP, resistant to proteolytic degradation by neutral endopeptidase, that retain the ability to stimulate signaling downstream of the CNP receptor, natriuretic peptide receptor B. The variants tested in vivo demonstrated significantly longer serum half-lives than native CNP. Subcutaneous administration of one of these CNP variants (BMN 111) resulted in correction of the dwarfism phenotype in a mouse model of ACH and overgrowth of the axial and appendicular skeletons in wild-type mice without observable changes in trabecular and cortical bone architecture. Moreover, significant growth plate widening that translated into accelerated bone growth, at hemodynamically tolerable doses, was observed in juvenile cynomolgus monkeys that had received daily subcutaneous administrations of BMN 111. BMN 111 was well tolerated and represents a promising new approach for treatment of patients with ACH.

Introduction
Achondroplasia (ACH), the most common form of human dwarfism with an estimated prevalence between 1 in 16,000 to 1 in 26,000 live births (Foldynova-Trantirkova et al., 2012), is an autosomal dominant condition with the majority of new cases (80%–90%) originating de novo from parents of normal stature.

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ABBREVIATIONS: ACH, achondroplasia; BMN 111, recombinant variant of C-type natriuretic peptide; BMN 1B2, chemically synthesized variant of C-type natriuretic peptide; BP, blood pressure; CNP, C-type natriuretic peptide; ECG, electrocardiography; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR3, fibroblast growth factor receptor-3; HR, heart rate; HSA, human serum albumin; IHC, immunohistochemistry; MAP, mean arterial pressure; MAPK, mitogen-activated protein kinase; MRI, magnetic resonance imaging; NEP, neutral endopeptidase; NPR, natriuretic peptide receptor; PBS, phosphate-buffered saline; PD, pharmacodynamics; PEG, polyethylene glycol; PEO, polyethylene oxide; PK, pharmacokinetics; sFGFR3, soluble fibroblast growth factor receptor-3; TDI, thanatophoric dysplasia type I; TDII, thanatophoric dysplasia type II.
regulation of chondrocyte proliferation and differentiation as well as decreased extracellular matrix synthesis (Murakami et al., 2004; Yasoda et al., 2004; Sebastián et al., 2011). In addition, stenosis of the foramen magnum and the spinal canal, caused by premature synchondrosis closure and fusion of ossification centers, is regulated by the same pathway (Hecht and Butler, 1990; Modi et al., 2008; Matsushita et al., 2009). Paracrine/autocrine factor C-type natriuretic peptide (CNP) signals through natriuretic peptide receptor (NPR) B and modulates the activity of FGFR3 through inhibition of the ERK/MAPK pathway at the level of rapidly accelerated fibrosarcoma protein kinase (RAF-1) (Krejci et al., 2005; Horton et al., 2007). CNP knockout mice, as well as those expressing mutant CNP receptors, exhibit dwarfism and have growth plates histologically similar to ACH (Rimoin et al., 1970; Naski et al., 1998; Chusho et al., 2001), whereas overexpression of CNP in mice (Kake et al., 2009) and humans (Bocciardi et al., 2007; Moncla et al., 2007) is characterized by skeletal overgrowth. The dwarfism in mice overexpressing Fgfr3 with a mutation analogous to human G380R (Fgfr3G72R/+) under the control of the type II collagen promoter is corrected by endogenous CNP overproduction (Yasoda et al., 2004) or the continuous infusion of exogenous CNP (Yasoda et al., 2009), giving credence to the hypothesis that systemic administration of CNP should stimulate growth in pediatric ACH patients with open growth plates.

CNP, expressed as a 126–amino acid protein precursor (prepro-CNP), is processed to an active 53–amino acid cyclic peptide by furin and further processed to a 22–amino acid peptide by unknown proteases (Potter et al., 2006). It has been reported that only the 17–amino acid cyclic domain residues (Cys6–Cys22 of CNP22), formed by an intramolecular disulfide linkage, are required for activity (Furuya et al., 1992). Native CNP (CNP22) is rapidly cleared from the circulation by the natriuretic clearance receptor (NPR C) and neutral endopeptidase (NEP) (EC 3.4.24.11; metalloendopeptidase; enkephalinase; neprilysin; CD10, CALLA) (Brandt et al., 1995, 1997). As a result, CNP22 has a short half-life in serum of less than 2 minutes in mice and humans, thereby requiring a lengthy infusion process to result in a pharmacological benefit (Hunt et al., 1994; Yasoda et al., 2009). In fact, mice given intravenous bolus or subcutaneous administrations of CNP22 demonstrated no pharmacological benefit.

We recently described the pharmacological activity of a 39–amino acid CNP variant (BMN 111; recombinant variant of C-type natriuretic peptide), which has an extended serum half-life due to its resistance to NEP digestion (Lorget et al., 2012). We demonstrated that daily subcutaneous administrations of BMN 111 in an ACH mouse model resulted in increased axial and appendicular skeletal lengths, improvements in dwarfism-related clinical features including flattening of the skull, straightening of the tibias and femurs, and correction of the growth plate defect. Here, we report the development of BMN 111, through in vitro and in vivo approaches, which is resistant to degradation by NEP and designed to elicit the growth-promoting effects of native CNP through a subcutaneous route of administration. We also examined the cardiovascular effects of BMN 111, since it is well established that natriuretic peptides, including CNP, induce vasodilation (Clavell et al., 1993; Charles et al., 1995; Igaki et al., 1998; Scotland et al., 2005; Pagel-Langenickel et al., 2007), and then evaluated the growth-potential at doses that were considered hemodynamically acceptable (<10% drop in blood pressure (BP) and <25% increase in heart rate (HR)) in mice and monkeys. This article focuses on the pharmacological effects of daily subcutaneous administrations of BMN 111 in mice (normal and ACH models) and normal juvenile cynomolgus monkeys.

Materials and Methods

Native CNP and Variants. Native CNP and variants were chemically synthesized using standard Fmoc chemistry (AnaSpec Inc., Fremont, CA; and GenScript USA Inc., Piscataway, NJ). Protein sequences for coded samples were as follows: NH2–GLSKGCFGLKLRDQSMSGLC–COOH (native CNP, CNP22), NH2–DLRVTSDKR–AAWARLQQEHPNarryKGANKKLCSGCFGLKLRDQSMSGLC–COOH (CNP53), NH2–GQEHPNARRYKGANKKLCSGCFGLKLRDQSMSGLC–COOH [BMN 1B2 chemically synthesized variant of C-type natriuretic peptide], NH2–GHKSEVAHRFKGANNKGLSCG–FGKGLKLRDQSMSGLC–COOH [chimeric peptide of human albumin (HSA) and CNP22; the HSA sequence (Ac P02768; amino acids 27–36) is underlined] [HSA27–36–CNP22], NH2–GQEHPNARRYKGANQQGLSKGCFLKLRDQSMSGLC–COOH [BMN 1B2 (QQ)], NH2–GERAFKAWARLSSLKGCFLKLRDQSMSGLC–COOH [chimeric peptide of human IgG and CNP22; the IgG sequence (Ac P02768; amino acids 231–245) is underlined] [HSA231–245–CNP22], NH2–GQERPAEQYVYTLPPGSLSKGCFLKLRDQSMSGLC–COOH [chimeric peptide of human IgG and CNP22; the IgG sequence (Ac P01857; amino acids 224–237) is underlined] [IgG224–237–CNP22], and NH2–GQERPAEQYVYTLPPGSKGCFLKLRDQSMSGLC–COOH [chimeric peptide of human IgG and CNP22; the IgG sequence (Ac P01857; amino acids 224–233) and the KK to QQ CNP22 variant sequence are underlined] [IgG224–233–QK224–237 (QQ) CNP27(QQ)]. BMN 111 was recombinantly expressed in Escherichia coli (Long et al., 2012) and has the following protein sequence: NH2–PGQEHPNARRYKGANKKLCSG–FGKGLKLRDQSMSGLC–COOH. CNP22 and all variant constructs have been oxidized to form one intramolecular disulfide bond. All peptides were ≥90% pure and masses were confirmed by liquid chromatography/mass spectrometry.

NEP Resistance. Native CNP (CNP22) and variants (100 μM) were incubated in the presence of purified recombinant human NEP (no. 1182-ZN-010, 1 μg/ml; R&D Systems, Minneapolis, MN) in phosphate-buffered saline (PBS) buffer at 37°C for 140 minutes. Throughout the incubation, a portion of the sample was removed and quenched with EDTA (10 mM). Reactions were reduced with dithiothreitol (10 mM) for 30 minutes at 37°C and then analyzed by liquid chromatography/mass spectrometry. All concentrations listed are final. Results were reported as percentage of intact peptide remaining compared with time zero. All assays were repeated at least once for candidates that demonstrated native potency.

Potency (cGMP) Assay. Potency was determined in a cell-based assay using murine NIH3T3 fibroblasts, which endogenously express NPR B and not the NPR A or NPR C receptors (Abby and Potter, 2003). Briefly, 50%–80% confluent fibroblasts were pretreated with a phosphodiesterase inhibitor (0.75 mM isobutylmethylxanthine) in Dulbecco’s modified Eagle’s medium/PBS (1:1) for 15 minutes at 37°C/5% CO2. Next, CNP22 or variants (10–11 M to 10–4 M) were added to the cells without media exchange in duplicate and incubated for an additional 15 minutes. Cells were detergent lysed (0.1% Triton X-100) and cGMP concentration was determined using a competitive immuno-based assay (CatchPoint; Molecular Devices, Sunnyvale, CA).

PEGylation. PEGylation reaction conditions were optimized to facilitate specific conjugation of polyethylene glycol (PEG) moiety at the NH2 terminus of CNP or its variant, such as CNP27. Briefly, N-hydroxysuccinimide–activated PEGs of varying size (NOF America Corporation, White Plains, NY and Thermo Fisher Scientific, Waltham, MA) were incubated with CNP22 or CNP27 at a 1:1 molar ratio in 0.1 M KPO4 pH 6 for 1 hour at room temperature. NH2-terminal lysines (i.e., nonring lysines) of CNP22 were changed to arginines to eliminate additional PEGylation sites without affecting NPR B binding and signaling activity (data not shown). Mono-PEGylated species were
purified by C5 reverse-phase high-performance liquid chromatography using an acetonitrile gradient containing 0.1% formic acid.

Pharmacokinetics. The pharmacokinetics (PK) profile of various CNP variants and their time courses of plasma cGMP concentrations were determined in 7- to 8-week-old male wild-type rats (Cry:CD (SD) IGS) or wild-type mice (FVB/nJ) (Charles River Laboratories, Inc., Wilmington, MA) after a single intravenous (20 nmol/kg in rats, n = 3; 25 nmol/kg in mice, n = 4) or subcutaneous (50 nmol/kg in rats, n = 3; 70 nmol/kg in mice, n = 4) administration. All peptides were formulated in 30 mM acetic acid pH 4.0 containing 10% sucrose and 1% benzyl alcohol. Plasma CNP immunoreactivity was determined using a competitive radioimmunoassay and a commercially available polyclonal antibody against the cyclic ring portion of CNP (Bachem, Bubendorf, Switzerland). Plasma cGMP concentration was determined by a competitive radioimmunoassay (Yamasa Corporation, Salem, OR).

Activity, Accumulation, and Clearance of BMN 111 at the Growth Plate. Mice were dosed and anesthetized at 15 minutes postdose, which was previously determined to coincide with the maximum cGMP response time, unless otherwise noted. Blood was collected from the heart via intracardiac puncture. Femurs with complete knee cartilage were harvested and immediately frozen in liquid nitrogen. Distal epiphysis sections from each mouse were separated for either cGMP or immunohistochemistry (IHC) experiments. For cGMP experiments, the epiphiyses were pulverized using a Covaris C200 cryoPREP tissue homogenizer (Covaris, Inc., Woburn, MA). cGMP was extracted from the frozen pulverized epiphiysis in PBS buffer containing 0.8 mM phosphodiesterase inhibitor (isosobutylmethylxanthine) and quantified by competitive enzyme-linked immunosorbent assay (CyclesPoint cGMP fluorescent assay kit; Molecular Devices). For IHC, tissues were fixed in 4% paraformaldehyde immediately after dissection, decalciﬁed in 10% formic acid/PBS until no calcium oxalate precipitate formed with 5% ammonium oxalate, then dehydrated, parafﬁn embedded, and sectioned at 7 μm. Sections were deparafﬁnized and rehydrated prior to antigen retrieval in 10 mM citrate (30 minutes, 80°C), then blocked (1% normal donkey serum, 0.1% bovine serum albumin, 0.1% NaNO3, 0.3% Triton X-100 in PBS; 1 hour, room temperature) and incubated in monoclonal CNP antibodies (4°C, overnight). Secondary donkey anti-mouse antibodies, conjugated to Alexa Fluor 488 were applied (1 hour, room temperature; Invitrogen, Carlsbad, CA). For quantification of signal intensity, confocal stacks were acquired using a Zeiss LSM 510 NLO laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with a 40× objective, 2× zoom, and a 0.53-μm z increment were used for IHC experiments. All experiments were performed in duplicate (n = 2).

Dose Regimen. Three-week-old wild-type (FVB/nJ; Charles River Laboratories, Inc.) male mice were given subcutaneous injections of BMN 111 (20 nmol/kg) daily on alternating weeks (weeks 1, 3, and 5) or vehicle [30 mM acetic acid pH 4.0 containing 10% sucrose (w/v) and 1% (w/v) benzyl alcohol] daily for 5 weeks (n = 10/group). Tail measurements were collected at study initiation. Growth was monitored during the in-life treatment period by weekly tail measurements. At necropsy, final X-ray and nose-ano tail and measurements were obtained. Long bones were collected and measured for length, and the femur and tibia were fixed for histology and archived.

Pharmacological Effects of CNP Variants in Wild-Type Mice. FVB/nJ wild-type mice (Charles River Laboratories, Inc.) were administered daily subcutaneous injections at varying dose levels (20–200 nmol/kg; n = 8/group) over 35 days. All CNP variants were formulated in vehicle [30 mM acetic acid buffer solution pH 4.0, containing 10% (w/v) sucrose and 1% (w/v) benzyal alcohol]. Mice (≥ 1 S.D. of the average body weight) were randomized at 3 weeks ± 2 days of age. Doses were given at approximately the same time each day, 2 hours prior to the dark cycle, and were based on the most recently collected body weight. The lengths of the tibia, femur, humerus, ulna, and lumbar vertebrae 5 were measured with a caliper. Treated groups were compared with the vehicle control group at common time points by analysis of variance with a post hoc Dunnett’s test (Dunnett and Crasafio, 1955) or other appropriate test.

Pharmacological Effects of BMN 111 in Fgfr3±/± mice. Fgfr3+/– mice were kindly provided by David M. Ornitz (Washington University in St. Louis, St. Louis, MO) and bred at Jackson Laboratories (West Sacramento, CA). Expression of activated Fgfr3 was targeted to growth plate cartilage using regulatory elements from the collagen 2 gene (Naski et al., 1998). Three-week-old Fgfr3+/– male mice (FVB/nJ, Fgfr3+/– JAX West, n = 8/group) were administered daily subcutaneous injections over 35 days (5, 20, and 70 nmol/kg). Fgfr3+/– mice and their wild-type littermates were anesthetized and randomized by body weight into treatment groups. Prior to the study, mice were monitored for body weight, general health, and tail length. On day 37, all mice were euthanized by terminal anesthesia. Left and right tibia, femur, humerus, and ulna were collected and measured using a digital caliper. The left bones were fixed in 10% neutral-buffered formalin overnight, and then stored in ethanol at 2–8°C.

Hemodynamic Effects of CNP Variants in Wild-Type Mice. Mouse studies were performed at LAB Research, Inc. (Durval, QC, Canada). An isoflurane gas–anesthetized mouse model was used to reduce background variability in hemodynamic readouts, and to provide greater sensitivity to reduction in BP by blunting the compensatory increase in HR. CNP variants were tested over a dose range of 20–200 nmol/kg (2000 nmol/kg additional dose for BMN 111). Mice (6- to 7-week-old FVB/nJ; Charles River Laboratories, Saint-Constant, QC, Canada) were anesthetized with isoflurane gas. A pressure-monitoring catheter connected to a telemetry transmitter (PA-C10 or PXT-C50; Data Sciences International, New Brighton, MN) was placed in the aorta for arterial BP measurements. The position of the catheter was confirmed by analysis of pressure tracings. Hemodynamic parameters were recorded continuously, and were allowed to stabilize for at least 15 minutes prior to subcutaneous administration of CNP variants or vehicle control. At least 30 minutes were allowed to elapse before administration of successive doses. The mean of parameter values in the 15 minutes before dosing was compared with the mean of parameter values in the 15 minutes immediately after dosing (n = 3–5/group).
tibial length and growth plate width were made by digital radiographs, proximal tibial growth plate volume and width were evaluated by magnetic resonance imaging (MRI), and lengths of limbs and tail were measured with a tape measure. One day after their last BMN 111 dose, the animals were euthanized and subjected to necropsy.

**MRI.** Sagittal, three-dimensional, fat-suppressed spoiled gradient recalled echo imaging sequences of each knee were acquired with an eight-channel knee coil, using a high-resolution 1.5 Tesla system (GE HDx, Mississauga, ON, Canada). Sequence parameters were as follows: echo time, 15 milliseconds; repetition time, 47 milliseconds; number of averages, 3; slice thickness/gap, 1.5 mm/0; matrix, 512 × 512; and field of view, 10 cm. All measures were performed by the same veterinary radiologist. The maximal height of the proximal physis of the right and left tibia was measured in its central third using OsiriX 3.7.1 software (Pixmeo, Geneva, Switzerland). Using the brush selection tool available in the software, the surface of the physis of the right proximal tibia was then selected to include the hyperintense layer between the adjacent hypointense bone. This was repeated on all consecutive images on which the growth plate was well demarcated and surrounded with hypointense layers of bone. This technique aimed to only select the plate itself and exclude the peripheral cartilage. To avoid inclusion of this peripheral cartilage, the selection solely included the portions of the plate that presented parallel borders and excluded more peripheral portions that presented diverging margins. When the surface of the growth plate was selected on all consecutive images, its volume was calculated using the automated volume calculation plug-in included in the software (n = 4/group).

**Radiographic Evaluation of Tibial Length.** Posteroanterior projections collimated to include each of the lower limbs and centered on the knees were performed with digital computed radiography (Agfa CR-DX, Toronto, ON, Canada) and taken while the animals were under general anesthesia. Mediolateral projections of the right tibia, centered on the proximal tibial physis, were also performed. Right tibial lengths (in millimeters) were measured manually on posterior–anterior projections with dedicated image analysis software (OsiriX 3.7.1; Pixmeo). The system was calibrated and the monkey legs were placed directly on the phosphorus plates to limit magnification effects. All images were interpreted and measured by the same veterinary radiologist who remained blinded to the treatment groups (n = 4/group).

**Postmortem Microcomputed Tomography of Lumbar Vertebrae.** Lumbar vertebrae 2, 3, and 4 were excised at necropsy, fixed in formalin, and scanned using the SkyScan 1176 microCT instrument (Micro Photonics, Inc., Allentown, PA), at a resolution of 35 μm, with the X-ray source set to 80 kV, 300 μA, and using a Cu + Al filter. Images were reconstructed by NRecon (Bruker MicroCT, Kontich, Belgium). To measure the foramen area of each vertebra, images were processed using the SkyScan-associated Data Viewer and the bone position was optimized. For each vertebra, the area was computed from the transaxial image corresponding to the narrowest part of the foramen in the coronal aspect (n = 4/group). The relevant transaxial image was saved as a single image and the foramen area measured using CTan software (Bruker MicroCT).

**Histomorphometric Analysis of the Growth Plate in Cynomolgus Monkeys.** For dynamic histomorphometry, calcein (10 mg/kg) was administered 14 days prior to necropsy, and oxytetracycline (40 mg/kg) was administered 6 days prior to necropsy. Left tibiae were dissected, formalin fixed, dehydrated, and embedded in methyl methacrylate. Five 7-μm sections were obtained from the 50% level of the bone for analysis of the proximal growth plates and trabecular bone. Tibias were stained with von Kossa, Goldner trichrome, and tartrate-resistant acid phosphatase stains. The combination of these three stains allowed analysis of the growth plate morphology, trabecular bone volume and architecture, quantification of unmineralized matrix (osteoid), and quantification of osteoblast and osteoclast numbers. Unstained sections were mounted for visualization of fluorescent labels for dynamic histomorphometry. Two operators measured total growth plate thickness of the right proximal tibial plate at six randomly chosen spots; 12 measurements were thereafter averaged for each sample. For each of the 12 fields, three columns of proliferating cells were assessed to determine the average number of proliferating cells per proliferating column. In addition, four regions of cuboidal chondrocytes in each field were assessed for mean cell volume of hypertrophic chondrocytes. For assessment of proliferating zone thickness and hypertrophic zone thickness, five measurements were made and averaged for each sample. Trabecular bone histomorphometry was evaluated within two 3500 μm × 3500 μm regions of interest by two operators.

All procedures described herein were conducted in accordance with the principles and procedures of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice and rats were humanely euthanized via anesthesia with carbon dioxide (performed in accordance with accepted American Veterinary Medical Association Guidelines on Euthanasia, June 2007). The monkeys were sedated with a combination of ketamine hydrochloride and acepromazine given intramuscularly, followed by an overdose of sodium pentobarbital, followed by exsanguination.

**Results**

**Rational Design and In Vitro Screening of Potential NEP-Resistant CNP Variants.** Watanabe et al. (1997) reported that proteolysis of CNP22 by NEP occurred after initial attack at the Cys6–Phe7 bond. To test this, we synthesized peptidomimetics of CNP22 that contained either a reduced or methylated amide bond between Cys6 and Phe7 (Cys-methylene and N-methyl-Phe7, respectively) of CNP22 and incubated in the presence of purified human NEP. Analysis of the digestion products revealed that the Cys–Phe peptidomimetic bond was resistant to NEP in both variants (data not shown). However, when measuring the rate of disappearance of the intact molecule, these variants were indistinguishable from CNP22, indicating that proteolysis occurred at other sites of CNP22 and does not depend on initial cleavage of the Cys–Phe bond (Table 1).

Ofner et al. (2000) proposed that the size-limited active site cavity of NEP restricts substrates based on their size (<3 kDa), a claim that is supported by natural substrate data (Kerr and Kenny, 1974; Erdös and Skidgel, 1989; Vijayaraghavan et al., 1990). To test this, we made larger variants of CNP through PEG conjugation, native CNP amino acid extensions, or by fusing CNP to other peptide sequences (chimeras). CNP variants, produced by chemically conjugating PEG units to the peptide NH2 terminus, exhibited size-dependent resistance to NEP proteolysis. Specifically, NEP resistance was observed in PEGylated CNP22 variants in which the molecular mass of the PEG unit was ≥1 kDa or when the total molecular mass of the PEG-CNP22 conjugate exceeded 3.2 kDa. However, these PEG-CNP conjugates were poor agonists of NPR B (≥16-fold increase in EC50). Interestingly, PEGylation of a longer native CNP sequence (CNP27) reclaimed the lost potency, while maintaining NEP resistance (Table 1).

Similar size-dependent results were observed by increasing the size of CNP22 through amino acid extensions. Here, we
TABLE 1
In vitro potency and NEP resistance for CNP variants

<table>
<thead>
<tr>
<th>Description</th>
<th>Molecular Mass</th>
<th>Potencya (EC50)</th>
<th>NEP Resistanceb</th>
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<tbody>
<tr>
<td></td>
<td>kDa</td>
<td>nM</td>
<td>% Intact</td>
</tr>
<tr>
<td>CNP22</td>
<td>2.2</td>
<td>13 ± 5.4</td>
<td>2.4 ± 1.8</td>
</tr>
<tr>
<td>CNP22, K4Rc</td>
<td>2.2</td>
<td>12 ± 1.4</td>
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<tr>
<td>CNP27, K4R, K5R, K9Rc</td>
<td>2.8</td>
<td>8.7 ± 1.8</td>
<td>&lt;5</td>
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<td>ANP28</td>
<td>3.1</td>
<td>&gt;2000</td>
<td>NT</td>
</tr>
<tr>
<td>CNP22, Cys6-methyleneed</td>
<td>2.2</td>
<td>44 ± 6.2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>CNP22, N-methyl-Phed</td>
<td>2.2</td>
<td>860 ± 380</td>
<td>&lt;5</td>
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<td>22</td>
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<td>84</td>
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<td>100</td>
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<td>3.2</td>
<td>640 ± 320</td>
<td>90</td>
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<td>210 ± 30</td>
<td>40</td>
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<td>CNP27, 2 kDa PEG</td>
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<td>&gt;2000</td>
<td>100</td>
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<td>3.8</td>
<td>16 ± 2.8</td>
<td>103 ± 27</td>
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<td>7.8 ± 1.4</td>
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<td>CNP36</td>
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<td>5.8 ± 3.5</td>
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<td>CNP37</td>
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<tr>
<td>BMN 1111*</td>
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<td>99 ± 0.6</td>
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<td>11 ± 3.2</td>
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<td>HSA27-36-CNP27g</td>
<td>4.0</td>
<td>6.9 ± 2.1</td>
<td>105 ± 6.4</td>
</tr>
</tbody>
</table>

NT, not tested.

*Mean EC50 (n = 2) of cGMP production in murine NIH3T3 fibroblasts after 15-minute exposure to CNP variants (10−10 M to 10−8 M), with nonlinear curve fit using the Hill equation (Erithacus Software).

†NEP resistance was determined by measuring the amount of intact peptide remaining after exposure to human NEP for 140 minutes in PBS at 37°C (n = 2), for variants with near native potency; n = 1 for all other variants. Peptide digests were analyzed by liquid chromatography/mass spectrometry.

‡Peptides used for PEGylation variants.

§Non-native Cys6-Phe7 peptide bond analogs were synthesized based on reported initial NEP cleavage site (Watanabe et al., 1997).

¶Biologic synthesis (all other analogs in this table were prepared by chemical synthesis).

⊥Chimeric sequences were synthesized on the amino terminus of CNP (IgG, Ac P01857, PDB ID2IWG; HSA Ac P02768, PDB ID 1BM0).

NT, not tested.

aMean EC50 (n = 2) of cGMP production in murine NIH3T3 fibroblasts after 15-minute exposure to CNP variants (10−10 M to 10−8 M), with nonlinear curve fit using the Hill equation (Erithacus Software).

bNEP resistance was determined by measuring the amount of intact peptide remaining after exposure to human NEP for 140 minutes in PBS at 37°C (n = 2), for variants with near native potency; n = 1 for all other variants. Peptide digests were analyzed by liquid chromatography/mass spectrometry.

cPeptides used for PEGylation variants.

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fChimeric sequences were synthesized on the amino terminus of CNP (IgG, Ac P01857, PDB ID2IWG; HSA Ac P02768, PDB ID 1BM0).
most NEP-resistant variants tested, with the exception that the polyethylene oxide (PEO) PEGylated variant (PEO24-CNP27) demonstrated a 3-fold longer serum half-life than the other variants after subcutaneous administration (Fig. 2B). Plasma cGMP profiles, a pharmacodynamics (PD) marker of NPR B activation (Wielinga et al., 2003), correlated well with the PK profiles of the CNP variants, demonstrating a clear PK/PD relationship (Fig. 2, C and D). Interestingly, cGMP concentration is not maintained for the PEGylated variant, despite the elevated exposure of this variant at the later time points (60–180 minutes; Fig. 2D). This could be caused by receptor desensitization of NPR B, which is known to occur upon prolonged exposure to CNP (Potter and Hunter, 2001). BMN 111, the recombinant version of BMN 1B2 containing one additional proline residue at the amino terminus, also demonstrated a prolonged half-life compared with CNP22 in wild-type murine studies (Fig. 2, E and F). Importantly, our data are consistent with a model whereby NEP functions as one of the major clearance pathways of CNP and supports our hypothesis that NEP-resistant variants should have longer serum half-lives.

**CNP Variant Selection Based on Stimulation of Bone Growth and Hemodynamic Effects in Wild-Type Mice.**

Studies in rat chondrocytes using the method developed by Krejci et al. (2005) indicated that daily 1-hour exposure to CNP22 significantly reversed the growth arrest induced by FGFR3 activation, comparable to cells continuously exposed to CNP22; these results support daily administration of CNP variants in wild-type mice (data not shown). Although PEO24-CNP27 demonstrated a superior PK profile, it failed to provide a significant growth benefit in wild-type mice compared with the placebo control in preliminary range-finding studies (data not shown). For this reason, we decided to evaluate a smaller, more potent PEG variant, PEO12-CNP27, in the comparative study.

Three-week-old wild-type FVB/nJ male mice (n = 3–9/group) were given daily subcutaneous injections of CNP variants BMN 1B2, BMN 111, PEO12-CNP27, or HSA(27–36)-CNP27 at 20, 70, or 200 nmol/kg or vehicle for 36 days. The growth of the appendicular and axial skeletons was dose related for most of the variants tested; however, growth effects were more pronounced in mice treated with BMN 111 (Fig. 3). A significant increase in naso-anal length was detected as early as 8 days after the start of BMN 111 treatment (data not shown). The PEGylated CNP variant, PEO12-CNP27, was the least pharmacologically active of the variants tested, potentially due to poor tissue bioavailability associated with PEGylated proteins (Veronese and Pasut, 2005; Ryan et al., 2008), and performed similarly to PEO24-CNP27 in our preliminary range-finding study. After 2 weeks of treatment, axial growth (naso-anal and tail length) was evident in mice treated with the chimeric CNP variant; however, the response was not sustained beyond 3 weeks (data not shown).

Additional studies designed to look at accumulation and clearance of BMN 111 at the growth plate demonstrated that consecutive daily administrations of BMN 111 augmented the cGMP levels in the distal femur growth plate, but not in the kidney, 15 minutes after the last injection (Fig. 4A). Consistent with this augmented cGMP response, immunoreactive CNP persists for several days after the last injection in wild-type mice (Fig. 4B, right). However, the accumulated BMN 111 appears to be inactive because the cGMP response was reduced to background levels by 24 hours after administration (Fig. 4B, left). On the basis of the augmented activity response we observed after consecutive daily administrations (Fig. 4A), it is unlikely that the immunoreactive BMN 111 has caused receptor desensitization. Rather, it is more likely that BMN 111 has been inactivated through a proteolytic event. In agreement with these data, in vivo dose regimen studies in wild-type mice demonstrated that accelerated growth was observed only during the week when mice received daily dosing. Discontinuation of treatment at 1-week intervals resulted in a return to normal growth rate (Fig. 5).

CNP produces hemodynamic effects in mice (Lopez et al., 1997), nonhuman primates (Seymour et al., 1996), rats, dogs, and humans (Barr et al., 1996); therefore, we decided to examine...
cardiovascular effects of the CNP variants (20–200 nmol/kg) in anesthetized wild-type FVB/nJ male mice fitted with a pressure-monitoring catheter connected to a telemetry transmitter. All variants showed similar BP-reducing and HR-increasing activity (Fig. 6). In most animals, effects were observed within 5 minutes of subcutaneous administration, with maximal drop in MAP occurring between 5 and 20 minutes postdose. This timing correlated well with the maximum concentration of the CNP
variants, and demonstrated a clear PK/PD relationship for this physiologic response. Because the hemodynamic responses were similar between the doses and variants tested, cardiovascular activity was not a differentiating property and no further experiments or statistical analyses were performed.

BMN 111 demonstrated an increased pharmacological activity compared with the PEGylated and chimeric CNP variants in wild-type mice, whereas the transient hemodynamic response was very similar for the non-PEGylated CNP variants. Histomorphometric analysis of long bones showed no observable changes in trabecular and cortical architecture associated with the 5-week daily treatment of BMN 111 (data not shown), indicating that although longitudinal growth was stimulated, de novo bone formation was unaffected and normal. Based on potency and similarity to native sequence, BMN 111 was selected for studies in ACH mice and cynomolgus monkeys.

### TABLE 2
PK parameters of CNP variants in wild-type rats [Crl:CD (SD) IGS] and wild-type mice [FVB/nJ]

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal</th>
<th>Dose (nmol/kg)</th>
<th>Route</th>
<th>Cmax (pmol/ml)</th>
<th>tmax (min)</th>
<th>t1/2 (min)</th>
<th>Bioavailability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP22</td>
<td>Rat</td>
<td>20</td>
<td>i.v.</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PEO24-CNP27</td>
<td>Rat</td>
<td>20</td>
<td>i.v.</td>
<td>7.3 (1.1)</td>
<td>1 (0)</td>
<td>22 (1.5)</td>
<td>22 (1.5)</td>
</tr>
<tr>
<td>BMN 1B2</td>
<td>Rat</td>
<td>20</td>
<td>i.v.</td>
<td>220 (86)</td>
<td>1.5 (1)</td>
<td>23 (3.4)</td>
<td>23 (3.4)</td>
</tr>
<tr>
<td>HSA(27–36)-CNP27</td>
<td>Rat</td>
<td>20</td>
<td>i.v.</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BMN 111</td>
<td>Mice</td>
<td>25</td>
<td>i.v.</td>
<td>9.0 (3.7)</td>
<td>5.0 (0.0)</td>
<td>10 (5.0)</td>
<td>10 (5.0)</td>
</tr>
<tr>
<td>BMN 1B2</td>
<td>Rat</td>
<td>50</td>
<td>s.c.</td>
<td>24 (1.9)</td>
<td>25 (8.7)</td>
<td>78 (16)</td>
<td>78 (16)</td>
</tr>
<tr>
<td>BMN 111</td>
<td>Mice</td>
<td>50</td>
<td>s.c.</td>
<td>15 (1.8)</td>
<td>12 (5.8)</td>
<td>25 (4.4)</td>
<td>25 (4.4)</td>
</tr>
<tr>
<td>BMN 111</td>
<td>Mice</td>
<td>50</td>
<td>s.c.</td>
<td>9.4 (2.2)</td>
<td>12 (5.8)</td>
<td>19 (4.3)</td>
<td>19 (4.3)</td>
</tr>
<tr>
<td>HSA(27–36)-CNP27</td>
<td>Rat</td>
<td>50</td>
<td>s.c.</td>
<td>22 (4.4)</td>
<td>5.0 (0.0)</td>
<td>25 (5.5)</td>
<td>25 (5.5)</td>
</tr>
<tr>
<td>CNP22</td>
<td>Mice</td>
<td>130</td>
<td>s.c.</td>
<td>10 (3.2)</td>
<td>2.8 (1.5)</td>
<td>15 (5)</td>
<td>15 (5)</td>
</tr>
<tr>
<td>BMN 111</td>
<td>Mice</td>
<td>70</td>
<td>s.c.</td>
<td>200 (140)</td>
<td>13 (5)</td>
<td>15 (5)</td>
<td>15 (5)</td>
</tr>
</tbody>
</table>

NA, not available.
Pharmacological Effects of BMN 111 in ACH Mice.

Targeted expression of an activated FGFR3 in the growth plate cartilage of mice was achieved using regulatory elements of the collagen 2 gene (Naski et al., 1998). Three-week-old Fgfr3ΔCH/male mice and their wild-type (FVB/nJ) littermates (n = 8–10/group) were given daily subcutaneous injections of BMN 111 at 5, 20, or 70 nmol/kg (20, 80, or 280 μg/kg) or vehicle for 36 days. Significant growth in the appendicular and axial skeletons was observed in BMN 111–treated Fgfr3ΔCH/+ mice (Fig. 7A). Although this Fgfr3ΔCH/+ mouse model represents a mild phenotype, naso-anal and femur lengths of Fgfr3ΔCH/+ mice were significantly shorter than wild-type mice at the start of the study (P < 0.05). Correction of the tail length was observed after 36 days of BMN 111 daily subcutaneous administrations at the 70 nmol/kg dose level. Naso-anal lengths were corrected at 20 nmol/kg after daily subcutaneous administration of BMN 111 for 36 days. Femur and tibia lengths were corrected at 5 and 20 nmol/kg by the end of the study. Histologic examination revealed a statistically significant increase in growth plate expansion in Fgfr3ΔCH/+ mice treated with 70 nmol/kg BMN 111 (Fig. 7B), including increased area and/or height in the zones of resting cartilage, proliferation, and hypertrophy (data not shown). These data indicate that BMN 111 activation of NPR B corrects growth plate abnormalities secondary to the Fgfr3 mutation that results in ACH dwarfism.

Hemodynamic Effects of BMN 111 in Cynomolgus Monkeys.

After initial dose-ranging studies were performed in mice (Fig. 6), a pilot study was performed in normal juvenile, anesthetized or conscious, cynomolgus monkeys after a single subcutaneous injection of BMN 111 at 5, 20, or 70 nmol/kg (20, 80, or 280 μg/kg) or vehicle for 36 days. Significant growth in the appendicular and axial skeletons was observed in BMN 111–treated Fgfr3ΔCH/+ mice (Fig. 7A). Although this Fgfr3ΔCH/+ mouse model represents a mild phenotype, naso-anal and femur lengths of Fgfr3ΔCH/+ mice were significantly shorter than wild-type mice at the start of the study (P < 0.05). Correction of the tail length was observed after 36 days of BMN 111 daily subcutaneous administrations at the 70 nmol/kg dose level. Naso-anal lengths were corrected at 20 nmol/kg after daily subcutaneous administration of BMN 111 for 36 days. Femur and tibia lengths were corrected at 5 and 20 nmol/kg by the end of the study. Histologic examination revealed a statistically significant increase in growth plate expansion in Fgfr3ΔCH/+ mice treated with 70 nmol/kg BMN 111 (Fig. 7B), including increased area and/or height in the zones of resting cartilage, proliferation, and hypertrophy (data not shown). These data indicate that BMN 111 activation of NPR B corrects growth plate abnormalities secondary to the Fgfr3 mutation that results in ACH dwarfism.
was to find a tolerable dose that yielded a \( \approx 10\)-mm Hg (approximately 10\%) decrease in MAP or a \( \approx 50\)-beats per minute increase (approximately 25\%) in resting HR. It was observed that HR increase was the most sensitive parameter, probably due to reflex tachycardia, and a dose of 7 nmol/kg gave an approximately 25\% increase in HR in conscious monkeys, with little or no effect on MAP (Fig. 8, B and D). The increase in HR was transient, with the maximal increase observed at 10–20 minutes postdose (Fig. 8, E and F). Multiple subcutaneous daily dosages (7 and 17.5 nmol/kg) for 7 consecutive days were well tolerated. ECG parameters were unaffected at any dose of BMN 111 tested (data not shown). The drop in MAP after BMN 111 administration was inconsistent and often somewhat less marked after subsequent doses (data not shown). On the basis of these data, the highest dose chosen for the long-term study was 8.25 nmol/kg. A lower dosage of 2.25 nmol/kg per day, which gave little or no cardiovascular effect, was also tested.

Pharmacological Effects of BMN 111 in Cynomolgus Monkeys. BMN 111 was administered subcutaneously to growing (2- to 4-year-old) cynomolgus male monkeys at 2.25 or 8.25 nmol/kg once daily for 6 months (\( n = 4 \)/group). Although BP was not monitored, no clinical signs of hypotension or distress were noted in any animal at any time during the study. The effect on proximal tibial growth plate size was observed by MRI imaging performed during the fourth week of dosing (Fig. 9A). Mean growth plate volume increased approximately 40\% for the high-dose group versus the pretreatment volume. This was the peak growth plate volume noted. Volume receded thereafter, but remained greater than baseline throughout the remainder of the 6-month study. Treatment with BMN 111 resulted in a dose-dependent increase in total tibial length (measured from digital radiographs) and rate of growth (Fig. 9b) as well as increased lengths of arms, legs, and tail when measured externally (data not shown). Treated animals maintained their height/length advantage through the end of the study period. Clinical chemistry and hematology parameters remained normal and unchanged throughout the 6-month study with the noted exception of increased serum levels of total and bone-specific alkaline phosphatase associated with the increase in bone formation (Fig. 9C).

Growth plate expansion, evaluated post mortem after 6 months of treatment, was evident at the histologic level (Fig. 10B, upper), with significant expansions in total growth plate thickness, proliferating zone thickness, and hypertrophic zone thickness, changes that are associated with inhibition of FGFR3 signaling (Iwata et al., 2000; Ornitz and Marie, 2002) (Table 3). Similar histologic and growth plate expansion results were observed in wild-type and Fgfr3\(^{ACH/+}\) murine studies (Fig. 10A; Table 3). Double fluorochrome labeling of newly formed mineralized bone performed during the final 14 days of the in vivo study illustrated that growth plate expansion in response to 8.25 nmol/kg per day BMN 111 translated into increased longitudinal growth of mineralized bone (Fig. 10B, lower panel). Static and dynamic measurements of trabecular bone architecture and turnover were not affected by BMN 111 treatment, indicating that normal bone was formed (Table 4).

To assess the effects of BMN 111 treatment on vertebral foramen area, post mortem microcomputed tomography was performed on excised lumbar vertebrae 2–4. For the high-dose group (8.25 nmol/kg per day), mean vertebral foramen area increased approximately 10\%–17\% going up the spine (L4 to L2) versus the vehicle control group, and was statistically significant in L2 (\( P = 0.03 \) versus vehicle by two-tailed \( t \) test) (Fig. 9D).

Discussion

In ACH, mutations in FGFR3 result in constitutive activation, suppressing the proliferation and differentiation of chondrocytes resulting in improper cartilage to bone conversion in the growth plate (Laederich and Horton, 2010). ACH is associated with significant morbidity and increased mortality, and current treatments are mostly surgical (Trotter and Hall, 2005; Wynn et al., 2007). BMN 111, a CNP variant, offers a potential treatment for ACH that addresses the underlying biochemical defect. By signaling through NPR B, BMN 111 suppresses downstream signals in normal and mutated FGFR3 pathways to enhance or restore chondrocyte proliferation and differentiation resulting in bone growth. Specifically, BMN 111 inhibits the ERK/MAPK pathway through phosphorylation of Raf-1 by cGMP-dependent protein kinase 2 (Krejci et al., 2005).
Because CNP is rapidly cleared from the circulation through receptor-mediated (NPR C) and proteolytic (NEP) pathways (Potter, 2011), CNP requires continuous infusion to be effective in ACH murine studies (Yasoda et al., 2009); however, this is not a desired therapy by physicians or patients. To overcome these limitations, we developed a CNP variant, BMN 111, which resists degradation by NEP at the site of subcutaneous administration and at the growth plate (Ruchon et al., 2000; Yamashita et al., 2000; Nakajima et al., 2012). Here, we demonstrate that BMN 111 is effective as a subcutaneous injectable therapeutic that promotes bone growth in juvenile wild-type mice and juvenile cynomolgus monkeys and corrects the ACH phenotype in Fgfr3\(^{ACH/+}\) mice.

**Fig. 7.** Fgfr3\(^{ACH/+}\) mice treated with BMN 111. (A) Growth of the appendicular and axial skeletons of Fgfr3\(^{ACH/+}\) mice after treatment with BMN 111. Three-week-old Fgfr3\(^{ACH/+}\) mice given daily subcutaneous administrations of BMN 111 (5, 20, or 70 nmol/kg) or vehicle for 5 weeks (n = 8/group). Wild-type vehicle controlled mice (FVB/nJ) were included to assess the degree of phenotype and normalization for each growth parameter (n = 8). The asterisk denotes statistical significance (P < 0.05) against vehicle-treated wild-type mice. The dagger denotes statistical significance against vehicle-treated Fgfr3\(^{ACH/+}\) mice (analysis of variance with post hoc Dunnett’s t test). (B) Distal femoral growth plates of mice treated with vehicle or BMN 111 (trichrome stained). Significant growth plate expansion was observed in Fgfr3\(^{ACH/+}\) mice treated with 70 nmol/kg BMN 111. Error bars indicate the S.D. Original magnification, 10\(\times\) in B. Veh, vehicle; WT, wild type.
Fig. 8. Effect of BMN 111 on BP and HR in cynomolgus monkeys. (A–D) In both anesthetized (A and C) and conscious monkeys (B and D), BMN 111 decreased MAP in a dose-dependent manner (n = 1–4/group). In conscious animals, there was a concomitant increase in HR. The HR response was blunted in the anesthetized animals. (A and B) Change in average HR over 10–20 minutes postdose (encompassing time of HR zenith) and baseline (15 minutes just prior to dosing). (C and D) Change in average MAP over 10–20 minutes postdose (encompassing time of MAP nadir) and baseline average (15 minutes just prior to dosing). (E and F) BP (E) and HR (F) after a single subcutaneous dose of BMN111 (17.5 nmol/kg) to a conscious monkey. Significant hypotension develops rapidly after administration, but begins to resolve within an hour. bpm, beats per minute.
native CNP in vitro activity. NEP resistance translated into improved serum half-lives in wild-type rat or murine studies ($t_{1/2}$, approximately 15 minutes for CNP variants versus 2–5 minutes for native CNP); however, the improved in vivo stability does not exclude the possibility that CNP variants are susceptible to other proteolytic pathways in addition to the known NPR clearance pathway (NPR C) present in the vasculature. BMN 111 was selected for ACH murine studies and larger animal studies based on its superior bone growth-promoting attributes in the wild-type murine studies. The lowest dosage tested in the wild-type murine screening study was 20 nmol/kg per day and this appeared to be well above the minimal effective dose. This dose also appeared to correct most growth deficits in the $Fgfr3^{ACH/+}$ mouse model. Importantly, we recently reported that BMN 111 stimulated bone growth in mouse models containing a stronger activating mutation of $Fgfr3^{Y367C/+}$, a mutation that results thanatophoric dysplasia type I (TDI) in humans (Lorget et al., 2012).

To test its effectiveness in larger animals, levels that had minimal effects on hemodynamic parameters were chosen and three cohorts of cynomolgus monkeys were dosed. Dose-dependent growth was observed in this 6-month study. The high-dose group showed measurable increases in growth plate expansion, rate of endochondral bone growth, and trends in

Fig. 9. Change in growth plate volume, tibial length, serum alkaline phosphatase levels, and lumbar vertebral foramen in cynomolgus monkeys treated with BMN 111. (A) Change in right proximal tibial growth plate volume with high-dose BMN 111 treatment, measured by MRI ($P = N.S.$ versus vehicle at all time points; $n = 4/group$). (B) Radiographic evaluation of cynomolgus tibias at several time points in animals treated with BMN 111. Dose-dependent change in rate of growth of tibial length. Right tibial lengths (in millimeters) were measured manually on posterior–anterior projections with dedicated image analysis software ($P = N.S.$ versus vehicle at all time points ($n = 4/group$)). (C) Increase in serum alkaline phosphatase with BMN 111 treatment. Known as markers of bone growth or deposition, changes in both total and bone-specific alkaline phosphatase (data not shown) were not statistically significant over prestudy values ($n = 4/group$). (D) Area of lumbar vertebral foramen of cynomolgus monkey assessed by microcomputed tomography. In vertebrae L2, L3, and L4, treatment with BMN111 at the high dose resulted in a trend toward greater area of vertebral foramen compared with vehicle controls. For L2, the increase was statistically significant (*$P = 0.03$ versus vehicle; $n = 4/group$). N.S., not significant.
Fig. 10. Cynomolgus monkey and wild-type mice growth plate histology after 6 months of treatment with BMN 111. (A) Distal femoral growth plates of mice treated with vehicle or BMN 111 (trichrome stained). Growth plate expansion was observed in mice treated with 20 and 70 nmol/kg BMN 111 (showing 70 nmol/kg). (B) The upper panel shows Goldner trichrome staining of growth plate (purple) and bone (green). The lower panel is the calcein label under UV (green) showing longitudinal growth rate in the last 14 days of treatment. The distal edge of growth plate is delineated with a dashed line, and longitudinal bone growth in 14 days prior to necropsy is represented with arrows (n = 4/group, showing one representative image from each group). Original magnification, 10× in A.
TABLE 3
Growth plate parameters and longitudinal growth rates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (n=4)</th>
<th>2.25 nmol/kg per day (n=4)</th>
<th>8.25 nmol/kg per day (n=4)</th>
<th>5 nmol/kg per day (n=4)</th>
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<tbody>
<tr>
<td>Longitudinal growth rate (m/day)</td>
<td>26 ± 7</td>
<td>26 ± 5 (N.S.)</td>
<td>26 ± 5 (N.S.)</td>
<td>26 ± 7 (N.S.)</td>
</tr>
<tr>
<td>Growth plate thickness (µm)</td>
<td>555 ± 61</td>
<td>594 ± 64 (N.S.)</td>
<td>682 ± 48 (&lt;0.05)</td>
<td>1594 ± 17 (0.0001)</td>
</tr>
<tr>
<td>Proliferating cell thickness (µm)</td>
<td>125 ± 10</td>
<td>139 ± 88 (N.S.)</td>
<td>162 ± 14 (&lt;0.001)</td>
<td>1594 ± 17 (0.0001)</td>
</tr>
<tr>
<td>Proliferating cell thickness (µm)</td>
<td>125 ± 10</td>
<td>139 ± 88 (N.S.)</td>
<td>162 ± 14 (&lt;0.001)</td>
<td>1594 ± 17 (0.0001)</td>
</tr>
<tr>
<td>Hypertrophic cell thickness</td>
<td>72 ± 26</td>
<td>89 ± 23 (N.S.)</td>
<td>128 ± 96 (&lt;0.005)</td>
<td>128 ± 96 (&lt;0.005)</td>
</tr>
<tr>
<td>Hypertrophic cell volume (µm³)</td>
<td>231 ± 30</td>
<td>295 ± 56 (N.S.)</td>
<td>295 ± 56 (N.S.)</td>
<td>295 ± 56 (N.S.)</td>
</tr>
</tbody>
</table>

Nevertheless, they were able to show stimulation of bone growth in wild-type and Fgfr3Ach/+- mice with their wild-type littermates. P3 as a postnatal therapy affected osteoclast activity, if at all.

Several other groups have reported potential therapeutic strategies that modulate the aberrant FGFR3 pathway. Garcia et al., 2013 demonstrated that a soluble fibroblast growth factor receptor-3 (sFGFR3) could act as a decoy receptor to prevent fibroblast growth factor (FGF) from binding to and signaling through the FGFR3. In vitro binding studies with fixed concentrations of FGF2, FGF9, and FGF18 demonstrated that sFGFR3 was required in 100-fold excess to reduce the concentration of these FGFs by one-half. Nevertheless, they were able to show stimulation of bone growth in wild-type and Fgfr3Ac/H+ murine studies. The long-term effects of continuous FGF depletion remain to be determined, but would be expected to impair wound repair and other developmental processes (Lynch et al., 1989; Kurtz et al., 2004). One question that comes to mind with this therapeutic strategy is whether sufficient amounts of this approximately 70-kDa sFGFR3 protein could diffuse through the highly negatively charged extracellular matrix of a larger human growth plate to compete for FGFs expressed locally as paracrine factors. Moreover, there is still no scientific consensus that FGF receptors require ligand for dimerization (He et al., 2011; Placone and Hristova, 2012).

In another report, Jin et al. (2012) discovered a 12-amino acid peptide through phage display, P3, which could bind to the extracellular domain of FGFR3 and partially block FGF2-mediated ERK1/2 phosphorylation. When pregnant Fgfr3Neo-K644E/1 mice (phenotypically normal thanatophoric dysplasia type II (TDII) carriers) were given daily peritoneal injections of P3 (100 µg/kg body weight) at E16.5 until birth, all TDII pups (Fgfr3Neo-K644E/1) survived, whereas all vehicle control TDII pups died. The TDII mice that survived had increased thoracic cavities, which rescued the postnatal lethality phenotype; however, the rescued mice still had smaller bodies and dome-shaped skulls compared with their wild-type littermates. P3 as a postnatal therapy for ACH, perhaps a more acceptable therapeutic regimen, was not tested in this study.

Matsushita et al. (2013) identified meclozine, an antihistamine used for motion sickness, as an antagonist of the expansion of the vertebral foramen. Although this study was not powered for significance, some statistically significant trends were observed; for example, growth plate thickness in the high-dose group, particularly in the proliferating and hypertrophic zones, was statistically larger than in vehicle-treated animals (P < 0.001 and P < 0.05, respectively), which is consistent with observations that suggest that FGFR3 inhibits both the proliferation and terminal differentiation of growth plate chondrocytes and the synthesis of extracellular matrix by these cells (Laederich and Horton, 2010). Double fluorochrome labeling of newly formed mineralized bone demonstrated that bone formation was increased in the high-dose group in accordance with the increased endochondral activity that caused growth plate expansion. Moreover, the achievement of this bone growth in the last 14 days of the study demonstrated the continued effectiveness of BMN 111 after chronic treatment, and suggested that the growth plate width, which receded after 4 weeks of treatment, was not associated with a reduction of BMN 111 activity. BMN 111-treated animals showed equivalent trabecular architecture parameters compared with vehicle-treated animals, suggesting that BMN 111 treatment did not significantly affect osteoclast activity, if at all.
FGFR3 pathway. They demonstrated that meclozine was able to attenuate FGF2-mediated ERK phosphorylation in rat chondrosarcoma cells, facilitate chondrocytic differentiation of ATDC5 cells expressing ACH or TDII mutant FGFR3, and promote tibial growth in FGF2-suppressed tissue explant studies. In explant studies, they compared CNP (0.2 μM) to meclozine (20 μM). Interestingly, meclozine demonstrated no statistically significant enhancement of tibial growth in the absence of FGF2, unlike CNP (Yasoda et al., 1998). Furthermore, meclozine was not tested in any of the available in vivo murine models for its ability to stimulate or correct growth. Thus, questions remain as to whether this is a viable therapeutic option.

In a recent article, Yamashita et al. (2014) demonstrated that statins could correct the degraded cartilage in both chondrogenically differentiated TDII and ACH-induced pluripotent stem cells. Interestingly, mRNA expression levels of FGFR3 were increased by lovastatin, but protein levels by immunoblot decreased, which led the authors to postulate that statins increase the degradation by lovastatin, but protein levels by immunoblot decreased, which led the authors to postulate that statins increase the degradation of FGFR3 in chondrogenically differentiated TDII induced pluripotent stem cells. In an 11-day ACH murine study (days 3–14), mice receiving daily injections of rosuvastatin demonstrated an increase in distal long-bone growth rate comparable to wild-type mice receiving vehicle. The effect beyond 14 days on final growth (6–8 weeks) was not assessed in this study. The mechanism is unclear but could be due to altering membrane dynamics, which may not be a good strategy given the frequency of known side effects of statins as well as the potential developmental consequences (Evans and Rees, 2002; Maji et al., 2013).

We believe that BMN 111 is a promising therapeutic option for children with ACH with open growth plates for a number of reasons. First, BMN 111, an NEP-resistant CNP variant, is a natural antagonist of the FGFR3 pathway, corrects the phenotype in Fgf3R3−/− mice, and attenuates the phenotype in stronger activating mutations of FGFR3 (TDI; Y367C/+) when given daily subcutaneously (Lorjet et al., 2012). Second, CNP and its receptor are expressed in the growth plate. Third, the amino acid content is basic (pi = approximately 10) and the peptide is small, which enable subcutaneous administered BMN 111 to target and diffuse through the anionic extracellular matrix barrier of the growth plate. Finally, unlike other small molecule strategies, BMN 111 will only target cells that express its cognate receptor, NPR B, which should mitigate many of the potential therapeutic options. BMN 111 is currently being investigated in children with ACH (Clinical-Trials.gov identifier NCT02055157).

In conclusion, through a series of in vitro and in vivo rodent studies, we identified five CNP variants comprising three types (PEGylated, chimeric, and natural amino acid extensions) that were resistant to NEP by virtue of size, retained native in vitro potency, and demonstrated prolonged half-lives in rats and mice. One CNP variant, BMN 111, was selected for further study based on potency and similarity to native CNP. When administered subcutaneously to normal mice, normal growing monkeys, or ACH mice, BMN 111 treatment resulted in growth of the axial and appendicular skeletons. In the 6-month daily dose study in juvenile monkeys, BMN 111 (administered at doses that did not cause an unacceptable hemodynamic effect) resulted in expansion of the proximal tibial growth plates, with widening of the hypertrophic zone, increased length and rate of limb growth, and increased area of the foramen of lumbar vertebrae. Concomitant increase in both total and bone-specific alkaline phosphatase levels may provide a biomarker of early BMN 111 activity. Transient, asymptomatic dose-dependent hemodynamic responses were observed in mice and monkeys at doses higher than needed to produce skeletal growth. These experiments indicate that growth in both normal and ACH juvenile animals is governed, at least in part, through the NPR B cGMP signaling pathway, and that BMN 111 affects this pathway. BMN 111 is being investigated as a potential therapeutic for pediatric patients with ACH.

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### Table 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>2.25 nmol/kg per day</th>
<th>8.25 nmol/kg per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>22 ± 5</td>
<td>27 ± 7</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Osteoid/bone surface (%)</td>
<td>33 ± 9</td>
<td>33 ± 6</td>
<td>33 ± 9</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td>133 ± 17</td>
<td>158 ± 24</td>
<td>132 ± 11</td>
</tr>
<tr>
<td>Trabecular number (mm−2)</td>
<td>1.6 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Trabecular spacing (μm)</td>
<td>501 ± 137</td>
<td>452 ± 124</td>
<td>339 ± 83</td>
</tr>
<tr>
<td>Osteoblasts/bone surface (n)</td>
<td>22 ± 2.4</td>
<td>23 ± 4</td>
<td>25 ± 3.8</td>
</tr>
<tr>
<td>Osteoclasts/bone surface (n)</td>
<td>1.8 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>Osteoid thickness (μm)</td>
<td>6.2 ± 2.1</td>
<td>9 ± 1.3</td>
<td>9.4 ± 1.4</td>
</tr>
<tr>
<td>Mineral apposition rate per day (μm/day)</td>
<td>1.6 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Bone formation rate/bone volume</td>
<td>0.013 ± 0.002</td>
<td>0.015 ± 0.003</td>
<td>0.011 ± 0.002</td>
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</tbody>
</table>
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C-Type Natriuretic Peptide Variant for FGFR3-Related Dwarfism 149


