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

Yi-Xiang Zhang1, Jolieke G. van Oosterwijk3, Ewa Sicinska2, Samuel Moss2, Stephen P. Remillard1, Tom van Wezel3, Claudia Bühnemann4, Andrew B. Hassan4, George D. Demetri1, Judith V.M.G. Bovee5, and Andrew J. Wagner1

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 (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 Llombart-Bosch, Valencia University, Valencia, Spain), and OLI-S-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. Sub-confluent 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.
Mice were randomized into statistically identical cohorts. Tumor xenograft histology and histochemistry 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 (Ser240/244) 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 (QImaging).

**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 permethanized 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) and antigen retrieval was conducted in a pressure cooker (Biocare Medical) for 2 minutes at 125°C followed by 10 minutes at 85°C. Nonspecific binding was blocked in PBS/Tween20 (0.5%) and 10% goat serum for 1 hour at room temperature. Primary rabbit anti-pS6 antibodies (1:100; New England Biolabs, UK; #4857) were incubated with the slides in a humidified chamber at 4°C overnight. After washing three times, a secondary goat anti-rabbit Alexa 594 antibody (1:300; Invitrogen) was added. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) and slides were mounted with Prolong Gold Antifade (Invitrogen). Images were acquired with an Olympus Fluoview FV1000 confocal microscope and a 60 × 0.9 oil objective (NA: 1.35). Each image had a size of 2048 pixels × 2048 pixels, a horizontal and vertical dimension of 211 μm × 211 μm and a thickness of 1.292 μm.

**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, EGF-R, MET, ERBB4, AXL, PDGFRα, RON, and RET were phosphorylated in CS-1 cells. All 4 EGF receptor (EGF-R) 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

![Image](https://example.com/image.png)
domain (Tyr$^{1234/1235}$) 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 IC$_{50}$ 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 (19, 20) decreased phosphorylation of IGF1R and INSR (Fig. 2A, right), and inhibited cell growth. siRNA-mediated knockdown of INSR also significantly reduced cell number (35.2% ± 4.0%) compared with control siRNA at 96 hours after transfection ($P<0.001$; Supplementary Fig. S1B and S1C). However, the IGF1R-specific inhibitor PQIP only showed mild effects as did siRNA-mediated knockdown of IGF1R and INSR. These findings suggest that IGF1R family kinases, and in particular INSR, are important regulators of the growth of OUMS-27 cells.

These results show that MET, EGFR, ERBB2, and INSR are important regulators of chondrosarcoma cell growth. Further studies are needed to identify small-molecule inhibitors or targeted therapies that specifically target these pathways.
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, left). 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 OUUMS-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 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 TMA samples 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 G0–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 μmol/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 AKT and the Thr<sup>308</sup> site of AKT at 10 and 100 nmol/L. At 300 nmol/L, BEZ235 treatment still potently inhibited the phosphorylation of Ser<sup>473</sup> at 24 hours, keeping AKT activity in a partially suppressed status, as phosphorylation of both Thr<sup>308</sup> and Ser<sup>473</sup> 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<sup>473</sup> site of AKT, but not Thr<sup>308</sup> 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 (Fig. 3B and Supplementary Fig. S4B).

In comparison with BEZ235, GDC-0941 effectively inhibited the phosphorylation of AKT at 300 nmol/L, but required a much higher concentration (1,000–10,000 nmol/L) to inhibit the phosphorylation of S6 and 4EBP1 (Supplementary Fig. S3C). Following 24-hour treatment with GDC-0941, the phosphorylation level of the Thr<sup>308</sup> site of AKT remained suppressed. A slight induction of the phosphorylation signal of S6 and Ser<sup>473</sup> site of AKT was observed when JJ012 and SW1353 cells were treated with 0.1% DMSO (control) or 300 nmol/L BEZ235. The location and names of capture antibodies are listed in Supplementary Fig. S3B. 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 Thr\textsuperscript{308} and Ser\textsuperscript{473} following rapamycin treatment (Supplementary Fig. S4B and S4D). However, in JJ012 cells rapamycin treatment enhanced the phosphorylation level of AKT only at Thr\textsuperscript{308} and not at Ser\textsuperscript{473} (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 Ser\textsuperscript{65} site was suppressed, and phosphorylation of Thr\textsuperscript{70} and Thr\textsuperscript{37/46} 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 Thr\textsuperscript{70} and Ser\textsuperscript{65} sites are critical for the release of 4E-BP1 (28, 29). BEZ235 treatment also decreased the phosphorylation of AKT at the Ser\textsuperscript{473} site, but the inhibition became less profound following the prolonged treatment of BEZ235. A slight increase in phosphorylation of Thr\textsuperscript{308} 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 IGF1R and/or INSR was observed (Fig. 3C and Supplementary Fig. S5A and S5B). We further checked the combinational effects of BEZ235 and the IGF1R/INSR inhibitor PQIP on AKT phosphorylation at the Thr308 site in OUMS-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 IGF1R/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). 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 IGF1R/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

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<th>Table 2. NRAS mutation analysis in chondrosarcoma clinical samples</th>
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Indeed, the PI3K/mTOR inhibitor BEZ235 dramatically and 
kinases, such as components of the PI3K/mTOR pathway. 

An alternative approach may be to target shared downstream 
potentially cumbersome approach to rare tumor subsets. An 
given individual tumor might suggest a specific kinase 

drosarcoma 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 

tumor growth. A combination of BEZ235 and IGF1R/INSR 

mTOR blockade by inducing cell apoptosis.

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 suppress 
ion 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 Novar-
tis. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Y.-X. Zhang, G.D. Demetri, J.V.M.G. Bovée, A.J. Wagner


Acquisition of data (provided animals, acquired and managed patients, 

Analysis and interpretation of data (e.g., statistical analysis, biosta-

Writing, review, and/or revision of the manuscript: Y.-X. Zhang, J.G. van Oosterwijk, S.P. Remillard, T. van Wezel, A.B. Hassan, J.V.M.G. Bovée, A.J. Wagner

Administrative, technical, or material support (i.e., reporting or orga-

izing data, constructing databases): Y.-X. Zhang, E. Sicinska, S. Moss, A.J. Wagner

Study supervision: G.D. Demetri, J.V.M.G. Bovée, A.J. Wagner

Acknowledgments

The authors thank N. Thanasou (Nuffield Department of Orthopaedic Surgery, University of Oxford, Oxford, United Kingdom), S. Daugaard (Department of Pathology, University Hospital Copenhagen, Copenhagen, Denmark), B. Liegel-Atzwanger (Department of Pathology, Medical University Graz, Graz, Austria), and P. Picci (Laboratory of Oncologic Research, Rizzoli Orthopaedic Institute, Bologna, Italy) for contributing cases of rare chondrosarcoma subtypes. In addition, authors also thank Maayke A.J.H. van Ruler for expert technical assistance and Daniëlle Meijer, Inge Briaire-de Bruijn, and Dorien van der Geest for construction of TMAs.
Grant Support
Dunkin Donuts Rising Star Award (to A.J. Wagner) and D.K. Ludwig Fund for Cancer Research supporting the Dana-Farber/Harvard Ludwig Center (to G.D. Demetri), the Netherlands Organization for Scientific Research (917-67-115; to J.G. van Oosterwijk and J.V.M.G. Bovee) and Dutch Cancer Society (UL10.2010-4873; to J.G. van Oosterwijk and J.V.M.G. Bovee). Leiden and Oxford are partners in the context of EuroSarc, an EC seventh framework programme under grant agreement no 278742.

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