Tyrosine kinase inhibitor NVP-BGJ398 functionally improves FGFR3-related dwarfism in mouse model

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Achondroplasia (ACH) is the most frequent form of dwarfism and is caused by gain-of-function mutations in the fibroblast growth factor receptor 3–encoding (FGFR3-encoding) gene. Although potential therapeutic strategies for ACH, which aim to reduce excessive FGFR3 activation, have emerged over many years, the use of tyrosine kinase inhibitor (TKI) to counteract FGFR3 hyperactivity has yet to be evaluated. Here, we have reported that the pan-FGFR TKI, NVP-BGJ398, reduces FGFR3 phosphorylation and corrects the abnormal femoral growth plate and calvaria in organ cultures from embryos of the Fgfr3Y367C/+ mouse model of ACH. Moreover, we demonstrated that a low dose of NVP-BGJ398, injected subcutaneously, was able to penetrate into the growth plate of Fgfr3Y367C/+ mice and modify its organization. Improvements to the axial and appendicular skeletons were noticeable after 10 days of treatment and were more extensive after 15 days of treatment that started from postnatal day 1. Low-dose NVP-BGJ398 treatment reduced intervertebral disc defects of lumbar vertebrae, loss of synchondroses, and foramen-magnum shape anomalies. NVP-BGJ398 inhibited FGFR3 downstream signaling pathways, including MAPK, SOX9, STAT1, and PLCγ, in the growth plates of Fgfr3Y367C/+ mice and in cultured chondrocyte models of ACH. Together, our data demonstrate that NVP-BGJ398 corrects pathological hallmarks of ACH and support TKIs as a potential therapeutic approach for ACH.

Introduction

Achondroplasia (ACH) is the most common form of dwarfism, which occurs with an estimated prevalence of between 1/16,000 and 1/25,000 live births (1, 2). ACH patients are characterized by disproportionate short stature, with a long trunk deformed by excessive lordosis. The head is large with frontal bossing and the midface is hypoplastic (2). ACH is an autosomal-dominant disorder, and patients with ACH have been found to have a gain-of-function mutation (p.Gly380Arg) localized in the transmembrane domain of the fibroblast growth factor receptor 3 (FGFR3), a member of the receptor tyrosine kinase family (3, 4). The FGFR3 mutation disturbs the balance of proliferation and differentiation in the growth plate. Several papers reported both decreased and increased proliferation related to stage-specific development (5–8). Chondrocyte differentiation is also impaired (9, 10) and seems to be controlled by SRY-box 9 (Sox9) expression in the growth plate (11). The ACH mutation activates the tyrosine kinase activity of the receptor and facilitates the transautophosphorylation of key tyrosine residues in the intracellular domain and activates mainly the downstream canonical MAP pathway. Other signaling pathways have been implicated in addition in ACH, e.g., STAT, Wnt/β-catenin, PI3K/AKT, and PLCγ pathways (9, 12, 13).

Potential therapeutic strategies have emerged for ACH over the last 5 years; these have aimed at reducing excessive activation of FGFR3 and its signaling pathways to stimulate linear bone growth. Our group conducted preclinical studies on a C-natriuretic peptide (CNP) analog (14), BMN111, and we showed that this treatment led to a substantial improvement of skeletal parameters in the Fgfr3Y367C/+ mouse, which is a preclinical model of ACH (15). BMN111 (vosoritide) is currently in a (phase 2) clinical trial in pediatric patients with ACH (ClinicalTrials.gov NCT02055157). Other preclinical studies using different strategies and various compounds, such as parathyroid hormone (PTH) (16), soluble FGFR3 (17), statin (18), and meclozine (19), reported changes in the dwarf Fgf3-/- mouse phenotype (7). For most of these strategies, the mechanism of action in the growth plate needs to be elucidated. However, the most direct therapeutic strategy would be to counteract the hyperactivity of FGFR3 using a tyrosine kinase inhibitor (TKI). NVP-BGJ398 was identified as a panspecific FGFR inhibitor that was equally active against FGFR1, FGFR2, and FGFR3 and less active against FGFR4 (20). Recently, cell-free kinase assays showed that NVP-BGJ398 was more selective for FGFR3 than the other FGFRs (21). This TKI is effective in reducing FGFR3 activation and has been used in preclinical murine models for the treatment of several FGFR-related pathological conditions, such as hypophosphatemic rickets (22), malignant rhabdoid tumors (23), and hepatocellular carcinoma (24). We therefore decided to investigate whether NVP-BGJ398 could penetrate into the car-

Conflict of Interest: I. Kramer, D. Graus-Porta, and M. Kneissel work for Novartis.
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Figure 1. NVP-BGJ398 inhibits the activation of FGFR3 and rescues ex vivo bone growth of Fgfr3 Y367C/+ mouse embryo femurs. (A) Phosphorylated FGFR3 expression in transfected human chondrocytes with FGFR3 (WT), FGFR3>G380R (ACH), FGFR3>Y373C (TDI), and FGFR3>K650E (TDII) constructs. (B) Phosphorylated FGFR3 expression in transfected HEK293 cells with FGFR3 (WT), FGFR3>Y373C (TDI), and FGFR3>K650M (SADDAN) constructs. Lanes were run on the same gel, but were noncontiguous. (C) Gain of length of E16.5 femurs in culture for 6 days (ΔD6-D0) with or without treatment (Fgfr3+/+; n = 6; untreated Fgfr3Y367C/+; n = 6; 10 nM Fgfr3Y367C/+; n = 5; 100 nM Fgfr3Y367C/+; n = 6; Fgfr3Y367C/+; n = 8. *P < 0.05, 1-way ANOVA. Scale bar: 1 mm. (D) H&E staining and immunohistochemistry for Col X and Ki67 on embryonic distal femur following 6 days of culture with or without treatment. Arrows indicate hypertrophic area. Scale bars: 200 μm. (E) Number of Ki67-labeled cells in treated and untreated Fgfr3Y367C/+ compared with Fgfr3+/+ embryonic femur (n = 6 per group). *P < 0.05, 1-way ANOVA. (F) Col X-labeled growth plate area in Fgfr3+/+ and Fgfr3Y367C/+ treated and untreated embryonic femur (n = 5 per group). *P < 0.05, 1-way ANOVA. Western blots are representative of 3 independent experiments. Data are expressed as mean ± SD.
tiltage and bone and improve the skeletal phenotype in ACH via its inhibitory action on FGFR3 and downstream signaling pathways. In our study, NVP-BGJ398 inhibited the hyperactivity of FGFR3 and the canonical MAPK in ACH human cartilage cells. NVP-BGJ398 rescued the anomalies of fetal calvaria and femur in organ cultures of tissue taken from the Fgfr3Y367C/+ mouse model of ACH. Moreover, NVP-BGJ398 treatment of Fgfr3Y367C/+ mice was able to correct the dwarf phenotype. The reduction in the activity of FGFR3 improved all pathological hallmarks of ACH in long bones, skull base, calvaria, intervertebral disc (IVD), and vertebral. The timing and type of the treatment were 2 important criteria for the improvement of bone growth in Fgfr3Y367C/+ mice. At cellular and tissue levels, chondrocyte proliferation was restored, as demonstrated by STAT pathway downregulation, and chondrocyte differentiation was improved, as demonstrated by SOX9 and MAPK downregulation. Our data suggest that TKIs may represent a pharmacological approach for the treatment of FGFR3 hyperactivation-related disorders.

Results

NVP-BGJ398 inhibits FGFR3 phosphorylation. We evaluated the ability of NVP-BGJ398 to inhibit FGFR3 phosphorylation in transiently transfected human control chondrocyte lines (Figure 1A) and HEK293-Vnr cells (Figure 1B). Cells were transfected with constructs expressing FGFR3WT and FGFR3 gain-of-function mutations localized in different domains of FGFR3 responsible for chondrodysplasias (FGFR3G380R [transmembrane domain; ACH], FGFR3Y337C [extracellular domain; thanatophoric dysplasia type 1 [TDI]], FGFR3G250R [transmembrane domain; thanatophoric dysplasia type 2 [TDII]] (25, 26). Twenty-four hours after transfection, phosphorylated FGFR3 was detected for each mutant (Figure 1, A and B). For all transfected cells expressing FGFR3 with activating mutations, NVP-BGJ398 reduced FGFR3 phosphorylation (Figure 1, A and B). No phosphorylated FGFR3WT was detected in HEK293-Vnr cells (Figure 1B).

NVP-BGJ398 restores bone growth in Fgfr3Y367C/+ embryonic femurs. In ACH, FGFR3-activating mutations disturb endochondral and membranous ossifications (27, 28). Our in vitro findings therefore prompted us to test the impact of NVP-BGJ398 on long bone and skull growth. To investigate the effect on endochondral ossification, we used ex vivo cultures of embryonic femurs isolated from Fgfr3Y367C/+ mice (E16.5) with various concentrations of NVP-BGJ398. At day 6, untreated Fgfr3Y367C/+ femurs exhibited a reduction of length (~16.7%) compared with WT femurs (Figure 1C and Table 1). Six days of ex vivo culture with 10 nM NVP-BGJ398 did not affect bone growth of WT femurs, whereas 100 nM NVP-BGJ398 rescued completely the bone growth defect of Fgfr3Y367C/+ femurs (Figure 1C and Table 1). At 1 μM, NVP-BGJ398 induced bone overgrowth of Fgfr3Y367C/+ femurs (+19.6% compared with WT femurs, +43.6% compared with untreated Fgfr3Y367C/+ femurs). These data show that the induced femoral elongation by NVP-BGJ398 was dose dependent, confirming the role of FGFR3 as a negative regulator of bone growth (5).

In Fgfr3Y367C/+ mice, the coordinated proliferation and differentiation of chondrocytes that ensure the continuous elongation of the growth plate are defective (29). We observed that in Fgfr3Y367C/+ explants cultured for 6 days, chondrocyte proliferation (i.e., Ki67 expression) was increased (7 times more than WT) as previously reported (ref. 26 and Figure 1, D and E). NVP-BGJ398 significantly reduced chondrocyte proliferation at 100 nM (~56.4% compared with untreated). Differentiated chondrocytes in the growth plate were assessed using an antibody against collagen X (Col X), and we observed that the growth-plate hypertrophic zone was reduced in Fgfr3Y367C/+ explants (~58.4% compared with WT) as previously reported (refs. 15, 30, and Figure 1, D and F). The area of the hypertrophic zone was increased (+167.6%) with NVP-BGJ398 treatment compared with vehicle alone, indicating that the defective differentiation of the chondrocytes was ameliorated (Figure 1, D and F).

To analyze the membranous ossification defect in the Fgfr3Y367C/+ skull vault, we used ex vivo cultures of embryonic mouse calvaria. Previously, we reported a calvarial defect in ACH and Fgfr3Y367C/+ mice, indicating a role of FGFR3 in skull bone growth (28). This defect gradually disappears during skull growth, as it does in mouse calvaria cultures. We observed that the calvarial defect, corresponding to a bone ossification delay, resolved faster and more completely in NVP-BGJ398–treated cultures (100 nM) compared with untreated Fgfr3Y367C/+ calvaria from culture day 4 (~20.1%) to culture day 14 (~46.1%) (Figure 2, A and B). These data confirm that the calvaria-formation defect is directly related to the overactivation of FGFR3.

Together, these results indicate that in ex vivo bone models of ACH, NVP-BGJ398 can rescue chondrocyte proliferation and differentiation defects and can reduce the bone-growth anomalies of the growth plate and the skull vault, thus modifying both chondrogenesis and osteogenesis during embryonic bone development.

NVP-BGJ398 improves growth of the appendicular skeleton in Fgfr3Y367C/+ mice. On the basis of those encouraging in vitro and ex vivo data, we treated Fgfr3Y367C/+ newborn mice (P1) daily with subcutaneous injections of NVP-BGJ398 (2 mg per kg body weight per day) for 15 days (protocol 1). The Fgfr3Y367C/+ mouse phenotype mimics human ACH in several respects; short stature, macrocephaly, prognatism, fused synchondroses, and foramen magnum (FM) stenosis (28, 29). Our objective was to evaluate whether and how NVP-BGJ398 inhibition of the overactivity of FGFR3 would affect bone growth in vivo. The treatment was well tolerated by Fgfr3Y367C/+ and Fgfr3Y367C/+ mice without noticeable modification of behavior.

X-ray analyses of the forelimbs (Figure 3A) and hind limbs (Figure 3B) showed in NVP-BGJ398–treated Fgfr3Y367C/+ mice a

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**Table 1. Improvement of femur lengths after NVP-BGJ398 treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Femur Length (μm)</th>
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<tr>
<td>Fgfr3+/+</td>
<td>5,604 ± 144 μm</td>
</tr>
<tr>
<td>Fgfr3Y367C/+</td>
<td>4,667 ± 104 μm</td>
</tr>
<tr>
<td>Fgfr3Y367C/++NVP-BGJ398, 10 nM</td>
<td>4,659 ± 61 μm</td>
</tr>
<tr>
<td>Fgfr3Y367C/++NVP-BGJ398, 100 nM</td>
<td>5,311 ± 127 μm</td>
</tr>
<tr>
<td>Fgfr3Y367C/++NVP-BGJ398, 1 μM</td>
<td>6,702 ± 151 μm</td>
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Size of femurs after 6 days of culture with NVP-BGJ398. Data are expressed as mean ± SD.
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Figure 2. NVP-BGJ398 reduces the ossification gap of Fgfr3Y367C/+ mouse embryo calvariae ex vivo. (A) Fetal calvaria gaps of Fgfr3Y367C/+ mice with or without treatment are indicated by white lines. Magnified images show the effects on suture. (B) The modifications of calvaria gap size of the Fgfr3Y367C/+ treated mice (n = 11) compared with the untreated Fgfr3Y367C/+ mice (n = 5) are indicated in the graph. *P < 0.05, Mann-Whitney U test. Scale bars: 1 mm. Data are expressed as mean ± SD.

A

B

Figure 2. NVP-BGJ398 reduces the ossification gap of Fgfr3Y367C/+ mouse embryo calvariae ex vivo. (A) Fetal calvaria gaps of Fgfr3Y367C/+ mice with or without treatment are indicated by white lines. Magnified images show the effects on suture. (B) The modifications of calvaria gap size of the Fgfr3Y367C/+ treated mice (n = 11) compared with the untreated Fgfr3Y367C/+ mice (n = 5) are indicated in the graph. *P < 0.05, Mann-Whitney U test. Scale bars: 1 mm. Data are expressed as mean ± SD.

distinct increase in size of these limbs compared with those of control Fgfr3+/+ littermates. At necropsy, long bones of treated Fgfr3Y367C/+ mice (NVP-BGJ398 and vehicle) and their Fgfr3+/+ control littermates were measured. In Fgfr3+/+ mice, the lengths of proximal and distal long bones were significantly higher with NVP-BGJ398 treatment than control treatment (femur +20.9%, humerus +11.9%, tibia +32.6%, ulna 22.3%, and radius +24.1%) (Figure 3C and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI83926DS1). μCT imaging confirmed these data and also showed that NVP-BGJ398 treatment expanded the total cross-sectional tissue area of Fgfr3Y367C/+ mouse distal femur metaphysis (+30% vs vehicle) (Figure 3, D–F). The long-bone growth of Fgfr3+/+ mice treated with NVP-BGJ398 was not affected (data not shown), and no premature death was observed, confirming the mild or null effect of the inhibitor on Fgfr3+/+ mice.

Histological analyses of the epiphyseal growth plate of long bones showed that NVP-BGJ398 improved growth-plate cartilage homeostasis in both distal and proximal femurs (Figure 4A) and tibias (Supplemental Figure 1B).

Chondrocyte proliferation was lower in Fgfr3Y367C/+ untreated mice (−59.9%) compared with Fgfr3+/+ mice, as measured with BrdU incorporation into the growth plate. By contrast, chondrocyte proliferation was significantly higher with NVP-BGJ398 treatment than control treatment in Fgfr3Y367C/+ mice (+162.1%) (Figure 4B).

Likewise, the impaired differentiation of hypertrophic chondrocytes in femurs and tibias of Fgfr3Y367C/+ mice was rescued by NVP-BGJ398 treatment, as demonstrated by Col X immunostaining (Figure 4C and Supplemental Figure 1B). In these treated mice, the expression of Col X was observed in the calcified cartilage, showing an active process of bone growth at the subtrabecular bone level (Figure 4C and Supplemental Figure 1B). Previously, we reported that angiogenesis was disturbed in Fgfr3 Y367C/+ mice (29). We hypothesized that the action of NVP-BGJ398 on cartilage could affect angiogenesis in the growth plate and thus activate mineralization reflected by the altered size of the secondary ossification center (Figure 4A, and Supplemental Figure 1B). In support of this hypothesis, CD34, a marker of immature endothelial cells, was higher in the chondroosseous junctions of Fgfr3 Y367C/+ mice with the NVP-BGJ398 treatment in comparison with the control treatment (Supplemental Figure 1C). These data suggest that NVP-BGJ398 treatment increased formation of endothelial sprouts and initiation of angiogenesis in Fgfr3Y367C/+ mice.
bar pedicles were shorter (~62.4%) in Fgfr3<sup>Y367C/+</sup> mice compared with Fgfr3<sup>+/+</sup> mice. After treatment with NVP-BGJ398 in Fgfr3<sup>Y367C/+</sup> mice, the lumbar pedicles were greater in size than with the control treatment (~21.8%) (Figure 5, B and C, and Supplemental Figure 2A). We noted a lower size of the L4–L6 segment in Fgfr3<sup>Y367C/+</sup> compared with Fgfr3<sup>+/+</sup> mice (~29%), and after 15 days of treatment with NVP-BGJ398, the size was greater than with the control treatment (~12.1%) (Figure 5D and Supplemental Figure 2A).

To complete these analyses, we focused on IVDs, where FGFR3 is expressed (Supplemental Figure 2B). The IVD contains the cartilage endplate (CEP) and fibrocartilaginous tissues, including the annulus fibrosus (AF) and the nucleus pulposus...
Figure 4. NVP-BGJ398 improves chondrocyte differentiation and proliferation in growth plate. (A) Safranin O (SO) staining on femur from Fgfr3^{+/+} and untreated or treated Fgfr3^{Y367C/+} mice. Scale bars: 100 μm. Asterisks show secondary ossification centers (Fgfr3^{+/+} treated Fgfr3^{Y367C/+} have these centers more developed compared with Fgfr3^{Y367C/+} vehicle). Arrowhead highlights appearance of columnar hypertrophic chondrocytes, while arrow indicates flattening of prehypertrophic cells in Fgfr3^{Y367C/+} treated mice. (B) BrdU immunohistology on distal femur growth plates from Fgfr3^{+/+} and untreated or treated Fgfr3^{Y367C/+}. Quantification of cell proliferation (BrdU-labeled cells/μm²) (n = 10 per group). *P < 0.05, 1-way ANOVA. Scale bar: 100 μm. (C) Immunohistochemistry for Col X on femur from Fgfr3^{+/+} and untreated or treated Fgfr3^{Y367C/+}. Scale bar: 100 μm. Images shown are representative of n = 6 animals per group. All data are from animals treated with protocol 1 (16 days old). Data are expressed as mean ± SD.
The NP is a gelatinous tissue located in the center of the IVD surrounded by the lamellar structured AF. First of all, we observed a lower width (−52.6%) and a greater length (+54.2%) of the NP in Fgfr3Y367C/+ mice compared with Fgfr3+/+ mice at 16 days (Figure 6, A and B). These defects were significantly ameliorated by NVP-BGJ398 treatment compared with control treatment for both the width (+73.3%) and length (−17.1%) in Fgfr3Y367C/+ mice.

In order to analyze the AF extracellular matrix (ECM), we studied Col I and II expression. The cartilaginous area (Col II) corresponds to the inner AF (IAF), and the fibrous area (Col I) forms the outer AF (OAF) (31). Interestingly, we observed for what we believe is the first time that the length of the IAF was higher in Fgfr3Y367C/+ mice (+72.4%) than in Fgfr3+/+ mice (Figure 6C and Supplemental Figure 2B). This IAF phenotype was significantly ameliorated by NVP-BGJ398 treatment compared with control treatment (−60%). As a consequence, the IAF/OAF ratio was also modified with the treatment.

In addition, we observed that the size of the CEP was lower in Fgfr3Y367C/+ mice (−18%) compared with Fgfr3+/+ mice, probably due to defective hypertrophic-chondrocyte differentiation (Supplemental Figure 3, A and B). This CEP phenotype was significantly ameliorated by NVP-BGJ398 treatment compared with control treatment (+29.3%) (Supplemental Figure 3, A and B).

Taking these data together, we demonstrate here that 15 days of NVP-BGJ398 treatment corrected the size of L4–L6 and pedicles and modified collagen expression in the IVD. These data suggest a possible improvement of the ACH spinal stenosis phenotype with treatment.

NVP-BGJ398 improves the growth of the craniofacial skeleton in Fgfr3Y367C/+ mice. The cranial base is severely affected in humans with ACH and in Fgfr3Y367C/+ mice and hence contributes greatly to the craniofacial phenotype (28).

Therefore, we assessed whether early treatment with NVP-BGJ398 could correct the cartilage defects of the skull base and thus prevent the premature loss of synchondroses. Imaging of the skull using μCT revealed that after 2 weeks of NVP-BGJ398 treatment compared with control treatment, the brachycephalic appearance of Fgfr3Y367C/+ mice was less obvious because the anterior-posterior length of the skull was greater (+5.9%) (Figure 7A). In addition, we observed that the size of nasal-bone length was lower in Fgfr3Y367C/+ mice (−33.8%) than in Fgfr3+/+ mice. This nasal-bone phenotype was significantly ameliorated by NVP-BGJ398 treatment compared with control treatment (+20.45%) (Figure 7A). NVP-BGJ398 treatment partially prevented the loss of 4 synchondroses: the sphenoccipital synchondrosis (SOS), the intersphenoidal synchondrosis (ISS), and the 2 interoccipital synchondroses (IOSs) in Fgfr3Y367C/+ mice (Figure 7, A and B). The efficacy of the NVP-BGJ398 treatment was more prominent on the IOSs (all treated mice had at least one IOS active) than on the SOS and ISS (Table 2).

The size of the FM is much smaller in ACH patients and Fgfr3Y367C/+ mice, causing cervicomedullary compression (28). In Fgfr3Y367C/+ mice, the size of the FM was significantly higher after...
NVP-BGJ398 treatment (area, +16.8%; transversal length, +15.8%; and sagittal length, +7.6% as assessed by μCT) (Figure 8, A and B). The rescue of the synchondroses phenotype presumably played a role in the amelioration of the FM phenotype.

These data indicate an impact of FGFR3 on both mesenchymal (synchondroses) and neural crest-derived osteoblasts (nasal bone) and suggest an important role of FGFR3 on both intramembranous and endochondral ossification processes.

**NVP-BGJ398 rescues defective FGFR3 signaling.** FGFR3 is expressed in proliferative and prehypertrophic zones of the growth plate and modulates both proliferation and differentiation of the chondrocytes (32). The activating mutation stabilizes the receptor in cells, as demonstrated by the high expression of the receptor in the growth plate. The treatment of NVP-BGJ398 had a beneficial effect on the turnover of the receptor and lowered its expression in chondrocytes (Supplemental Figure 4A). In our Fgfr3Y367C/− mice, as expected, another tyrosine kinase, FGFR1, was expressed in the hypertrophic zone of the growth plate, and its expression was not modified with NVP-BGJ398 treatment (Supplemental Figure 4B).

FGFR3 negatively regulates chondrocyte proliferation and differentiation via several signaling pathways. The 2 principal signaling pathways are the MAPK pathway, which regulates matrix production and chondrocyte differentiation, and the STAT1 pathway, which controls proliferation (32). The FGFR3 canonical downstream MAPK signaling pathway is more active in FGFR3-related disorders (33, 34). Consistent with decreased FGFR3 phosphorylation, the activation of MAPK and PLCγ pathways was reduced with NVP-BGJ398 dosing in HEK293-Vnr cells and human chondrocytes transiently transfected with constructs encoding activation mutants of FGFR3 (Supplemental Figure 5, A and B). These results were confirmed using human immortalized ACH (p.Gly380Arg/+) and TD (p.Ser249Cys/+) chondrocytes (ref. 25 and Supplemental Figure 5C) and using human (ACH, control) and mouse primary chondrocytes (Figure 9, A and B). With these cell cultures, we also observed a NVP-BGJ398 dose-dependent decrease in the level of phosphorylated ERK1/2 (Supplemental Figure 5C).

High levels of phosphorylated ERK1/2 were observed in Fgfr3Y367C/+ chondrocyte cultures and in the growth plate of Fgfr3Y367C/+ mice in vivo, thus confirming the constitutive activation of the MAPK pathway in those mice (Figure 9C). Interestingly, ERK1/2 phosphorylation was undetectable in NVP-BGJ398-treated chondrocyte cultures, and ERK1/2 phosphorylation was lower in growth-plate cartilage after NVP-BGJ398 treatment compared with control treatment in Fgfr3Y367C/+ mice (Figure 9, A–C, and Supplemental Figure 5, A–C). These data show that NVP-BGJ398 inhibited FGFR3 activity in a RAS-ERK-dependent fashion.
The role of the transcription factor SOX9 in chondrogenic differentiation is well recognized (35, 36). Previously, it was reported that activated FGFR3 prevents the downregulation of transcription factor SOX9 in differentiated chondrocytes (11). This mechanism was also observed in Fgfr3Y367C/+ mice where the high expression of SOX9 in the prehypertrophic chondrocytes was lowered with NVP-BGJ398 treatment (Figure 9D). Also, the downregulation of SOX9 correlated with the modification of the size and the shape of hypertrophic chondrocytes (Figure 4, A and C).

In FGFR3-related disorders, it has been demonstrated that the STAT1 pathway regulates chondrocyte proliferation (37, 38). In the Fgfr3Y367C/+ growth plate, the level of phosphorylated STAT1 is higher than in Fgfr3+/- mice, and the level of phosphorylated STAT1 is lowered by NVP-BGJ398 treatment (Supplemental Figure 5D). Hence, these data provide an underlying molecular explanation for the higher rate of chondrocyte proliferation with NVP-BGJ398 treatment in Fgfr3 Y367C/+ mice, as shown with BrdU data (Figure 4B).

Overall, the data suggest that NVP-BGJ398 treatment in Fgfr3Y367C/+ mice restores the activity of FGFR3 downstream-signaling pathways to that of levels observed in Fgfr3+/- mice.

**Effect of timing and type of treatment on the improvement of bone growth.** Bone elongation relies on an active process controlled by proliferation and differentiation of chondrocytes within the growth plate. In humans, this process comes to a halt at puberty and corresponds to the disappearance of the growth plate. Therefore, in ACH patients, treatment effectiveness may be better if the treatment commences before puberty. For this reason, we chose to treat Fgfr3Y367C/+ mice from day 1 for 15 days (protocol 1). However, in order to compare the efficacy of NVP-BGJ398 with that shown in previously published data, we also tested a second protocol (protocol 2), similar to the one used for BMN111 (start at day 7 for 10 days) (15). The gain of growth of femurs and tibias was 50% higher in animals treated earlier and longer with NVP-BGJ398 (from day 1) than those treated from day 7 (Supplemental Figure 6 and Table 3). When NVP-BGJ398 treatment was compared with BMN111 treatment in Fgfr3Y367C/+ mice using protocol 2, we observed a greater amelioration of several aspects of the dwarfism pheno-

**Table 2. Active synchondroses**

<table>
<thead>
<tr>
<th></th>
<th>Fgfr3+/+</th>
<th>Fgfr3Y367C/+</th>
<th>Fgfr3Y367C/+ + NVP-BGJ398</th>
</tr>
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<tbody>
<tr>
<td>ISS</td>
<td>100% (9/9)</td>
<td>0% (0/10)</td>
<td>37.5% (3/8)</td>
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<tr>
<td>SOS</td>
<td>100% (9/9)</td>
<td>0% (0/10)</td>
<td>25% (2/8)</td>
</tr>
<tr>
<td>Both ISS+SOS</td>
<td>100% (9/9)</td>
<td>0% (0/10)</td>
<td>75% (6/8)</td>
</tr>
<tr>
<td>1 IOS only</td>
<td>–</td>
<td>0% (0/10)</td>
<td>25% (2/8)</td>
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Percentage of active synchondroses. All data are from animals treated with protocol 1 (16 days old).
signaling through the control of SOX9 (32) and chondrocyte proliferation is suppressed by STAT1 activation (12).

One therapeutic strategy to rescue the defective skeletal development caused by \(FGFR3\) gain-of-function mutations is to reduce or counteract the hyperactivity of \(FGFR3\). Among the putative therapeutic approaches for ACH, the use of TKIs seems the most suitable. Although TKIs have been successfully used in cancer (45–47), TKIs have so far not been applied in skeletal disorders. Here, we demonstrate that NVP-BGJ398, a pan-FGFR inhibitor (20), was able to reduce the increased phosphorylation of \(FGFR3\) and the activation of its downstream signaling pathways in vitro, in human and mouse \((Fgfr3^{Y367C/+})\) chondrocyte models of ACH, and to rescue the growth-plate phenotype in vivo in \(Fgfr3^{Y367C/+}\) mice. We believe the result obtained with NVP-BGJ398 is novel, as previous in vivo experiments using TKIs failed to demonstrate any rescue of the dwarf phenotype (48). Previously, NVP-BGJ398 was tested in animal models for cancers, including hepatocellular carcinoma (24) and malignant rhabdoid tumors (23) as well as \(FGF23\)-dependent hypophosphatemic rickets (22). The effective doses administrated in these preclinical studies for cancer were 10 to 100 times higher than the doses used in this present study to correct the dwarfism phenotype. Other therapeutic strategies were explored for ACH, with protocols using soluble \(FGFR3\) (17), statin (18), or meclozine (19) in another mouse model of ACH \((Fgfr3^{Ach})\) in which the \(Fgfr3\) transgene with the ACH mutation is expressed under the control of \(Col2a1\) (7). The comparison of those results with the results from our study is made complicated by the differences among the mouse models (genotypes, skeletal type with NVP-BGJ398 than with BMN111 (femur, tibia, tail, L4–L6) (Supplemental Figure 6, Table 3, and ref. 15). In conclusion, NVP-BGJ398 treatment of \(Fgfr3^{Y367C/+}\) mice appeared more effective when administered earlier postnatally and for a longer period. NVP-BGJ398 treatment also appeared more effective than BMN111 treatment, suggesting that directly targeting \(FGFR3\) with a TKI represents a better approach than targeting \(FGFR3\) downstream-signaling pathways.

**Discussion**

Elevated kinase activity of the \(FGFR3\) receptor due to germinal or somatic mutations disrupts several biological functions, as demonstrated in \(FGFR3\)-related disorders (ACH, TD, hypochondroplasia, Muenke syndrome) (1, 2, 39) and numerous cancers (bladder cancers and multiple myeloma) (40–42). Hyperactive \(FGFR3\) signaling causes dwarfism, including ACH, which is characterized by anomalies in bone development, and the gain-of-function \(Fgfr3^{Y367C/+}\) mutation can be used to model ACH in mice (6, 28, 29, 43). Conversely, a total impairment of the \(FGFR3\) signaling pathway can cause bone overgrowth, confirming that \(FGFR3\) is a negative regulator of bone growth (5). On a functional level, \(FGFR\) activation affects various cell behaviors via the downstream activation of the MAPK intracellular signaling cascade. The MAPK pathway plays an important role in bone development, as demonstrated in MEK1 gain-of-function mice (12) and ERK1 and ERK2 null mice (44), leading to a dwarf or skeletal overgrowth phenotype, respectively. The underlying mechanisms in \(FGFR3\)-related disorders suggest that chondrocyte differentiation is regulated by MAPK signaling through the control of SOX9 (32) and chondrocyte proliferation is suppressed by STAT1 activation (12).

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Here, we demonstrate that the axial skeleton was affected in Fgfr3Y367C/+ mice and the size of the lumbar vertebral bodies and pedicles was reduced. In addition, we showed that the three components (NF, AF, CEP) of the IVD were affected. One of the findings of this study is that NVP-BGJ398 treatment remarkably corrected the lumbar vertebral body and pedicle size defects and modified the components of the ECM of the IVD. These data demonstrate that overactivation of FGFR3 disturbs cartilage, IVDs, and bone-forming lumbar rachis, thus explaining the spinal stenosis, herniated disc, and kyphoscoliosis phenotypes of ACH patients. The positive impact of NVP-BGJ398 treatment is particularly noteworthy in this context: an improvement of axial skeletal development in ACH could reduce the need for decompressive laminectomy surgery, a procedure required for treating severe spinal stenosis (52).

The duration of the NVP-BGJ398 treatments were probably too short for the amelioration of the defective bone structure of the long bones in Fgfr3Y367C/+ mice (43). Longer treatments might be necessary to improve the bone architecture, as demonstrated previously with 2 months of PTH injection in an Fgfr3-related phenotypes, and FGFR3 expression pattern (17–19). Interestingly, NVP-BGJ398 treatment for 10 days improved the bone growth 2 to 3 times more than what had been reported for BMN111 (15). NVP-BGJ398 (2 mg/kg) treatment of WT mice did not affect skeletal growth (data not shown), and treatment of Fgfr3Y367C/+ mice did not modify the expression of FGFR1 in the hypertrophic zone of the growth plate. Thus, in this preclinical study, NVP-BGJ398–mediated effects were mainly due to FGFR3 inhibition, with no other gross side effects being observed.

The rescue of the growth-plate disorganization, the defective chondrocyte proliferation, and the delay of differentiation and secondary ossification were remarkable. The disrupted downstream FGFR3 signaling pathways were corrected with the NVP-BGJ398 treatment, confirming the key role of ERK1/2, SOX9, and STAT1 signaling during skeletal development (32).

Of major importance, NVP-BGJ398, when applied from birth onwards, increased the size of the reduced FM and inhibited the premature fusion of synchondroses. In addition, the size of the neural crest–derived nasal bone was increased, reflecting that intramembranous ossification is also affected in ACH (28). These treatment-related effects on the skull suggested the potential of correcting facial appearance in ACH patients and, most importantly, the potential of reducing the risks related to the cervicomedullary compression and early death that can occur in ACH children (1, 49, 50).

The spine anomalies of children and adults with ACH are kyphosis at the thoracolumbar junction (51) and spinal stenosis (52), eventually leading to leg paralysis and neurogenic claudication (2). Here, we demonstrate that the axial skeleton was affected in Fgfr3Y367C/+ mice and the size of the lumbar vertebral bodies and pedicles was reduced. In addition, we showed that the three components (NF, AF, CEP) of the IVD were affected. One of the findings of this study is that NVP-BGJ398 treatment remarkably corrected the lumbar vertebral body and pedicle size defects and modified the components of the ECM of the IVD. These data demonstrate that overactivation of FGFR3 disturbs cartilage, IVDs, and bone-forming lumbar rachis, thus explaining the spinal stenosis, herniated disc, and kyphoscoliosis phenotypes of ACH patients. The positive impact of NVP-BGJ398 treatment is particularly noteworthy in this context: an improvement of axial skeletal development in ACH could reduce the need for decompressive laminectomy surgery, a procedure required for treating severe spinal stenosis (52).

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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tail</th>
<th>Femur</th>
<th>Tibia</th>
<th>L4–L6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP-BGJ398 2 mg/kg (protocol 1)</td>
<td>26.5%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.9%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.6%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.1%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NVP-BGJ398 2 mg/kg (protocol 2)</td>
<td>25.8%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.1%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.5%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.6%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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Gain in length (percentage) for tail, femur, tibia, and L4–L6 of NVP-BGJ398–treated mice compared with vehicle 1. A best improvement is observed with animals treated at day 1 (protocol 1). The dwarfism improvement is more important with NVP-BGJ398 than with BMN111 (protocol 2 compared with data from ref. 15). <sup>a</sup>P < 0.05 by 1-way ANOVA test.
dwarf mouse model (16). Because short-term NVP-BGJ398 treatment improved the chondroosseous junction in the long bones of the growing Fgfr3Y367C/+ mice, there is potential that longer NVP-BGJ398 treatments could lead to a secondary positive effect on the resulting bone structure.

The different skeletal phenotypes and molecular pathways evaluated in our study provide a wide range of markers that can be monitored for further optimization of dosing schedules of TKI in addition to prolongation, such as reductions in dose quantity or frequency (e.g., intermittent injections every 2 or 3 days).

In summary, we have shown that NVP-BGJ398 inhibits FGFR3 and its downstream signaling pathways, which are active in cartilage in ACH. As a consequence, NVP-BGJ398 rescues the endochondral and membranous ossification processes in a mouse model of ACH. Our results provide a rationale for targeting FGFR3 with a specific TKI for the treatment of children with ACH.

**Methods**

**Transient transfections of cDNA FGFR3 constructs.** Human control chondrocyte lines (25) and human embryonic kidney (HEK) cells (26) at 80%-90% confluence were transiently transfected with FGFR3 and its downstream signaling pathways, which are active in cartilage in ACH. As a consequence, NVP-BGJ398 rescues the endochondral and membranous ossification processes in a mouse model of ACH. Our results provide a rationale for targeting FGFR3 with a specific TKI for the treatment of children with ACH.

**FGFR3K650M** obtained as previously reported (26) using JetPrime (Polyplus-transfection) following the manufacturer’s instructions. Transfected cells were incubated with NVP-BGJ398 (Novartis Basel) at concentrations of 100 nM, 1 nM, or 10 μM overnight. Transfected cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5% NP40, and 0.25% sodium deoxycholate, supplemented with protease and phosphatase inhibitors; Roche). Immunoprecipitations were performed by incubating 3 μl rabbit anti-FGFR3 (Sigma-Aldrich F0425)/500 μg protein with protein A–agarose (Roche). Primary and immortalized human chondrocytes and mouse chondrocytes cultures. Primary and immortalized mutant cells were obtained as previously described (25). The protocol used for mouse chondrocytes was previously reported (24). Human and mouse cells were incubated with NVP-BGJ398 (1, 10, 50, 100, 250, and 500 nM) overnight.

**Immunoblotting.** Whole-cell lysates and immunoprecipitated proteins were subjected to NuPAGE 4%-12% bis-tris acrylamide gels (Life Technologies). Blots were probed with antibodies using standard protocols: anti-FGFR3 polyclonal antibody (Sigma-Aldrich FO425; 1:1,000 dilution), anti-phospho-tyrosine P-Tyr-100 monoclonal antibody (Cell Signaling catalog 9411; 1:1,000 dilution), anti-phospho-ERK1/2 monoclonal antibody (Cell Signaling catalog 4270; 1:1,000 dilution), anti-phospho-ERK1/2 polyclonal antibody (Sigma-Aldrich M670; 1:1,000 dilution), anti-phospho-PLCγ polyclonal antibody (Cell Signaling catalog 2821; 1:1,000 dilution), and anti-PLCγ polyclonal antibody (Cell Signaling catalog 2822; 1:1,000 dilution).

**Mouse model and drug treatment.** Fgfr3Y367C/+ mice were described previously (29). All mice were on a C57BL/6 background. Cartilage and bone analyses were performed in 16-day-old mice. Protocol 1 was as follows: Fgfr3Y367C/+ mice were 1 day old at treatment initiation and received daily subcutaneous administrations of NVP-BGJ398 for 15 days (2 mg/kg body weight) or vehicle (HCl 3.5 mM, DMSO 5%) for 10 days; this protocol is similar to the BMN111 protocol previously described (15).

**Genotyping.** Genomic DNA was isolated from tail tips by proteinase K digestion and extracted with NucleoSpin Tissue (Macherey-Nagel) according to the manufacturer’s instructions. PCR was performed using primers as previously described (29).

**Femoral culture system.** Ex vivo femur cultures were conducted as described previously (30). Right femur was cultured with NVP-BGJ398 at a concentration of 10-100 nM to 1 μM and compared with the untreated left femur. The bone length was measured at the beginning (before treatment) and at the end of time course. Each experiment was repeated at least 5 times. Images were captured with an Olympus SZX12 stereo microscope. The size of the femur was measured using cellSens software (Olympus).

**Calvarial culture system.** Ex vivo calvarial cultures were conducted as described previously (28). Calvaria (E18.5) were placed in 24-well plates on top of 250 μl of Matrigel (BD Biosciences) previously placed at the bottom of the well. Calvaria were cultured in α-MEM (Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies), 100 μg/ml ascorbic acid (Sigma-Aldrich), and 1% penicillin/streptomycin (Gibco, Life Technologies) and NVP-BGJ398 at 100 nM over 14 days. Images were captured with an Olympus SZX12 stereo microscope. The size of the calvaria defect was measured using ImageJ software.

**Histological and immunohistochemical analyses.** Fetal femur explants, long bones (femurs, tibia), and L4-L6 vertebrae were fixed in 4% paraformaldehyde, decalcified with EDTA 0.4 M, and embedded in paraffin. Serial sections of 5 μm were stained with H&E and safranin O. For immunohistochemistry, sections were labeled with antibodies against Ki67 (monoclonal antibody, Abcam ab92353; 1:3,000 dilution), Col X (BIOCYN, N.2031501005; 1:50 dilution), CD34 (monoclonal antibody, Abcam ab1858; 1:100 dilution) and Col I (monoclonal antibody, Novotec, catalog 20151; 1:1,000 dilution), Col II (monoclonal antibody, Novotec, catalog 20251; 1:1,000 dilution), SOX9 (polyspecific antibody, Santa Cruz Biotechnology Inc., catalog D0609; dilution 1:75), phospho-ERK1/2 (monoclonal antibody, Cell Signaling, catalog 4370; 1:100 dilution), phospho-Stat 1 (monoclonal antibody, Abcam, catalog ab19932; 1:200 dilution), FGFR1 (polyclonal antibody, Abcam, catalog ab63601; 1:75 dilution), and FGFR3 (polyclonal antibody, Sigma-Aldrich, catalog FO425; 1:250 dilution) using the Dako Envision Kit. Proliferation was analyzed with the BrdU Envision Kit. Images were captured with an Olympus DP70-IX2-UBC microscope and cellSens software. Labeled cells were counted using ImageJ software.

**X-rays and CT scanner.** The whole skeletons of the mice were radiographed using Faxitron (MX-20). μCT analyses were performed using a μCT40 Scanco vivaCT42 (Scanco Medical) instrument and the following settings: integration time, 300 ms (skull) or 175 ms (femur); 45 kVp; 177 μA. For determination of femoral structural parameters, a segmentation threshold of 270 was applied and a Gaussian filter (σ = 0.7, support of 1 voxel) was used to suppress the noise. The evaluated volume was proportional to the femoral length, as measured on plain radiographs using a specimen radiography system (Faxitron LX-60), and corresponded to an average of 142 μm (metaphysis) or
120 μm (diaphysis) for vehicle-treated and 169 μm (metaphysis) or 143 μm (diaphysis) for compound-treated Fgf3−/− mice.

Statistics. Differences between experimental groups were assessed using ANOVA with Tukey’s post hoc test or Mann-Whitney U test. The significance threshold was set at P ≤ 0.05. Statistical analyses were performed using GraphPad PRISM (v5.03). All values are shown as mean ± SD.

Study approval. Experimental animal procedures and protocols were approved by the French Animal Care and Use Committee.

Author contributions
DKE, ED, IK, AM, and LLM designed the research. DKE, ED, CBL, NK, CLG, LM, and MBD performed the experiments. DKE, IK, FDR, and MBD analyzed the scanners. PB, FB, and DGP analyzed NVP-BGJ398 activity. DKE prepared the figures. DKE, IK, MK, MBD, and LLM wrote the manuscript.

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