Functional Profiling of Receptor Tyrosine Kinases and Downstream Signaling in Human Chondrosarcomas Identifies Pathways for Rational Targeted Therapy

Yi-Xiang Zhang, Jolieke G. van Oosterwijk, Ewa Sicinska, Samuel Moss, Stephen P. Remillard, Tom van Wezel, Claudia Bühnemann, Andrew B. Hassan, George D. Demetri, Judith V.M.G. Bovee, and Andrew J. Wagner

Abstract

Purpose: Chondrosarcomas are notoriously resistant to cytotoxic chemotherapeutic agents. We sought to identify critical signaling pathways that contribute to their survival and proliferation, and which may provide potential targets for rational therapeutic interventions.

Experimental Design: Activation of receptor tyrosine kinases (RTK) was surveyed using phospho-RTK arrays. S6 phosphorylation and NRAS mutational status were examined in chondrosarcoma primary tumor tissues. siRNA or small-molecule inhibitors against RTKs or downstream signaling proteins were applied to chondrosarcoma cells and changes in biochemical signaling, cell cycle, and cell viability were determined. In vivo antitumor activity of BEZ235, a phosphoinositide 3-kinase (PI3K)/mTOR inhibitor, was evaluated in a chondrosarcoma xenograft model.

Results: Several RTKs were identified as critical mediators of cell growth, but the RTK dependencies varied among cell lines. In exploration of downstream signaling pathways, strong S6 phosphorylation was found in 69% of conventional chondrosarcomas and 44% of dedifferentiated chondrosarcomas. Treatment with BEZ235 resulted in dramatic reduction in the growth of all chondrosarcoma cell lines. Tumor growth was similarly inhibited in a xenograft model of chondrosarcoma. In addition, chondrosarcoma cells with an NRAS mutation were sensitive to treatment with a mitogen-activated protein kinase/extracellular signal–regulated kinase (MEK) inhibitor. Functional NRAS mutations were found in 12% of conventional central chondrosarcomas.

Conclusions: RTKs are commonly activated in chondrosarcoma, but because of their considerable heterogeneity, targeted inhibition of the PI3K/mTOR pathway represents a rational therapeutic strategy. Chondrosarcomas with NRAS mutations may benefit from treatment with MEK inhibitors.

Introduction

Chondrosarcomas, mesenchymal tumors with cartilaginous differentiation, are biologically and clinically heterogeneous. Complete surgical resection of localized disease remains the only known curative treatment. No systemic treatments have been proven to be effective in the metastatic or unresectable setting. Therefore, there is an urgent need to identify therapeutic targets and to develop novel treatment strategies for patients with this disease (1–4).

Deregulated expression and/or function of receptor tyrosine kinases (RTK) by gene amplification, mutation, or translocation has been found to be important for cancer cell proliferation, survival, motility, and invasion, as well as tumor angiogenesis and resistance to chemotherapy (5, 6). Given their pivotal role in tumor initiation and progression, RTKs have become one of the most prominent target families for drug development, and more than 10 inhibitors or antagonistic antibodies have been approved for the treatment of cancer (7, 8).

In this study, we used phospho-RTK arrays to simultaneously assess the phosphorylation status of more than 40 RTKs in chondrosarcoma cells under conditions of serum depletion. We found that although several RTKs are constitutively activated, this occurs in differing patterns among different human tumor-derived cell lines. Several RTKs were identified...
Translational Relevance

Chondrosarcomas are notoriously resistant to conventional chemotherapy. There is an urgent need to identify therapeutic targets and to develop novel treatment strategies for this disease. We found multiple receptor tyrosine kinases (RTK) to be activated in chondrosarcoma cells and to have critical roles in mediating cell growth. Strong phosphorylation of S6 was detected in 69% of conventional chondrosarcoma and 44% of dedifferentiated chondrosarcoma clinical samples and is likely due to RTK activation. Inhibition of phosphoinositide 3-kinase (PI3K) and mTOR, signaling proteins downstream of RTKs and upstream of S6, potently blocked the growth of chondrosarcoma cells in vitro and in vivo. NRAS mutations were identified in 12% of conventional central chondrosarcoma tumor tissues. An NRAS mutation-harboring chondrosarcoma cell line was sensitive to treatment with a mitogen-activated protein kinase/extracellular signal–regulated kinase kinase (MEK) inhibitor. Our findings provide new insights into the genetics and the heterogeneity of chondrosarcomas, and have implications for the clinical development of PI3K/mTOR or MEK inhibitors in this disease.

Materials and Methods

Cell lines and culture conditions

Human chondrosarcoma cell lines included SW1353 (American Type Culture Collection), CS-1 (gift of Dr. Francis J. Hornicek, Massachusetts General Hospital, Boston, MA), JJ012 (gift of Dr. Joel A. Block, Rush University, Chicago, IL), CH-2879 (kindly provided by Prof. Antonio Llobart-Bosch, Valencia University, Valencia, Spain), and OLMS-27 (kindly provided by Dr. M. Namba, Okayama University Medical School, Okayama, Japan; refs. 9–12). Cells were cultured in RPMI-1640 supplemented with 10% FBS and 1 × penicillin–streptomycin–glutamine (10378-016; Invitrogen) at 37°C in a humidified incubator with 95% air and 5% CO2. Cell line identity was verified by high-resolution short-tandem repeat (STR) profiling with Promega PowerPlex 1.2 system.

Phospho-RTK array

First-generation phospho-RTK arrays (#ARY001; R&D Systems) were used for assessing the phosphorylation status of 42 RTKs in chondrosarcoma cells under serum-depleted condition. Phospho-RTK analyses were conducted as recommended by the manufacturer. Subconfluent cells were washed once with serum-free media, and incubated in serum-depleted medium for 24 hours before harvest. A total of 450 μg of protein was used for the assay.

For qualitative assessment of signal, pixel densities on developed X-ray film were analyzed using a transmission mode scanner and the Adobe Photoshop software. The pixel densities of the areas (49 pixels × 26 pixels, width × height) surrounding the pair of duplicate dots were determined. The pixel density of the PBS-negative control was used as a background value and subtracted from each read. RTKs with a signal greater than the positive controls were scored as “+++”; RTKs with a signal level similar to positive controls were scored as “++”; and RTKs with a signal less than positive controls, but 5-fold higher than antibody isotype-negative controls were scored as “+”. RTKs with signal less than 5-fold higher than the antibody isotype-negative control were labeled as “−”.

We used second-generation phospho-RTK arrays (#ARY001B; R&D Systems) to analyze the effects of long-term treatment of BEZ235 on the phosphorylation of RTKs because the first-generation RTK arrays were no longer available. The new arrays include 49 RTK capture antibodies but no longer contain antibody isotype negative controls. The pixel density was measured as described earlier. The changes in the phosphorylation level of RTKs were determined by comparing the intensity of RTK signals in BEZ235-treated samples to the 0.1% dimethyl sulfoxide (DMSO)–treated control samples.

Antibodies, inhibitors, and siRNAs

Detailed information is provided in the Supplementary Materials and Methods.

Cell viability assays

Cells were plated in 96-well plates at 1,000 to 2,000 cells per well in 100 μL of medium containing 10% FBS. After 24 hours, cells were exposed to increasing concentrations of compounds. Each treatment was tested in triplicate. Cell viability was determined after 72 hours using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) with a modification in the protocol in that the CellTiter-Glo reagent was diluted 1:3 with PBS. The relative luminescence units (RLU) were measured using the FLUOstar Optima plate reader (BMG Labtech GmbH) and relative cell number was calculated by normalization to the RLU of the control-treated cells.

Cell-cycle analysis

Cells were exposed to inhibitors or 0.1% DMSO for 24 hours and harvested. After washing with ice-cold PBS, cells were fixed in 70% ethanol at 4°C for at least 2 hours. Fixed cells were stained in PBS containing 10 μg/mL RNase A and 20 μg/mL propidium iodide (Sigma) in the dark. DNA content analysis was conducted by flow cytometry.
Mutation analysis

Genomic DNA was extracted from chondrosarcoma cell lines using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s protocol. The coding sequences of selected genes in the PI3K/mTOR and RAS/mitogen-activated protein kinase (MAPK) pathways were amplified from genomic DNA by PCR with primers listed in the Supplementary Table S1 (sheet 1). PCR was conducted in 50 μL reactions containing Platinum PCR SuperMix (Invitrogen), 200 ng DNA, 0.3 μmol/L forward and reverse primers using GeneAmp PCR System 9700 (Applied Biosystems). The PCR product was purified using QIAquick PCR Purification Kit (Qiagen), and sequenced using BigDye Terminator v3.1 chemistry in combination with an Applied Biosystems 3730xl Sequencer.

For chondrosarcoma primary tumor tissues, mutation analysis for PIK3CA (13) and NRAS was conducted using DNA available from 89 chondrosarcomas. Hydrolysis probes assay was used to specifically screen for the PIK3CA c.1624G>A (p.E542K), c.1633G>A (p.E545K), and c.3140A>G (p.H1047R) and the NRAS c.35G>A (p. G12D), c.183A>T (p.Q61H), c.181C>A (p.Q61K), c.182A>T (p.Q61L), and c.182A>G (p.Q61R) hotspot mutations as described previously (13). Primer and probe sequences for NRAS mutation analysis are provided in Supplementary Table S1 (sheet 2).

Tumor xenografts in nude mice

JJ012 cells (1 × 10⁶) were suspended in PBS, mixed 1:1 with Matrigel (BD Biosciences), and subcutaneously injected into nude female mice (Nu/Nu; Charles River Laboratories) in a final volume of 100 μL. Treatment began when tumors reached an average size of approximately 50 mm³. Mice were randomized into statistically identical cohorts (≥6 mice/group). BEZ235 was freshly prepared in 10:90 (v/v) N-methyl pyrrolidone-polyethylene glycol 300 (Fluka #69118 and #81160), as described previously (14), and was administered daily at 35 mg/kg by oral gavage. Tumor xenografts were measured twice a week by ultrasound imaging (VisualSonics Vevo 770), and animal weight was recorded every 3 to 4 days. Following drug administration, tumors were harvested and fixed for histologic and immunohistochemical analysis or snap-frozen for immunoblot analysis. All procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA).

Tumor xenograft histology and immunohistochemistry

Hematoxylin and eosin (H&E) staining as well as immunohistochemistry were conducted on 5-μm sections of formalin-fixed paraffin-embedded (FFPE) samples from tumors resected from mice. Tissue sections were deparaffinized, rehydrated, and microwaved in 10 mmol/L citrate buffer (pH 6.0) in a 750 W microwave oven for 15 minutes. Anti-phospho-S6 ribosomal protein (Ser 240/244) primary antibody (Cell Signaling Technology; #2215) was added at a dilution of 1:100 and incubated overnight at 4°C. Sections were further processed with horseradish peroxidase–conjugated secondary antibody. The reaction was detected by 3,3-diaminobenzidine and hematoxylin staining. Images were taken by using Olympus CX41 microscope with QCapture software (QL imaging).

Tissue microarray immunohistochemistry

Tissue microarrays (TMA) containing 157 conventional chondrosarcomas and 25 dedifferentiated chondrosarcomas were described previously (15, 16). All specimens were handled according to the ethical guidelines described in “Code for Proper Secondary Use of Human Tissue in The Netherlands” of the Dutch Federation of Medical Scientific Societies. TMAs contained 2-mm cores of all samples in triplicate. After dewaxing and rehydrating, TMAs were permethylated with TBS/0.5% Tween20 for 30 minutes at room temperature and washed several times in distilled water. For antigen demasking, slides were immersed in citrate buffer (pH 6.0) in a 750 W microwave oven for 15 minutes. For antigen demasking, slides were immersed in citrate buffer (pH 6.0). Anti-phospho-S6 ribosomal protein (Ser 240/244) primary antibody (Cell Signaling Technology; #2215) was added at a dilution of 1:100 and incubated overnight at 4°C. Sections were further processed with horseradish peroxidase–conjugated secondary antibody. The reaction was detected by 3,3-diaminobenzidine and hematoxylin staining. Images were taken by using Olympus CX41 microscope with QCapture software (QL imaging).

Statistical analysis

Drug concentrations required to inhibit cell growth by 50% (IC₅₀) were calculated by dose–response curve fitting with Prism version 5.0 (GraphPad Software). Comparisons between groups were made using the unpaired t test. Differences in mean ± SEM with P < 0.05 were considered statistically significant.

Results

Coactivation of RTKs in chondrosarcoma cells

We used phospho-RTK arrays to detect the phosphorylation status of 42 RTKs in five human tumor-derived chondrosarcoma cell lines, and we observed that multiple RTKs were phosphorylated in cells under serum-depleted conditions (Fig. 1A and B). For example, EGFR, MET, ERBB4, AXL, PDGFRα, RON, and RET were phosphorylated in CS-1 cells. All 4 EGF receptor (EGFR) family members were phosphorylated in JJ012 cells. IGF-IR, INSR, EGFR, and EphA7 were phosphorylated in OUMS-27 cells.
To further validate the phospho-RTK array data, we analyzed by immunoblot the expression and phosphorylation status of the 9 RTKs which were most strongly phosphorylated in the respective chondrosarcoma cell lines. As shown in Fig. 1C and Supplementary Fig. S1A, the phospho-RTK array data were confirmed: EGFR (site Tyr1068) was highly phosphorylated in CS-1 cells and less so in the four other cell lines; ERBB2 (site Tyr1221/1222) and ERBB3 (site Tyr1289) were highly phosphorylated in JJ012 cells; phosphorylation of MET was detected in CS-1, JJ012 and SW1353 cells, with the highest level in CS-1 cells; insulin receptor β (INSRβ) and insulin-like growth factor I receptor β (IGF1Rβ) were highly phosphorylated in OUMS-27 cells; the highest phosphorylation level of AXL and EphA2 was observed in CS-1 and SW1353 cells, respectively; and phosphorylation of PDGFRα was detected in CS-1 and CH-2879 cells.

Moreover, most kinases remained constitutively phosphorylated under conditions of serum depletion at levels similar to that seen in the serum-containing media (Fig. 1C). Although the precise mechanism(s) of kinase activation remains unknown, it seems to be cell-autonomous and independent of exogenous growth factors.

Taken together, the immunoblot data confirmed that RTKs are constitutively activated in chondrosarcoma cells, and that the patterns of activation vary among cell lines.

**Effects of RTK inhibition on the growth of chondrosarcoma cells**

To explore the involvement of the above-noted highly activated RTKs on the growth and survival of chondrosarcoma cells, we applied small-molecule inhibitors and/or siRNAs targeting corresponding RTKs to cells (Fig. 2). We examined the effects of the MET inhibitor PHA665752 (17) on MET signaling pathways and cell growth in the CS-1 cell line because of its high level of constitutive phosphorylation. MET phosphorylation at key residues in the kinase...
domain (Tyr1234/1235) was greatly suppressed by treatment with 200 nmol/L PHA665752 in the presence of serum (Fig. 2A, left). Correspondingly, PHA665752 induced a dramatic reduction in cell number with 43% inhibition at 125 nmol/L (Fig. 2B, left). We also applied MET siRNAs to exclude the possibility of off-target effects of the small-molecule inhibitor. As shown in Supplementary Fig. S1B, the expression of MET was dramatically decreased by MET siRNA. Cells transfected with MET siRNA had an obvious decrease (~30%) in cell viability in comparison with control siRNA-transfected cells after 72 hours (Supplementary Fig. S1C). These results show that MET is involved in the regulation of CS-1 chondrosarcoma cell growth. There was no significant effect of MET pathway inhibition in cell lines JJ012 and SW1353 that have low basal phospho-MET levels (Fig. 2B, left). In addition, we determined the effects of the EGFR inhibitor gefitinib and the anti-EGFR antibody cetuximab on the viability of CS-1 cells because of the high phosphorylation level of EGFR, but no significant changes were observed (data not shown).

In JJ012 cells, 4 members of the EGFR kinase family were phosphorylated. The irreversible EGFR/ERBB2 inhibitor BIBW-2992 (18) decreased phosphorylation of ERBB2 and ERBB3 (Fig. 2A, middle), and inhibited cell growth in a dose-dependent manner with an IC50 of 0.38 ± 0.02 μmol/L (Fig. 2B, middle). siRNA-mediated knockdown of ERBB3 also significantly reduced cell number (37.7% ± 6.1%) compared with control siRNA at 96 hours after transfection (P < 0.001; Supplementary Fig. S1B and S1C). However, the EGFR-specific inhibitors gefitinib and erlotinib only showed mild effects as did siRNA-mediated knockdown of EGFR and ERBB2 (Supplementary Fig. S1C and S1D). These findings suggest that EGFR family kinases, and in particular ERBB3, are important regulators of the growth of JJ012 cells.

In OUMS-27 cells, IGF1R and INSR are highly phosphorylated. Treatment with the IGF1R/INSR inhibitor PQIP showed a reduction in phosphorylation of IGF1R and INSR (Fig. 2A, right). Immunoblot analysis showed that TKIs reduced phosphorylation of specific downstream effectors. IP, immunoprecipitation analysis; IB, immunoblot analysis.

Figure 2. Effects of RTK inhibition in chondrosarcoma cells. A, effects of MET inhibitor PHA665752 (left, at 2 hours), EGFR/ERBB2 inhibitor BIBW-2992 (middle, at 2 hours) and IGF1R/INSR inhibitor PQIP (right, at 6 hours) on the phosphorylation of RTKs and their downstream effectors were evaluated by immunoblot. B, effects of RTK inhibitors on cell viability were evaluated by CellTiter-Glo assay at 72 hours. IP, immunoprecipitation analysis; IB, immunoblot analysis.
Effects of RTK inhibition on the activity of PI3K/mTOR and MAPK pathways in chondrosarcoma cells

We further examined the effects of RTK inhibition on the PI3K/mTOR and MAPK pathways. In MET-dependent CS-1 cells, treatment with the MET inhibitor PHA665752 for 2 hours led to a dose-dependent decrease in the phosphorylation of AKT at Thr308 and Ser473, S6 at Ser235/236 and p44/42 MAPK at Thr202/Tyr204 (Fig. 2A, right). In JJ012 cells, phosphorylation of AKT and S6 was partially reduced by BIBW-2992 treatment, but no effect on MAPK phosphorylation was observed (Fig. 2A, middle). In OUMS-27 cells, IGF1R/INSR inhibitor PQIP treatment decreased AKT and S6 phosphorylation in a dose-dependent manner, with complete inhibition at 1.25 μmol/L, but had no effect on MAPK phosphorylation (Fig. 2A, right). The inhibition of phosphorylation in the presence of the above inhibitors was continued for at least 24 hours, with the level of phosphorylation at 24 hours even somewhat lower (Supplementary Fig. S1E). No significant inhibition on the phosphorylation of 4EBP1 was observed following the treatment of RTK inhibitors for 1 or 24 hours (Supplementary Fig. S1E).

Chondrosarcomas have PI3K/mTOR activation and are sensitive to inhibitors in vitro

The above data suggest that RTKs are important mediators of chondrosarcoma cell growth. However, the heterogeneity of implicated pathways poses a considerable challenge to the clinical evaluation of any single tyrosine kinase inhibitor for the treatment of this disease. Targeting common pathways downstream of RTKs may instead be a method to inhibit cell growth irrespective of the particular upstream signaling molecule.

We hypothesized that the PI3K/mTOR pathway might commonly mediate signaling from heterogeneous RTK activation, and thus we examined the activity of this pathway in human primary chondrosarcoma tissue specimens. As an established surrogate of PI3K/mTOR pathway activity, we analyzed the S6 phosphorylation status in chondrosarcoma TMAs by immunohistochemical staining. In total, 73 of 106 (69%) conventional chondrosarcomas and 11 of 25 (44%) dedifferentiated chondrosarcomas were positive (Table 1 and Supplementary Fig. S2).

To determine whether PI3K/mTOR signaling and S6 phosphorylation were relevant to chondrosarcoma growth, we tested the effects of BEZ235, a dual PI3K/mTOR inhibitor (14). Treatment with BEZ235 potently inhibited the growth of all of the chondrosarcoma cell lines, with IC50 values below 10 nmol/L (Fig. 3A, left). Cell-cycle analysis showed a substantial increase in the proportion of cells in the G1 phase of the cell cycle after treatment with the inhibitor (Supplementary Fig. S3A). No induction of apoptosis was observed (data not shown).

We also tested the effects of the PI3K inhibitor GDC-0941 and the mTOR inhibitor rapamycin on cell growth. Each were significantly less potent than BEZ235, with GDC-0941 inhibiting the growth of chondrosarcoma cell lines with IC50 values of 0.9 to 2.5 nmol/L and rapamycin causing 22% to 49% growth inhibition at a concentration of 1000 nmol/L (Supplementary Fig. S3B).

Functionally important mutations in the PI3K/mTOR pathway have been reported as potential predictors of sensitivity to the treatment of PI3K/mTOR pathway inhibitors (21, 22). We sequenced selected hotspot sites, including PIK3CA (exon 9 and 20), AKT1 (exon 2), and PTEN (exon 5). However, no mutations were detected in these 5 chondrosarcoma cell lines (data not shown). Moreover, PIK3CA hotspot mutations were absent in all 88 chondrosarcomas tested using hydrolysis probe assays (data not shown).

Sustained suppression of phosphorylation of S6 and 4EBP1, and feedback induction of AKT phosphorylation after prolonged treatment with BEZ235 in vitro

We used the phosphorylation state of AKT, S6 [a major substrate of p70 S6 kinase 1 (S6K1)], and 4EBP1 as markers to monitor the activity of PI3K/mTOR pathway inhibitors. The Thr308 site and Ser473 sites of AKT are regulated by PDK1 (a major downstream effector of PI3K kinase) and the mTOR complex 2 (mTORC2), respectively. The mTORC1 complex catalyzes the phosphorylation of S6K1 and 4EBP1 (23–25).

Following 2-hour treatment of JJ012 with BEZ235, the phosphorylation of S6 and the Ser473 site of 4EBP1 were suppressed at 1 and 30 nmol/L, respectively, and the phosphorylation of IGF1Rβ and INSRβ (Fig. 2A, right), and inhibited growth in a dose-dependent manner with an IC50 of 1.42 ± 0.08 μmol/L (Fig. 2B, right). These data suggest that the insulin receptor kinase family is involved in the growth of OUMS-27 cells.

Table 1. Phosphorylated S6 staining in chondrosarcoma clinical samples

<table>
<thead>
<tr>
<th>Type</th>
<th>Number positive for pS6a (%)</th>
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<tbody>
<tr>
<td>Enchondroma</td>
<td>7 (51)</td>
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<tr>
<td>Osteochondroma</td>
<td>6 (83)</td>
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<tr>
<td>Conventional chondrosarcoma</td>
<td>106 (69)</td>
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<tr>
<td>Central chondrosarcoma</td>
<td>80 (73)</td>
</tr>
<tr>
<td>Grade 1</td>
<td>37 (73)</td>
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<tr>
<td>Grade 2</td>
<td>30 (67)</td>
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<tr>
<td>Grade 3</td>
<td>13 (85)</td>
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<tr>
<td>Peripheral chondrosarcoma</td>
<td>26 (58)</td>
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<tr>
<td>Grade 1</td>
<td>14 (64)</td>
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<tr>
<td>Grade 2</td>
<td>9 (56)</td>
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<td>Grade 3</td>
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<tr>
<td>Dedifferentiated</td>
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<tr>
<td>chondrosarcoma</td>
<td>25 (44)</td>
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*pS6: phosphorylated S6 staining as conducted on TMAs.

20) decreased phosphorylation of IGF1Rβ and INSRβ (Fig. 2A, right), and inhibited growth in a dose-dependent manner with an IC50 of 1.42 ± 0.08 μmol/L (Fig. 2B, right). These data suggest that the insulin receptor kinase family is involved in the growth of OUMS-27 cells.
phosphorylation of AKT and the Thr\textsuperscript{37/46} and Thr\textsuperscript{70} sites of 4EBP1 was inhibited at 100 nmol/L (Supplementary Fig. S3C). Following 24-hour treatment, the inhibition of S6 and 4EBP1 phosphorylation was well sustained or even strengthened (Fig. 3B, left), whereas the initial inhibition on the AKT phosphorylation was dramatically reversed at 24 hours with an increase in phosphorylation level at the Thr\textsuperscript{308} site of AKT at 10 and 100 nmol/L. At 300 nmol/L, BEZ235 treatment still potently inhibited the phosphorylation of Ser\textsuperscript{473} at 24 hours, keeping AKT activity in a partially suppressed status, as phosphorylation of both Thr\textsuperscript{308} and Ser\textsuperscript{473} sites is required for full AKT activation (refs. 26, 27; Fig. 3B, left). We further examined the effects of BEZ235 treatment on the PI3K/mTOR signaling in the other 4 chondrosarcoma cell lines, and similar results were observed: the phosphorylation of S6, 4EBP1 and the Ser\textsuperscript{473} site of AKT, but not Thr\textsuperscript{308} site, were effectively inhibited by 300 nmol/L BEZ235 treatment at 24 hours (Supplementary Fig. S4A–S4D). The effects of BEZ235 on MAPK phosphorylation were also examined, and a slight increase in its phosphorylation level was observed when JJ012 and SW1353 cells were treated with 100 to 1,000 nmol/L BEZ235. The location and names of capture antibodies are listed in Supplementary Fig. S5B. The IGF1R kinase is highlighted with a rectangle. D, combinational treatment with BEZ235 and IGF1R/INSR inhibitor PQIP prevented AKT from reactivation as evaluated by immunoblot at 24 hours. IB, immunoblot analysis.
observed in OUMS-27 and CS-1 cells, respectively (Supplementary Fig. S4A and S4D).

Rapamycin treatment blocked S6 phosphorylation but only slightly decreased the phosphorylation of 4EBP1 at 10 nmol/L (Fig. 3B and Supplementary Fig. S3C). It has previously been shown that mTORC1 inhibition can lead to feedback activation of PI3K in cancer cells (25). In SW1353 and OUMS-27 cells, we also observed a dramatic and lasting increase in the phosphorylation level of AKT at Thr308 and Ser473 following rapamycin treatment (Supplementary Fig. S4B and S4D). However, in JJ012 cells rapamycin treatment enhanced the phosphorylation level of AKT only at Thr308 and not at Ser473 (Fig. 3B). There were no obvious changes observed in CS-1 and CH-2879 cells (Supplementary Fig. S4A and S4C).

**In vivo effects of PI3K/mTOR inhibitor BEZ235 on chondrosarcoma tumor growth and PI3K/mTOR signaling**

The antitumor activity of BEZ235 was studied in a mouse xenograft model of the JJ012 cell line treated for 21 days with drug or vehicle control. As shown in Fig. 4A, BEZ235 significantly suppressed tumor growth (P < 0.01). No significant weight loss of the mice was observed (data not shown). Histologic analysis showed a marked decrease in tumor cell viability as well as in the phosphorylation of S6 protein (Fig. 4B). We also explored the effects of short-term (2 hours) and long-term (21 days + 2 hours) treatment of BEZ235 on PI3K/mTOR signaling in JJ012 xenografts by immunoblots (Fig. 4C). The phosphorylation signal of S6 was diminished in all of the xenografts samples treated with 35 mg/kg BEZ235. The multiple phosphorylation sites of 4EBP1 were differentially dephosphorylated: phosphorylation of Ser65 site was suppressed, and phosphorylation of Thr70 and Thr37/46 sites were partially decreased, which may enable 4EBP1 to partially block the function of the eukaryotic translation initiation factor 4E (eIF4E), as the phosphorylation of Thr37 and Ser46 sites are critical for the release of 4E-BP1 from eIF4E (28, 29). BEZ235 treatment also decreased the phosphorylation of AKT at the Ser473 site, but the inhibition became less profound following the prolonged treatment of BEZ235. A slight increase in phosphorylation of Thr308 of AKT was observed.

**BEZ235-induced hyperphosphorylation of IGF1R family kinases and feedback activation of AKT in chondrosarcoma cells**

Because it has been reported that rapamycin can induce feedback activation of AKT through RTK-dependent...
mechanisms (25), we used RTK arrays to explore the changes in RTK phosphorylation following 24-hour treatment with BEZ235. A common increase in the phosphorylation levels of IGFR1 and/or INSR was observed (Fig. 3C and Supplementary Fig. S5A and S5B). We further checked the combinational effects of BEZ235 and the IGFR1/INSR inhibitor PQIP on AKT phosphorylation at the Thr308 site in OU1MS-27 and SW1353 cells, and found that this remained suppressed following combination treatment with BEZ235 and PQIP for 24 hours (Fig. 3D). These data suggest that BEZ235 induces feedback activation of IGFR1/INSR, which further induces AKT phosphorylation in chondrosarcoma cells.

NRAS-mutated chondrosarcoma cell line is sensitive to inhibition of MAPK pathway

In contrast to BEZ235, the MEK inhibitor ARRY-142886 (30) only inhibited the growth of SW1353, and had minimal effects on the other four chondrosarcoma cell lines (Fig. 3A, right). Sequencing of hotspot mutation-containing exons of HRAS, KRAS, NRAS (exon 2 and 3), and BRAF (exon 15) genes (31, 32) revealed a heterozygous c.181C>A (Q61K) mutation in NRAS in SW1353 cells (Supplementary Fig. S6A). No mutation was found in these exons in the other 4 cell lines.

We additionally conducted hotspot mutation analysis for NRAS exons 2 and 3 in DNA from frozen chondrosarcoma clinical samples. NRAS mutations were found to be specific for a subset of conventional central chondrosarcomas: 6 samples (12%) harbored NRAS mutations, including 2 c.181C>A (Q61K) mutations, and 4 c.182A>T (Q61H) mutations (Table 2). The other chondrosarcoma subtypes were negative.

We examined the effects of ARRY-142886 on the MAPK and PI3K/mTOR pathways in SW1353 cells. Treatment with ARRY-142886 for 1 hour led to a dose-dependent decrease in the phosphorylation of MAPK with complete inhibition at 300 nmol/L, but had no obvious effect on the phosphorylation of AKT, S6, or 4EBP1 (Fig. 3B, right and Supplementary Fig. S6B). A dramatic decrease in S6 phosphorylation and a partial inhibition of AKT phosphorylation were observed following 24-hour treatment with ARRY-142886 (Fig. 3B, right). The mechanism is unclear.

We also examined the combinational effects of BEZ235 and ARRY-142886 on MAPK and AKT signaling pathways in SW1353 cells, and found stronger inhibition in comparison with BEZ235 treatment alone (Supplementary Fig. S6C). Correspondingly, the combination of BEZ235 and ARRY-142886 had additive inhibitory effects on the cell viability in SW1353 cells (Supplementary Fig. S6D). However, apoptosis was not observed following the combination treatment.

Discussion

Sarcomas constitute a heterogeneous family of mesenchymal tumors with divergent lineages of differentiation. Some sarcomas have well-defined pathogenic genetic lesions, such as activating kinase mutations in gastrointestinal stromal tumors (GIST) or translocations that yield aberrant chimeric transcription factors in Ewing sarcoma, for example. Many other subtypes of sarcomas have no known dominant molecular pathogenic lesions to explain disease initiation, maintenance, or progression. Chondrosarcomas fall in this latter category (1, 4, 33) although recent reports indicate that approximately half have mutations in IDH1 or IDH2 (34), which are considered to be an early event and of which the functional consequences remain to be established. Chondrosarcomas are notoriously resistant to conventional chemotherapeutic agents and currently no effective systemic therapies exist for metastatic or unresectable disease. A better molecular and biochemical understanding of this malignancy may yield novel and effective treatment approaches.

We explored the potential role of RTKs in driving chondrosarcoma cell survival and proliferation. We identified constitutive, serum-independent activation of the MET, EGFR family, and IGFR1/INSR family kinases in multiple cell lines through as of yet unidentified mechanisms. Inhibition of RTK signaling by treatment with small-molecule kinase inhibitors or by suppressing RTK expression with siRNA led to alterations in phosphorylation of downstream pathway members and a concomitant decrease in cellular proliferation. The incomplete

<table>
<thead>
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<th>Table 2. NRAS mutation analysis in chondrosarcoma clinical samples</th>
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<td><strong>Chondrosarcoma</strong></td>
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<td>--------------------</td>
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<tr>
<td>Conventional central</td>
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block of cell growth suggests that other parallel pathways are also likely to be important.

Consistent with our data showing RTK activation, strong and common phosphorylation of the downstream signaling proteins AKT, MEK, and S6 kinase was previously shown using kinase substrate peptide arrays and extracts from chondrosarcoma cell lines and primary cultures (35). Here, we have linked RTK activation in chondrosarcoma cells to PI3K/AKT/mTOR signaling by showing that RTK inhibitors suppress AKT and S6 phosphorylation. Moreover, high-level phosphorylation of S6 was found in approximately 70% of chondrosarcoma tumors, suggesting that activation of the PI3K/AKT/mTOR pathway in cell lines is clinically relevant. We also identified functional mutations of NRAS in a chondrosarcoma cell line and in clinical samples; these may contribute to the activation of the MAPK signaling pathway.

Notably, the heterogeneous pattern of RTK and NRAS activation among the varying human tumor-derived chondrosarcoma cell lines poses a challenge for the potential clinical development of tyrosine kinase inhibitors for this disease. Molecular profiling of signaling pathways in any given individual tumor might suggest a specific kinase inhibitor as a rational agent to test in that specific individual patient. Accordingly, such drugs could be studied in selected groups of patients, but this is a highly personalized and potentially cumbersome approach to rare tumor subsets. An alternative approach may be to target shared downstream kinases, such as components of the PI3K/mTOR pathway. Indeed, the PI3K/mTOR inhibitor BEZ235 dramatically and potently blocked the growth of all chondrosarcoma cell lines in vitro and inhibited tumor growth in vivo. These data suggest that PI3K/mTOR pathway inhibitors, many of which are currently in clinical development (36–38), should be studied for their efficacy in the treatment of advanced chondrosarcoma.

In chondrosarcoma cells, BEZ235 exhibited more potent inhibition on cell growth than the mTORC1 inhibitor rapamycin and PI3K inhibitor GDC-0941. A detailed comparison of the effects of these three inhibitors on PI3K/mTOR signaling showed that BEZ235 is a more potent inhibitor of the phosphorylation of S6 and 4EBP1, important regulators of global protein synthesis and cap-dependent protein translation, respectively (39). Increases in the overall rate of protein synthesis as well as enhanced translation of oncoproteins are often driven by the hyperactivation of RTKs and their downstream effectors, allowing uncontrolled growth and survival of cancer cells (40). The ability of BEZ235 to inhibit S6 and 4EBP1 phosphorylation both in vitro and in vivo may account for its potent antitumor activity in chondrosarcoma as well as in other cancer types (41, 42).

In the models presented here, the major effect of BEZ235 treatment was primarily cell-cycle inhibition and a delay in tumor growth. A combination of BEZ235 and IGF1R/INSR inhibitor prevented AKT from reactivation, but still failed to induce apoptosis. High expression of BCL2 family members was recently shown to play an important role in chemoresistance of chondrosarcoma, and inhibition of BCL2 was shown to repair the apoptotic machinery, rendering chondrosarcoma cells chemosensitive (43). Inhibition of BCL2 may further supplement the observed outcome of PI3K/mTOR blockade by inducing cell apoptosis.

Recently, growth-inhibitory effects of BEZ235 were reported in osteosarcoma, Ewing sarcoma, and rhabdomyosarcoma model systems (44). However, aside from their common mesenchymal origin, these sarcoma subtypes are generally chemosensitive and are unrelated to chemo- and radioresistant chondrosarcomas, strongly differing in their biology and clinical behavior.

In our study, an activating NRAS mutation was identified in 12% of clinical chondrosarcoma samples. Two of these 6 chondrosarcoma samples also contain an IDH1 R132C mutation (45). Similarly, SW1353 carries IDH2 (45) and NRAS mutations, indicating that these mutations are not mutually exclusive. SW1353 cells were found to be sensitive to MEK inhibitors as well as to a PI3K/mTOR inhibitor. A combination of these two inhibitors had additive suppression of SW1353 cell viability but did not induce apoptosis, consistent with reported effects of the combination in colorectal carcinomas with RAS mutations (46).

Taken together, our study identifies the heterogeneity of RTK activation present in chondrosarcoma cell lines, and suggests that inhibition of the PI3K/mTOR pathway, a common signaling pathway downstream of RTKs, may be a rational therapeutic strategy for the treatment of advanced chondrosarcoma. Identification of NRAS mutations show that a subset may be particularly sensitive to MEK inhibitors, and these should also be tested in selected patients with chondrosarcoma.

Disclosure of Potential Conflicts of Interest

G.D. Demetri is a consultant/advisory board member of Infinity, Merck, and Novartis. A.J. Wagner is a consultant/advisory board member of Novartis. No potential conflicts of interest were disclosed by the other authors.

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