NF449 Is a Novel Inhibitor of Fibroblast Growth Factor Receptor 3 (FGFR3) Signaling Active in Chondrocytes and Multiple Myeloma Cells*§

From the §Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048, the ¶Department of Animal Physiology and Immunology, Institute of Experimental Biology, Masaryk University, 61377 Brno, Czech Republic, the ¶Department of Cytokinetics, Institute of Biophysics, Academy of Sciences of the Czech Republic, 61265 Brno, Czech Republic, the ¶Department of Orthopaedics, Case Western Reserve University, Cleveland, Ohio 44106, the ¶Department of Chemistry, Utrecht University, NL-3584 CH Utrecht, The Netherlands, and the ¶¶Department of Pediatrics, UCLA School of Medicine, Los Angeles, California 90095

The FGFR3 receptor tyrosine kinase represents an attractive target for therapy due to its role in several human disorders, including skeletal dysplasias, multiple myeloma, and cervical and bladder carcinomas. By using molecular library screening, we identified a compound named NF449 with inhibitory activity toward FGFR3 signaling. In cultured chondrocytes and murine limb organ culture, NF449 rescued FGFR3-mediated extracellular matrix loss and growth inhibition, which represent two major cellular phenotypes of aberrant FGFR3 signaling in cartilage. Similarly, NF449 antagonized FGFR3 action in the multiple myeloma cell lines OPM2 and KMS11, as evidenced by NF449-mediated reversal of ERK MAPK activation and transcript accumulation of CCL3 and CCL4 chemokines, both of which are induced by FGFR3 activation. In cell-free kinase assays, NF449 inhibited the kinase activity of both wild type and a disease-associated FGFR3 mutant (K650E) in a fashion that appeared non-competitive with ATP. Our data identify NF449 as a novel antagonist of FGFR3 signaling, useful for FGFR3 inhibition alone or in combination with inhibitors that target the ATP binding site.

NF449 (fibroblast growth factor receptor 3) is a transmembrane tyrosine kinase that serves as a receptor for the members of the fibroblast growth factor (FGF) family and functions in many biological processes, including cell proliferation, differentiation, migration, and survival. To date, activating mutations in FGFR3 have been associated with several human disorders, such as skeletal dysplasias, multiple myeloma, and cervical and bladder carcinomas (1–4).

Among the skeletal dysplasias, activating FGFR3 mutations cause achondroplasia, the most common form of human skeletal dysplasia, and thanatophoric dysplasia, the most common form of lethal skeletal dysplasia (1). Long bones of individuals suffering from FGFR3-related skeletal dysplasias show markedly shortened zones of chondrocyte proliferation and differentiation, whereas Fgr3 knock-out mice and humans without functional FGFR3 demonstrate skeletal overgrowth, together implying the role of FGFR3 as a negative regulator of bone growth (5).

Apart from cartilage, ~15% of patients suffering from multiple myeloma markedly up-regulate FGFR3 as a consequence of a t(4;14)(p16.3;q32) translocation, with a fraction of patients also harboring activating mutations in FGFR3, identical to those found in the skeletal dysplasias (2, 3). Ectopic expression of FGFR3 enhances multiple myeloma cell proliferation and survival, demonstrating the oncogenic potential of FGFR3 (6).

Given its role in human disease, FGFR3 signaling represents an attractive target for therapy. There is no treatment available for achondroplasia at present, thus inciting the development of novel approaches to target FGFR3. The aim of this study was to identify novel inhibitors of FGFR3 signaling in cellular environments relevant to FGFR3-related disorders. We recently established a chondrocyte cell-based reporter assay suitable for identification of inhibitors of FGFR3 signaling via molecular library screening (7).

Here, we used this assay to identify and characterize the compound named NF449 as a novel antagonist of FGFR3 signaling active in both chondrocytes and multiple myeloma cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Growth Assays—Rat chondrosarcoma (RCS) chondrocytes, Chinese hamster ovary (CHO) cells, and multiple

signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PLC, phospholipase C.

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myeloma cell lines OPM2 and KMS11 were propagated in Dulbecco’s modified Eagle’s medium, Opti-MEM, or RPMI medium (Invitrogen), supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, GA) and antibiotics. The RCS cell growth experiments utilizing crystal violet staining to quantify cell growth are described in detail elsewhere (7). For the RCS growth arrest experiments here, ∼1 × 10^4 cells were seeded in 24-well tissue culture plates (Costar, Cambridge, MA), treated as indicated for 72 h, and counted. FGF2 was obtained from R&D Systems (Minneapolis, MN); C-natriuretic peptide, SU5402, suramin, NF023, and NF449 were from Calbiochem; NF007, NF110, NF157, NF279, ATP, MethyleneATP, and BzATP were from Tocris Bioscience (Ellisville, MO).

Bone Explant Culture—For explant culture, tibiae were harvested from wild type mouse embryos at embryonic day 16.5. Tibiae were dissected out under the microscope, placed in non-treated polystyrene 96-well plates, and cultured in α-minimal essential medium (Invitrogen), supplemented with 0.2% bovine serum albumin, 1 mM β-glycerophosphate, and 50 µg/ml ascorbic acid. Tibiae were further treated with FGF2 in the presence or absence of NF449 for 6 days, fixed in 10% formaldehyde in phosphate-buffered saline, demineralized with 0.5 M EDTA, and embedded in paraffin. Sections were stained with hematoxylin, eosin, and Alcian blue. Images were taken with a Leica microscope and Leica suite software (Leica, Bannockburn, IL). The length of the proximal epiphysis and bone marrow space was measured using PhotoFiltre image editing software.

Alcian Blue Staining and [35S]Sulfate Labeling Assays—For Alcian blue staining, growing RCS cultures were treated with FGF2 (5 ng/ml) alone or in the presence of NF449 (20 µM) for 72 h, fixed with 4% paraformaldehyde in phosphate-buffered saline, dehydrated with 0.5 M EDTA, and embedded in paraffin. Sections were stained with hematoxylin, eosin, and Alcian blue. Images were taken with a Leica microscope and Leica suite software (Leica, Bannockburn, IL). The length of the proximal epiphysis and bone marrow space was measured using PhotoFiltre image editing software.

Preparation of Cell Extracts, Western Blotting (WB), Immunoprecipitation, Signaling, and Kinase Studies—Cells were lysed in ice-cold immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 25 mM NaF, 10 mM Na_2VO_4) supplemented with proteinase inhibitors. Protein samples were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The following antibodies were used: ERK1/2, phospho-ERK1/2(Thr202/Tyr204), phospholipase Cγ1 (PLCγ1), phospho-PLCγ1(Tyr783), MEK1/2, phospho-MEK1/2(Ser217/218), STAT1, phospho-STAT1(Tyr701), cavelin 1, lamin A/C, ID2, FMS, PDGFRα, AXL, IGF1R, FLT3, and c-Kit (Cell Signaling, Beverly, MA); actin, FGFR1, FGFR2, FGFR3, FGFR4, and TRKA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). WB signal was quantified by determining the integrated optical density of a given band using Scion Image software (Scion Corp., Frederick, MA). For cell signaling studies, cells were either treated with NF449 for 30 min to 1 h prior to the FGF2 addition (OPM2 and KMS11) or pretreated with NF449 for 12–24 h before the FGF2 addition (RCS). Heparin (1 µg/ml; Invitrogen) was used together with FGF2 for RCS chondrocyte treatment, and the cell cartilaginous matrix was removed by collagenase (type II; Invitrogen) treatment before the addition of NF449. Immunoprecipitations and FGFR3 kinase assays were performed as described (8). Briefly, the pRK7 vector encoding C-terminally FLAG-tagged K650E-FGFR3 was transfected into CHO cells using FuGENE reagent according to the manufacturer’s protocol (Roche Applied Science). 48 h after transfection, cells were treated with 20 ng/ml FGF2 for 15 min, and K650E-FGFR3 was immunoprecipitated from ∼800 µg of cell lysate protein using 4 µg of anti-FLAG antibody (Sigma). Cells transfected with a plasmid encoding green fluorescent protein (pcDNA3) served as a negative control for the immunoprecipitation. Immuno complexes were washed two times with kinase buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl_2, 3 mM MnCl_2), and the kinase reactions were performed for 30 min at 30 °C in the presence of 2.5 µg of polyethylene glycol, 10 µM ATP, 200–300 ng of recombinant STAT1 (Enzo Life Sciences, Plymouth, PA) or Prolias (Rockville, MD) as a substrate, and inhibitors were added directly to the kinase reaction. Kinase assays utilizing the recombinant tyrosine kinase (TK) domains of FGFR1 to -4, FMS, PDGFRα, TRKA, AXL, IGF1R, FLT3, epidermal growth factor receptor, c-Kit, and DDR2 (SignalChem, Richmond, Canada) were carried out similarly, with 200–300 ng of kinase used in a 50-µl reaction, and inhibitors were added directly to the kinase reaction. Kinase-mediated phosphorylation of STAT1 was detected by WB with phospho-STAT1(Tyr701) antibody.

Real-time Reverse Transcription-PCR—Total RNA was isolated using the RNeasy mini-kit (Qiagen, Valencia, CA), and poly(dT)-primed cDNA was synthesized from 3 µg of RNA using the Omniscript RT kit (Qiagen). Real-time reverse transcription-PCRs were carried out as described in detail elsewhere (9), using Dynamo SYBR Green qPCR chemistry (Finnzymes, Espoo, Finland). The PCR primers were the following (5’ to 3’, followed by product size): CCL3, CACATTCCGTACCTGGTCCAG and TGCGCTCTCGTCTCAAGAATGTC, 192 bp; CCL4, TCTCTAGCCAATGGGCTCAG and CACGTTAGTTCCAGGTTACACAG, 214 bp; actin, GATTCCTATGTGGGCGAG and ATGGCTGGGTGGTAGAGGGTCTC, 245 bp. The results are expressed as -fold difference relative to control (2^ddCt).

RESULTS

NF449 Inhibits FGFR3 Signaling in Chondrocytes—The RCS is an FGFR3-expressing chondrocytic cell line that represents a well characterized in vitro cellular model for FGFR3-related skeletal dysplasias (10). RCS chondrocytes respond to FGFR3 activation (via exogenous FGF2 addition) with potent growth arrest, loss of the cartilage-like extracellular matrix, and marked alteration of their cellular shape (9, 11, 12). We have taken advantage of this response and adapted the FGF2/FGFR3-mediated growth arrest for rapid screening of chemical compounds for their activity against FGFR3 signaling (7).

We used the RCS growth arrest assay to screen a molecular library consisting of 1120 molecules (Tocris Bioscience). Initial screenings performed at 5, 10, and 20 µM scales identified a
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![Graph showing NF449 inhibits FGFR3-mediated growth arrest in chondrocytes](image)

**FIGURE 1.** NF449 inhibits FGFR3-mediated growth arrest in chondrocytes. A, RCS chondrocytes were treated with 5 ng/ml FGF2 and various concentrations of NF449 (upper graph) or with 15 μM NF449 and various FGF2 concentrations (lower graph; samples containing no FGF2 were given an artificial value of 0.8 on the logarithmic x axis) for 72 h and counted. Data represent an average from four wells with the indicated S.D. (error bars). Note the potent growth arrest induced by FGF2 as well as the NF449-mediated reversal of this phenotype. Also note the lack of significant NF449 toxicity throughout its active range (8–30 μM; upper graph). B, RCS chondrocytes were treated as indicated, grown for 72 h, and counted. Note the cumulative effect of both C-natriuretic peptide (CNP) and NF449 on the FGF2-mediated growth arrest. Data represent an average from four wells with the indicated S.D. Statistical differences are indicated (Student’s t test; *, p < 0.01).

A compound named NF449 as a potent antagonist of the FGF2/FGFR3-inhibitory effect on RCS proliferation. This effect was confirmed in detailed cell growth experiments, where 20 μM NF449 caused significant rescue of the growth arrest phenotype without apparent cellular toxicity throughout its active concentration range (~6–30 μM; Fig. 1A). This activity of NF449 is remarkable, considering the robust nature of the RCS growth arrest phenotype (7).

We next compared the NF449 effect with that of C-natriuretic peptide, which is a recently discovered inhibitor of FGFR3 signaling (12, 13). Fig. 1B shows that C-natriuretic peptide caused a ~20% rescue of the FGF2-mediated RCS growth arrest, consistent with our previous data (12). This effect was significantly exceeded by NF449, which caused a ~50% rescue of the growth arrest phenotype. When used together, C-natriuretic peptide and NF449 acted synergistically, rescuing nearly 80% of the growth arrest phenotype.

Apart from the growth inhibition, aberrant FGFR3 signaling in cartilage results in the loss of the extracellular matrix, rich in sulfated proteoglycans (12). Growing RCS cultures produce abundant amounts of cartilage-like sulfated proteoglycan, which is shed into the extracellular matrix, as demonstrated by Alcian blue staining (Fig. 2A). This staining is lost after the 72 h of FGF2 treatment as a result of both inhibition of matrix production and induction of matrix metalloproteinase-mediated degradation (12). In cells treated with FGF2 together with NF449, the intensity of Alcian blue staining equaled that of the control cells, suggesting that NF449 rescued the FGF2-mediated loss of the extracellular matrix (Fig. 2A).

To further confirm this observation, we incubated RCS cells with [35S]sulfate for 72 h to label the extracellular matrix and determined the effect of FGF2 and NF449 on the cell-bound radioactivity. [35S]Sulfate incorporation was significantly inhibited by FGF2, and this effect was completely rescued by NF449 (Fig. 2B). Altogether, these data demonstrate that NF449 rescues the FGFR3-mediated effects on both chondrocyte proliferation and extracellular matrix loss in vitro.

We next determined the effect of NF449 on FGFR3-mediated inhibition of cartilage development in bone organ culture system employing limb rudiments isolated from developing murine embryos. Such rudiments maintain their growth in culture for several days, which can be attenuated by continuous stimulation of endogenous FGFR3 via the addition of the FGF ligand, thus exploiting the role of FGFR3 as a negative regulator of cartilage growth (5, 14). Here, the addition of exogenous FGF2 inhibited growth of tibiae isolated from embryonic day 16.5 wild type embryos. When used together with FGF2, NF449 rescued the FGF2-mediated growth inhibition in all three experiments carried out (Figs. 2, C and D, and supplemental Fig. S1). Tibia histology showed that NF449 rescued the FGF2 effect by increasing the length of both growth plate cartilage and bone elements (Fig. 2, E and F).

NF449 Inhibits FGFR3 Signaling in Multiple Myeloma Cell Lines OPM2 and KMS11—Because multiple myeloma represents another important area of pathological FGFR3 signaling, we asked whether NF449 inhibits FGFR3 signaling in a multiple myeloma cellular environment. OPM2 and KMS11 represent multiple myeloma-derived cells that up-regulate FGFR3 carrying the strongly activating mutations K650E or Y373C, respectively (15). As a surrogate for FGFR3 activation, we used the activatory, Thr202/Tyr204 phosphorylation of ERK MAPK, which is induced by the addition of exogenous FGF2 to the OPM2 or KMS11 cells (16). Fig. 3A demonstrates that FGF2 treatment led to ERK phosphorylation, which was almost completely blocked by NF449. Next, we analyzed the effect of
NF449 on transcription of the genes induced by FGFR3 signaling in multiple myeloma cells. Because the CCL3 (MIP-1α) and CCL4 (MIP-1β) chemokines were previously identified as transcriptional targets of FGFR3 signaling in multiple myeloma (17),3 we determined the effect of NF449 on the FGF2-mediated accumulation of the CCL3 and CCL4 transcripts. Real-time reverse transcription-PCR analysis showed the partial NF449-mediated rescue of the CCL3 and CCL4 accumulation induced by FGF2 treatment in the OPM2 cells (Fig. 3B).

NF449 Inhibits Tyrosine Kinase Activity of FGFR3—To gain better insight into the mechanism of NF449-mediated inhibition of FGFR3 signaling, we focused on its effect on the mediators of downstream FGFR3 signaling as well as other molecular markers characteristic of FGFR3-mediated RCS chondrocyte growth arrest. Fig. 4A shows that FGF2-mediated activation of PLCγ as well as two kinases belonging to the Ras-ERK MAPK pathway (i.e. MEK and ERK) was sensitive to NF449. Furthermore, we analyzed the effect of NF449 on FGF2-mediated modulation of the senescence markers in RCS chondrocytes. The induction of caveolin 1 and lamin A/C and suppression of ID2 expression, which is triggered by 24 h of FGF2 treatment, was rescued by NF449 (Fig. 4B).

Because NF449 was originally designed as an inhibitor of P2X adenine receptors (29), we next asked whether P2X receptors are involved in growth-inhibitory FGFR3 signaling. Treatment of RCS chondrocytes with three different P2X agonists (i.e. ATP, MethyleneATP, and BzATP (1, 10, and 50 μM scale)) failed to inhibit RCS proliferation or sensitize cells to FGF2 in a growth arrest assay (data not shown), suggesting that NF449 action might be independent of P2X receptors. To test whether NF449 inhibits the kinase activity of FGFR3, we used a cell-free FGFR3 kinase assay that employs a recombinant TK domain of FGFR3 as a kinase and STAT1 as a substrate (18). In the initial experiment, we used a 5–30 μM NF449 concentration range and found that FGFR3-mediated tyrosine phosphorylation of STAT1 was inhibited at all concentrations. In a broader 0.1–30 μM range, NF449 caused some inhibition of FGFR3 kinase activity at all concentrations, although ≥2 μM NF449 was necessary to achieve more than 50% inhibition (Fig. 5, A and B).
The K650E substitution is one of the most strongly activating FGFR3 mutations known to date, present in both multiple myeloma and thanatophoric dysplasia (1–3). Because the OPM2 cells express K650E-FGFR3 and their FGFR3 signaling is inhibited by NF449 (Fig. 3), we used the cell-free kinase assay to determine whether NF449 inhibits the kinase activity of K650E-FGFR3. As described in detail elsewhere (8), FLAG-tagged full-length K650E-FGFR3 was expressed in CHO cells for 48 h, activated by brief FGF2 treatment, and purified by immunoprecipitation. Immunocomplexes were subjected to a kinase assay with STAT1 as a substrate and NF449 added directly to the kinase reaction. Fig. 5C shows that NF449 inhibits K650E-FGFR3 kinase activity with an efficiency similar to TK-FGFR3 (Fig. 5A).

We next determined the activity of NF449 against other receptor tyrosine kinases. NF449 inhibited FGFR1 kinase activity but showed very weak activity against FGFR2, demonstrating some selectivity of NF449 action within the FGFR family of kinases (Fig. 5, D and E). We were unable to evaluate NF449 activity against FGFR4 because the recombinant FGFR4 used here showed no activity in a kinase assay (data not shown). We next probed a panel of nine other receptor tyrosine kinases for their phosphorylation of STAT1 in a kinase assay and measured sensitivity of this phosphorylation to NF449. Recombinant FMS, PDGFRA, TRKA, AXL, IGF1R, FLT3, epidermal growth factor receptor, and c-Kit induced strong STAT1(Tyr701) phosphorylation in a kinase assay, which was very weakly inhibited by NF449 in the case of TRKA, AXL, and FLT3. In contrast, IGF1R-mediated phosphorylation of STAT1 was strongly inhibited by NF449, comparable with FGFR3 (supplemental Fig. S2A). As recombinant DDR2 used here did not phosphorylate STAT1 in a kinase assay, we employed DDR2 autophosphorylation as a reporter of its kinase activity. Supplemental Fig. S2B shows that NF449 did not inhibit DDR2 autophosphorylation in contrast to FGFR3 autophosphorylation, which was inhibited by NF449 similarly to FGFR3-mediated phosphorylation of STAT1 (Fig. 5, A and B, and supplemental Fig. S2A).
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Suramin Analogs Inhibit FGFR3 Kinase Activity—Finally, we evaluated other compounds that share structural similarity to NF449 (i.e. suramin and its analogs NF007, NF023, NF110, NF157, and NF279) (Fig. 7) for their activity against FGFR3. In the RCS growth arrest assay, suramin, NF110, and NF279 rescued the FGF2-mediated inhibition of cell proliferation in a manner similar to NF449, whereas NF007, NF023, and NF110 had no effect (Fig. 8A). Similar data were obtained in a kinase assay, where suramin, NF110, NF279, and NF449 but not NF007, NF023, and NF110 inhibited FGFR3 kinase activity (Fig. 8B).

DISCUSSION

Activating mutations in the FGFR3 receptor tyrosine kinase account for more than 10 human conditions, including syndromes affecting the skeleton (hypochondroplasia, achondroplasia, thanatophoric dysplasia, SADDAN, Muenke syndrome), skin (epidermal nevi, seborrheic keratosis, acanthosis nigricans), and cancer development (multiple myeloma, prostate, bladder cancer, seminoma) (1, 20–24). Although its role in human disease makes FGFR3 an attractive candidate for therapy, there is no treatment for FGFR3 disorders available to date, thus warranting development of novel approaches to target FGFR3 signaling (25–27). Here, we took advantage of RCS chondrocytes, which represent the most extensively studied cell model for FGFR3-related skeletal dysplasia (8–12). We used the well characterized growth-inhibitory response of RCS chondrocytes to FGFR3 activation as a reporter for compound library screening aimed at identification of novel inhibitors of FGFR3 signaling (7). A molecular library consisting of 1120 molecules was screened, leading to identification of several potential antagonists of FGFR3 signaling.3 In this article, we characterized the inhibitory effect of a compound named NF449 on FGFR3 signaling.

In RCS chondrocytes, NF449 rescued two major cellular phenotypes of pathological FGFR3 signaling in skeletal dysplasias (i.e. the growth arrest and extracellular matrix loss), as evidenced by cell counting experiments, matrix visualization by Alcian blue staining, and matrix proteoglycan labeling by [35S]sulfate (Figs. 1 and 2, A and B). Importantly, NF449 also rescued the inhibitory effect of FGF3 activation on murine limb development in organ culture (Fig. 2, C–F, and

NF449 Cooperates with SU5402 in FGFR3 Inhibition—To test whether NF449 targets the ATP binding site on FGFR3, we performed cell-free FGFR3 kinase assays with a range of ATP concentrations (1–50 μM) in the presence of 20 μM NF449. The amounts of STAT1(Tyr701) phosphorylation were detected by WB, quantified by densitometry, and graphed. In the presence of NF449, increasing amounts of ATP did not elevate the rate of the kinase reaction to the control levels (no NF449 in the reaction) (Fig. 6A), suggesting that NF449 inhibits FGFR3 non-competitively with ATP. This opened the possibility that NF449 could act synergistically with the well established FGFR inhibitors that target the ATP binding site, such as SU5402 (19). Indeed, NF449 and SU5402 had additive effects on the FGFR3 kinase assay (Fig. 6B). Similar data were achieved in the RCS growth arrest assay, where NF449 and SU5402 together caused a significantly greater rescue of the FGF2-mediated growth arrest compared with each inhibitor alone (Fig. 6C).

NF449 cooperates with SU5402 in FGFR3 inhibition. A and B, cell-free kinase assays were carried out as described under “Experimental Procedures,” with the recombinant TK domains of FGFR1 (A) or FGFR3 (B) as a kinase, recombinant STAT1 as a substrate, and NF449 added directly to the kinase reaction. FGFR3-mediated phosphorylation of STAT1 was detected by WB with phospho-STAT1(Tyr701) antibody. The membrane was reprobed with FGFR3 and STAT1 antibodies to control for kinase and substrate quantity. The sample with ATP omitted serves as a negative control for the kinase reaction. Note the FGFR3-mediated phosphorylation of STAT1 that is inhibited by NF449. The phospho-STAT1 and STAT1 signal was quantified by densitometry and expressed as a ratio between P-STAT1 and STAT1 signal for each given sample (Fig. 5). C–F, the cell-free FGFR3 kinase assay was carried out using a full-length FLAG-tagged K650E-FGFR3 mutant that was expressed in CHO cells, purified by immunoprecipitation with FLAG antibody, and subjected to a kinase reaction with recombinant STAT1 as a substrate and NF449 added to the kinase reaction. Cells transfected with green fluorescent protein-encoding plasmid serve as a negative control for the immunoprecipitation. D and E, cell-free kinase assays were carried out as described in A, except that the recombinant TK domains of FGFR1 (D) or FGFR2 (E) were used as a kinase. Note the poor NF449 inhibition of FGFR2 kinase activity when compared with both FGFR1 and FGFR3.

FIGURE 5. NF449 inhibits FGFR3 kinase activity in a cell-free kinase assay. A and B, cell-free kinase assays were carried out as described under “Experimental Procedures,” with the recombinant TK domain of FGFR3 as a kinase, recombinant STAT1 as a substrate, and NF449 added directly to the kinase reaction. FGFR3-mediated phosphorylation of STAT1 was detected by WB with phospho-STAT1(Tyr701) antibody. The membrane was reprobed with FGFR3 and STAT1 antibodies to control for kinase and substrate quantity. The sample with ATP omitted serves as a negative control for the kinase reaction. Note the FGFR3-mediated phosphorylation of STAT1 that is inhibited by NF449. The phospho-STAT1 and STAT1 signal was quantified by densitometry and expressed as a ratio between P-STAT1 and STAT1 signal for each given sample (A). C–F, the cell-free FGFR3 kinase assay was carried out using a full-length FLAG-tagged K650E-FGFR3 mutant that was expressed in CHO cells, purified by immunoprecipitation with FLAG antibody, and subjected to a kinase reaction with recombinant STAT1 as a substrate and NF449 added to the kinase reaction. Cells transfected with green fluorescent protein-encoding plasmid serve as a negative control for the immunoprecipitation. D and E, cell-free kinase assays were carried out as described in A, except that the recombinant TK domains of FGFR1 (D) or FGFR2 (E) were used as a kinase. Note the poor NF449 inhibition of FGFR2 kinase activity when compared with both FGFR1 and FGFR3.
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supplemental Fig. S1), thus demonstrating its capacity to inhibit FGFR3 signaling in chondrocytes both in vitro and in vivo. To test whether this is also the case in the multiple myeloma-cellular environment, we determined the effect of NF449 on FGFR3 signaling in OPM2 and KMS11 cell lines, which represent well-characterized models of pathological FGFR3 signaling in multiple myeloma. OPM2 and KMS11 cells were established from patients suffering from multiple myeloma and up-regulate FGFR3 as a result of t(4;14)(p16.3;q32) translocation. In addition, OPM2 and KMS11 harbor highly activating FGFR3 mutations (K650E and Y373C, respectively), common in severe forms of FGFR3-related skeletal dysplasia and cancer (1, 2). In agreement with previous studies, FGFR3 activation in OPM2 and KMS11 cell lead to activation of ERK MAPK and transcriptional induction of CCL3 and CCL4 chemokines (16, 17), with both phenotypes being vulnerable to NF449 (Fig. 3). Our data demonstrate that NF449 is not only capable of inhibiting FGFR3 signaling in the multiple myeloma cellular environment but also capable of inhibiting the signaling of FGFR3 carrying highly activating mutations.

Because NF449 inhibited the FGF2-mediated phosphorylation of PLCγ, a direct substrate of the FGFR3 kinase (Fig. 4A), we hypothesized that NF449 targets FGFR3 itself, either extracellularly or at the intracellular kinase domain. NF449 exhibits close structural similarity with suramin, a polysulfonlated naphthyl urea derivative (Fig. 7). Suramin is known to inhibit FGFR activation via formation of suramin-FGF multimers that are incapable of binding FGFR (32). In addition, several studies using smaller structural units of suramin (such as sulfonlated naphtyl urea) indicated that the suramin can bind to the heparin binding pocket in FGF (32–36). Because formation of binary FGF-FGFR complex requires participation of proteoglycans, such as heparin (37–39), the competitive binding of suramin to the heparin binding site would interfere with the FGF-FGFR interaction, thus preventing subsequent FGFR activation (40). In line with these findings, we observed reversal of the FGFR3-mediated RCS growth arrest that was originally described as an antagonist of both G-proteins (selective to the Gαs subunit) and P2X receptors (28, 29). Although these findings open the attractive possibility of involvement of G-proteins or P2X receptors in FGFR3 signaling, the lowest NF449 concentration active in the RCS growth arrest assay (∼6 μM; Fig. 4A) was significantly higher than that needed to inhibit Gαs or P2X receptors (∼0.1 μM for Gαs, subnanomolar for P2X) (28, 29). In addition, NF023 and NF110, which are both potent inhibitors of P2X receptors (30), showed no activity in the RCS growth arrest assay (Fig. 8A). Because a treatment with three P2X receptor agonists (i.e. ATP, MethyleneATP, and BzATP) did not inhibit growth or sensitize RCS chondrocytes to FGF2 (data not shown), we concluded that NF449 inhibits FGFR3 signaling independent of P2X receptors. Interestingly, the same conclusion was reached by Guzmán-Arángués et al. (31), who recently reported the inhibitory effect of another P2X receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt, on the signaling of FGFR3 with an achondroplasia mutation (G380R) in chondrogenic RCJ3.1 cells.

How does the NF449 inhibit FGFR3 signaling? Little is known about the biological activities of NF449, which was originally described as an antagonist of both G-proteins (selective to the Gαs subunit) and P2X adenosine receptors (28, 29). Although these findings open the attractive possibility of involvement of G-proteins or P2X receptors in FGFR3 signaling, the lowest NF449 concentration active in the RCS growth arrest assay (∼6 μM; Fig. 4A) was significantly higher than that needed to inhibit Gαs or P2X receptors (∼0.1 μM for Gαs, subnanomolar for P2X) (28, 29). In addition, NF023 and NF110, which are both potent inhibitors of P2X receptors (30), showed no activity in the RCS growth arrest assay (Fig. 8A). Because a treatment with three P2X receptor agonists (i.e. ATP, MethyleneATP, and BzATP) did not inhibit growth or sensitize RCS chondrocytes to FGF2 (data not shown), we concluded that NF449 inhibits FGFR3 signaling independent of P2X receptors. Interestingly, the same conclusion was reached by Guzmán-Arángués et al. (31), who recently reported the inhibitory effect of another P2X receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt, on the signaling of FGFR3 with an achondroplasia mutation (G380R) in chondrogenic RCJ3.1 cells.

Because NF449 inhibited the FGF2-mediated phosphorylation of PLCγ, a direct substrate of the FGFR3 kinase (Fig. 4A), we hypothesized that NF449 targets FGFR3 itself, either extracellularly or at the intracellular kinase domain. NF449 exhibits close structural similarity with suramin, a polysulfonlated naphthyl urea derivative (Fig. 7). Suramin is known to inhibit FGFR activation via formation of suramin-FGF multimers that are incapable of binding FGFR (32). In addition, several studies using smaller structural units of suramin (such as sulfonlated naphtyl urea) indicated that the suramin can bind to the heparin binding pocket in FGF (32–36). Because formation of binary FGF-FGFR complex requires participation of proteoglycans, such as heparin (37–39), the competitive binding of suramin to the heparin binding site would interfere with the FGF-FGFR interaction, thus preventing subsequent FGFR activation (40). In line with these findings, we observed reversal of the FGFR3-mediated RCS growth arrest that was originally described as an antagonist of both G-proteins (selective to the Gαs subunit) and P2X receptors (28, 29). Although these findings open the attractive possibility of involvement of G-proteins or P2X receptors in FGFR3 signaling, the lowest NF449 concentration active in the RCS growth arrest assay (∼6 μM; Fig. 4A) was significantly higher than that needed to inhibit Gαs or P2X receptors (∼0.1 μM for Gαs, subnanomolar for P2X) (28, 29). In addition, NF023 and NF110, which are both potent inhibitors of P2X receptors (30), showed no activity in the RCS growth arrest assay (Fig. 8A). Because a treatment with three P2X receptor agonists (i.e. ATP, MethyleneATP, and BzATP) did not inhibit growth or sensitize RCS chondrocytes to FGF2 (data not shown), we concluded that NF449 inhibits FGFR3 signaling independent of P2X receptors. Interestingly, the same conclusion was reached by Guzmán-Arángués et al. (31), who recently reported the inhibitory effect of another P2X receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt, on the signaling of FGFR3 with an achondroplasia mutation (G380R) in chondrogenic RCJ3.1 cells.
interference with assembly of the heparin-FGF2-FGFR3 complexes essential for proper FGFR3 activation).

However, our findings reveal an alternative mechanism of FGFR3 inhibition by both NF449 and suramin. As demonstrated by cell-free kinase assays utilizing both recombinant and immunopurified FGFR3 as a kinase and recombinant STAT1 as a substrate (18), both NF449 and suramin inhibited FGFR3 kinase activity (Figs. 5, 6, and 8 and supplemental Figs. S2 and S3). It is important to note that in the majority of these experiments, both FGFR3 kinase and STAT1 substrate components were recombinant proteins, and, in addition, the FGFR3 kinase was a truncated form that lacked the extracellular, FGF, and heparin-binding domains. Our data thus indicate that NF449 inhibits FGFR3 via directly targeting its intracellular tyrosine kinase domain. Although the exact mechanism of this inhibition is not known and is a subject of ongoing investigation, our data suggest that NF449 targets the kinase domain of FGFR3 at a site other than the ATP binding site (Fig. 6A).

Fig. 1B demonstrates that NF449-mediated rescue of FGF2-mediated growth arrest in RCS chondrocytes significantly exceeds the similar activity of C-natriuretic peptide. C-natriuretic peptide is a recently discovered potentially therapeutic antagonist of FGFR3 signaling that suppresses the pathological FGFR3 signaling in cartilage via inhibition of one of its intermediates, the ERK MAP kinase pathway (12, 13). Because NF449 and C-natriuretic peptide target FGFR3 signaling at different levels (FGFR3 kinase versus activation of the ERK pathway) (12), their combination could prove useful to achieve stable therapeutic inhibition of FGFR3 signaling. Similarly, NF449 might be combined with SU5402, which represents a well-established inhibitor of FGFR kinases that acts via binding the ATP pocket of FGFR (19). When compared directly with NF449, SU5402 proved to be a more potent inhibitor of FGFR3 kinase activity, achieving nearly complete inhibition at the 10 μM scale (supplemental Fig. S3A). A similar effect was achieved in a growth arrest experiment, where SU5402 rescued the FGFR3-mediated RCS growth arrest more potently than NF449 (supplemental Fig. S3B). Unlike NF449, however, SU5402 showed significant cellular toxicity throughout its active range, potentially limiting its therapeutic use. Because both inhibitors target FGFR3 differently (i.e., via the ATP-binding pocket (SU5402) and independent of ATP (NF449)), we asked whether NF449 and SU5402 can synergistically inhibit FGFR3. Fig. 6 demonstrates that potent FGFR3 inhibition can be achieved by both inhibitors used together at low concentration, thus overcoming the toxic effects evident at higher SU5402 concentrations.

As a suramin analog, NF449 belongs to a large group of chemicals defined as symmetric polysulfonates with a central urea bridge (Fig. 7) (41, 42). Using both the RCS growth arrest assay and cell-free FGFR3 kinase assay, we determined the activity of several such compounds, including NF007, NF023,
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NF110, NF157, and NF279. We found differential activities among the tested compounds, with NF157 and NF279 inhibiting FGFR3 signaling similarly to NF449 or suramin, whereas NF007, NF023, and NF110 were virtually inactive (Fig. 8). Based on their structure, the studied compounds can be divided into three groups. First, NF007, the “one half” of the suramin molecule, corresponds essentially to the active moiety (i.e. 3,1-phenylenecarbonylimino-1,3,5-naphthalenetricarboxylic acid of suramin). The fact that NF007 does not possess any inhibitory activity toward FGFR3 (Fig. 8) implies that the isolated active moiety of suramin has insufficient selectivity with respect to FGFR3 binding site.

In the second group, consisting of suramin, NF157, and NF279, the basic phenylcarbonyl urea core is symmetrically substituted with either two 3,1-phenylenecarbonylimino-1,3,5-naphthalenetricarboxylic acid groups (NF279) or two 3,1-phenylenecarbonylimino-1,3,5-naphthalenetricarboxylic acid groups (suramin, NF157). The only difference between suramin and NF157 is a CH₃ or F substituent in the 3,1-phenylenecarbonylimino moiety at position 4 (Fig. 7). Because these two compounds display similar activity toward FGFR3 (Fig. 8), it is unlikely that the nature or position of substituents contributes to the activity and/or specificity of suramin, NF157, and NF279 toward FGFR3.

The third group studied consists of NF023, NF110, and NF449, characterized by symmetrical substitution of the basic phenylcarbonyl urea core directly with mono- and disulfonic acids of imino-benzene (NF110 and NF449) or trisulfonic acids of imino-naphthalene (NF023). The most obvious difference when compared with the first group is the absence of 4,1-phenylenecarbonylimino linker, typical for suramin, NF157, and NF279 (Fig. 7). Although NF023 and NF110 showed no FGFR3-inhibitory activity, the NF449 activity was comparable with the activities of suramin, NF157, and NF279 (Fig. 8). As demonstrated by Hausmann et al. (43), selectivity of suramin analogs to P2X receptors is primarily determined by the location of sulfonic acid groups. Three-dimensional structural modeling suggests that the location of sulfonic acid groups is also responsible for the different activities toward FGFR3. The NF449 model indicates that four of its eight sulfonic acid groups have a spatial arrangement virtually identical to those in suramin, NF157, and NF279 (supplemental Figs. S4 and S6). In contrast, the inactive suramin analogs have either an insufficient number (NF110) or inappropriate spatial orientation of the sulfonic acid groups (NF023) (supplemental Figs. S5 and S6). Correspondingly, modeling indicates that the binding of suramin, NF157, NF279, and NF449 to FGFR3 is mediated by four sulfonic groups having a rectangular arrangement separated roughly by 5 and 10 Å (supplemental Fig. S6). However, not only the number and spatial separation of sulfonic acid groups but also their mutual orientation appears critical for selectivity toward FGFR3, as illustrated by supplemental Fig. S6.

Taken together, our data suggest that both the number and exact location of negatively charged sulfonic groups are important for FGFR3-inhibitory activity. Symmetrical suramin analogs based on 4,4′-carboxylbis(imino-3,1-phenylenecarbonylimino)bis-1,3-benzenesulfonic acid might thus represent attractive, novel lead compounds in the development of FGFR3 inhibitors.

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