Afatinib Is a New Therapeutic Approach in Chordoma with a Unique Ability to Target EGFR and Brachyury

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Abstract

Chordomas are rare bone tumors with no approved therapy. These tumors express several activated tyrosine kinase receptors, which prompted attempts to treat patients with tyrosine kinase inhibitors. Although clinical benefit was observed in phase II clinical trials with imatinib and sorafenib, and sporadically also with EGFR inhibitors, therapies evaluated to date have shown modest activity. With the goal of identifying new drugs with immediate therapeutic potential for chordoma patients, we collected clinically approved drugs and other advanced inhibitors of MET, PDGFRβ, and EGFR tyrosine kinases, and assessed their antiproliferative activity against a panel of chordoma cell lines. Chordoma cell lines were not responsive to MET and PDGFRβ inhibitors. U-CH1 and UM-Chor1 were sensitive to all EGFR inhibitors, whereas the remaining cell lines were generally insensitive to these drugs. Afatinib was the only EGFR inhibitor with activity across the chordoma panel. We then investigated the molecular mechanisms behind the responses observed and found that the antiproliferative IC50s correlate with the unique ability of afatinib to promote degradation of EGFR and brachyury, an embryonic transcription factor considered a key driver of chordoma. Afatinib displayed potent antitumor efficacy in U-CH1, SF8894, CF322, and CF365 chordoma tumor models in vivo. In the panel analyzed, high EGFR phosphorylation and low AXL and STK33 expression correlated with higher sensitivity to afatinib and deserve further investigation as potential biomarkers of response. These data support the use of afatinib in clinical trials and provide the rationale for the upcoming European phase II study on afatinib in advanced chordoma. Mol Cancer Ther; 17(3): 603–13. ©2017 AACR.

Introduction

Chordomas are primary malignant bone tumors that arise along the axial skeleton, usually in the sacrum or skull-base, but also with low frequency in the mobile spine. Chordomas are rare tumors, with an incidence below 1:1,000,000, and account for 1% to 4% of all primary bone malignancies. They are typically late onset tumors with a peak incidence between the fifth and sixth decades of life, but can also occur in children and young adults.

Chordomas are slow growing tumors, but are characterized by a high recurrence rate even after complete surgical resection of the primary tumor. Distant metastases occur in 20% to 30% of cases (1), but local recurrences affect 50% of patients (2). In case of relapse, surgery or radiotherapy become challenging and patients usually die of their disease. Due to the location of these tumors along the neuro-axis, patients commonly experience physical dysfunctions and significant pain requiring morphine derivatives and steroids (1, 3). No standard medical therapy is currently available, and chordomas are resistant to cytotoxic chemotherapy.

Chordomas arise from embryonic notochordal remnants and are characterized by expression of the “T” gene product “brachyury”, a notochord-specific transcription factor essential for mesodermal specification and differentiation during development (4). The anomalous expression of brachyury in adult notochordal remnants is believed to play a major role in the onset and maintenance of chordoma (5, 6). Brachyury silencing in chordoma cell lines was shown to impair cell proliferation and induce senescence, and attempts to target brachyury-expressing cells through a vaccine are currently ongoing (7–9). Multiple studies have shown that chordomas commonly exhibit expression and activation of tyrosine kinase receptors and downstream signaling molecules, with MET (HGF receptor), PDGFRβ (PDGFRB), and EGFR as the most widely expressed, and HER2 (erbB2), KIT (SCFR), and VEGFR (KDR) also expressed (10–14).

The availability of clinically approved drugs targeting EGFR and PDGFR has prompted the evaluation of imatinib and lapatinib in phase II clinical trials for chordoma patients selected for expression of corresponding drug targets (15–19). Imatinib demonstrated some clinical benefit, although not achieving dimensional and long-lasting responses (16),
whereas lapatinib did not show a real clinical benefit (19). However, anecdotal responses to other EGFR inhibitors have been reported, suggesting that EGFR inhibitors might have therapeutic potential for these tumors (20–24). More recently, a phase II clinical trial with sorafenib was conducted on chordomas, based on the in vitro activity of this drug on VEGFR and PDGFR family members. In this study, patients were not selected or characterized for expression of the corresponding receptors. Notably, sorafenib actually achieved a longer progression-free survival compared with imatinib (25).

Systematic preclinical studies to identify drugs active in chordomas have been limited in the past by the paucity of biological models, as U-Ch1 and U-Ch2 cell lines represented the only two available validated chordoma cell lines (26). More recently, a few additional bona fide chordoma cell lines have become available through the Chordoma Foundation, a patients’ advocacy organization (www.chordomafoundation.org).

With the aim of identifying kinase inhibitor drugs that might have therapeutic interest for chordoma patients, we assembled a representative panel of chordoma cell lines and assessed their sensitivity to EGFR, PDGFR, and MET inhibitors, including approved drugs and other representative compounds.

Materials and Methods

Cell lines and culture conditions

U-Ch1, U-Ch2, JHC7, and UM-Chor1 cell lines were obtained from Chordoma Foundation. MUG-Chor1 and U-Ch2 (ATCC) cell lines were purchased from the ATCC. Chor-IN-1 cell line was established in house from a surgical sample (27). Chordoma cell lines were cultured in collagen-coated plates with IMDM/RPMI1640 4:1 Medium (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO2. Media were supplemented with 10% (v/v) heat-inactivated FBS (Euroclone). All cell lines were cultured in collagen-coated plates with IMDM/RPMI1640 4:1 Medium (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO2. Media were supplemented with 10% (v/v) heat-inactivated FBS (Euroclone). All cell lines were cultured in collagen-coated plates with IMDM/RPMI1640 4:1 Medium (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO2. Media were supplemented with 10% (v/v) heat-inactivated FBS (Euroclone). 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Sequence Detector User Bulletin 2, https://www2.appliedbiosystems.com). All procedures were performed according to the manufacturer’s instructions.

**EGFR**; fw 5'-AAGTCCCACGTGATCTGCT-3', rev 5'-TGCCCTTGCTCTGGAAATT-3';

- brachyury: fw 5'-GTATGAGCGTCAAGGACTC-3', rev 5'-GATAGGCTGTCGACCAAG-3';

- STK33: fw 5'-CGAATACGTGAGGCTATGGA-3', rev 5'-TCTCTCTGAGCGTCCGCAA-3';

- AXL: fw 5'-GCCCTCCCTGCGATCTTTC-3', rev 5'-CTCGAGTCCAGCCACATGGCC-3';

- GUSB: fw 5'-GCATCCAAAAGACGCACTTC-3', rev 5'-CACCAGTACCTCAAGCTTG-3';

- PPA: fw 5'-CACCACCTGTTTCTTCGACCAT-3', rev 5'-TTTCT-CTGCTTTTGGGACCTT-3';

- 18S rRNA: fw 5'-AAGTCCCCCAGTGACTGCT-3', rev 5'-TGGCTT-TAGTGACAAAACACTTCGTT-3';

- GAPDH: fw 5'-GTATGAGGCTGTCGACCAAG-3', rev 5'-CTCTCTGAGCGTCCGCAA-3';

**Results**

We assembled a panel of chordoma cell lines which included cells of sacral origin like the widely used U-CH1 (28) and U-CH2 (26), the recently established MUG-Chor1 (30) and JHC7 (8), and the first available clival cell line UM-Chor1 (31). In addition, we tested Chor-IN-1, a new cell line derived in house from a sacral chordoma surgical sample shown to meet validation criteria for chordoma cell lines (27). All cell lines were first authenticated by STR fingerprinting. The STR profile of U-CH2 cells obtained from two sources differed at a single locus, suggesting the acquisition of little differences during their propagation (Supplementary Table S1). Both clones were therefore tested in parallel.

Immunoblot analysis showed that all seven cell lines expressed the "T" gene product brachyury, considered a defining marker of chordoma (Fig. 1).

**In vivo studies**

Preclinical studies were conducted through the Chordoma Foundation Drug Screening Pipeline at South Texas Accelerated Research Therapeutics (START) under International Animal Care and Use Committee-approved protocols.

Antitumor activity was tested in:

- U-CH1: xenograft of the U-CH1 cell line (28).

- SF8934: PDX model (29).

- CF322: new chordoma PDX model generated from a clival recurrent tumor of a 42-year-old male.

- CF365: new chordoma PDX model generated from a clival poorly differentiated metastatic tumor of a 11-year-old male.

For all models, athymic nude mice (Charles River Labs) between 6 and 8 weeks of age were implanted subcutaneously with tumor fragments from host animals. Once tumors reached approximately 150 to 250 mm³, animals were matched by tumor volume (TV) and randomized to control and treatment groups. All groups were dosed at 20 mg/kg PO daily, U-CH1 and SF9984 group for 28 days, and CF322 and CF365 groups to end. Initial dosing began at day 0. Animals were observed daily and weighed twice a week. TV and animal weight data were collected electronically using a digital caliper and scale; tumor dimensions were converted to volume using the formula TV (mm³) = width² (mm²) × length (mm) × 0.52. Endpoints were a mean control final tumor measurements. Statistical analysis was performed using a two-way ANOVA followed by the Dunnett multiple comparisons test.

EGFR immunoprecipitation

Cell lysates were precleared by rocking at 4°C for 20 minutes with Protein G Sepharose previously washed 4X with ice-cold PBS and 1X with CLB buffer. Preloaded resin/antibodies were incubated at 4°C rocking for 3 hours with the cell lysate. Samples were transferred on cytospin filter (CytoSignal cat. C00-100), washed 3X with CLB buffer, and eluted at 95°C in Laemmli-SDS buffer 2X.
MET and EGFR were shown to display similar expression levels across the different cell lines, and HER2 was barely detectable. PDGFRβ expression was more heterogeneous, ranging from very low (U-CH1) to high expression (U-CH2 and UM-Chor1). The downstream signaling molecules AKT (PKB), MAPK (MAPK1/ MAPK3), and STAT3 were differentially activated across the different cell lines, with no specific correlation to receptor expression (Fig. 1).

To evaluate the importance of these tyrosine kinase receptors for the growth of chordoma cells, we assembled a broad panel of potent MET, PDGFRβ, and EGFR inhibitors, including approved drugs as well as other reference inhibitors. The antiproliferative activity of compounds was determined after 144-hour incubation, to allow two population doublings, considering the slow growth rate of chordoma cells (>60-hour doubling time). In parallel, the inhibitors were profiled on reference cell lines: gastric adenocarcinoma MKN-45 with MET amplification (32), NSCLC NCI-H1703 with PDGFRα amplification (33), and epidermoid carcinoma A431 with EGF amplification were used as positive controls, whereas ovarian carcinoma A2780 cell line was used as general negative control, for which no target-dependent activity was expected.

Crizotinib (34, 35) and cabozantinib (36), two approved MET inhibitor drugs, and the MET-selective inhibitor PHA-667572 (35, 37) did not display considerable antiproliferative activity against chordoma cell lines, with I_{50,8} higher than those measured for the A2780 control cell line (Supplementary Table S2A).

A similar result was observed with several PDGFR inhibitors, including the approved drugs sunitinib and imatinib (35, 38, 39) as well as crenolanib (ref. 40; Supplementary Table S2B).

We then tested the clinically approved EGFR inhibitor drugs erlotinib, gefitinib, afatinib, and lapatinib, which possess different potency and selectivity within the EGFR/HER2 family (35, 44). All compounds were active on U-CH1 and UM-Chor1 cell lines, although with different potency (Table 1A). In particular, afatinib was very potent against both cell lines, with I_{50,8} of 0.014 and 0.023 μmol/L, respectively, whereas the other three drugs exhibited I_{50,8} in the 0.1 – 0.8 μmol/L range. Moreover, afatinib was the only drug displaying activity, although with different potency, against all cell lines (I_{50} < 0.7 μmol/L), with the exception of JHC7. The other EGFR inhibitors erlotinib, lapatinib, and gefitinib, despite being active on U-CH1 and UM-Chor1 in the submicromolar range, did not generally show considerable antiproliferative activity against the other chordoma cell lines.

To investigate whether these results were correlated with the potency and/or the covalent mechanism of EGFR inhibition of afatinib, we also tested the covalent EGFR inhibitors neratinib (35, 45) and dacomitinib (46). Both inhibited U-CH1 and UM-Chor1 in the nanomolar range, but had poor activity on the other cell lines, with comparable or higher I_{50,8} than those observed for the A2780 control cell line (Table 1B).

Interestingly, dacomitinib displayed I_{50} values comparable with afatinib in U-CH1 and UM-Chor1, but it was not generally active in the other cell lines, therefore indicating that the activity of afatinib across the chordoma panel is not just related to its biochemical potency against EGFR. Of note, the T790M EGFR mutant-selective covalent inhibitors osimertinib (AZD-9291; ref. 47) and rociletinib (48) were inactive or poorly active in all chordoma cell lines, as expected in the absence of EGFR mutations (13, 27, www.chordomafoundation.org).

These data suggest that the antiproliferative effect observed with afatinib against chordoma is linked to EGFR inhibition, but is not just related to its potency or covalent mechanism of action.

To obtain a dynamic view of the effects induced by treatment with afatinib, we performed a kinetic live-cell analysis (see Supplementary Methods) using a multiwell automated microscope (Incucyte ZOOM). U-CH1 cells were treated with afatinib or doxorubicin standard, and a time-lapse movie was generated integrating the sequence of live-cell images acquired through 144-hour treatment. Cell confluence was progressively decreased over time with increasing concentrations of afatinib or doxorubicin, while increasing in the untreated cells (Supplementary Fig. S1A). At active compound concentrations, graphs bottomed out on April 23, 2021. © 2018 American Association for Cancer Research. mct.aacrjournals.org Downloaded from mct.aacrjournals.org on April 23, 2021. © 2018 American Association for Cancer Research.

Table 1. Antiproliferative activity of EGFR inhibitors in chordoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>U-CH1 (μmol/L)</th>
<th>U-CH1 (μmol/L)</th>
<th>U-CH2 (μmol/L)</th>
<th>U-CH2 (μmol/L)</th>
<th>U-CH2 (μmol/L)</th>
<th>U-CH2 (μmol/L)</th>
<th>U-CH2 (μmol/L)</th>
<th>U-CH2 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Approved EGFR inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afatinib</td>
<td>0.014</td>
<td>0.023</td>
<td>0.258</td>
<td>0.494</td>
<td>0.531</td>
<td>0.668</td>
<td>1.346</td>
<td>0.026</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>0.049</td>
<td>0.069</td>
<td>0.377</td>
<td>0.746</td>
<td>0.947</td>
<td>1.984</td>
<td>2.156</td>
<td>0.187</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>0.065</td>
<td>0.156</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.562</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>0.071</td>
<td>0.751</td>
<td>6.241</td>
<td>6.259</td>
<td>5.936</td>
<td>9.040</td>
<td>7.010</td>
<td>0.335</td>
</tr>
<tr>
<td>B. Other advanced EGFR inhibitors</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Neratinib</td>
<td>0.038</td>
<td>0.150</td>
<td>1.946</td>
<td>1.926</td>
<td>3.253</td>
<td>2.009</td>
<td>2.893</td>
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<tr>
<td>Dacomitinib</td>
<td>&lt;0.019</td>
<td>&lt;0.019</td>
<td>2.075</td>
<td>2.400</td>
<td>2.126</td>
<td>0.418</td>
<td>1.820</td>
<td>0.043</td>
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<tr>
<td>Osimertinib</td>
<td>0.045</td>
<td>0.569</td>
<td>1.129</td>
<td>2.250</td>
<td>1.165</td>
<td>0.384</td>
<td>0.897</td>
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<tr>
<td>(T790M)</td>
<td>0.120</td>
<td>0.143</td>
<td>0.228</td>
<td>0.085</td>
<td>0.134</td>
<td>0.473</td>
<td>0.021</td>
<td>0.110</td>
</tr>
<tr>
<td>(T790M)</td>
<td>0.484</td>
<td>0.586</td>
<td>1.520</td>
<td>1.552</td>
<td>0.884</td>
<td>1.250</td>
<td>1.683</td>
<td>1.527</td>
</tr>
</tbody>
</table>

NOTE: In bold, registered EGFR inhibitors. The average I_{50} (μmol/L) were determined as described under the Materials and Methods. All values were derived from technical duplicates and confirmed in multiple (n > 6) independent biological experiments. A431 and A2780 cell lines are shown for comparison. Doxorubicin is used as reference standard.
to a background signal, representing the image analysis of cell debris. Conversely, caspase 3 (CASP-3) and caspase 7 (CASP-7) induction (Supplementary Methods), measured with a specific fluorescent dye, was evident upon doxorubicin treatment, but was not detectable upon treatment with afatinib (Supplementary Fig. S1B and S1C). This experiment demonstrated that afatinib inhibits U-CH1 cell proliferation and induces cell death in a time- and dose-dependent manner, and indicated that the main mechanism of cell death is not apoptosis.

We then studied the dose- and time-dependent biomarker modulation following treatment with afatinib, erlotinib, or lapatinib in U-CH1 cells, responsive to all EGFR inhibitors (Fig. 2). AKT and MAPK pathways were modulated in a dose-dependent manner by the three inhibitors, both after short (2-8-24 hours) and longer (48 hours) incubation times, at doses consistent with antiproliferative IC50s. STAT3 activation instead was not dependent upon EGFR signaling in these cells. Caspase 3 cleavage was not observed up to 48 hours, again suggesting the involvement of mechanisms other than apoptosis in the antiproliferative effect of these inhibitors (Fig. 2; Supplementary Fig. S1B). Interestingly, treatment with afatinib and erlotinib induced a decrease in the total level of both the

**Figure 2.**
Biomarker modulation upon treatment of U-CH1 cell line with different EGFR inhibitors. Immunoblot analysis of U-CH1 cells treated with the indicated doses of inhibitors for 2-8-24 hours (top) or 48 hours (bottom). Protein cell extracts were resolved on SDS-PAGE gel and membranes probed with the indicated antibodies. IC50 of the different inhibitors are reported.
cytoplasmic and membrane-bound forms of LC3B (MAP1LC3B), hinting a potential involvement of autophagic pathways.

A striking difference in the mechanism of action of the three compounds was the effect on brachyury and EGFR protein levels at the latest time points. After 48-hour incubation, afatinib induced not only a strong decrease in the total level of EGFR, but also a dose-dependent decrease of brachyury protein. EGFR depletion was observed but only to a lesser extent upon treatment with erlotinib, and was absent after treatment with lapatinib. Brachyury expression was not affected by treatment with erlotinib or lapatinib.

To assess whether the afatinib-induced EGFR and brachyury downmodulation was linked to stronger potency or covalent mechanism of inhibition, we analyzed the effect of dacomitinib, which displayed similar IC50s on U-CH1 and UM-Chor1, but was generally inactive on the other cell lines (Table 1). Treatment of U-CH1 cells with dacomitinib induced a dose-dependent downmodulation of P-AKT levels, but, differently from afatinib, did not affect total levels of EGFR and brachyury up to 5 μmol/L (Supplementary Fig. S2). These data support the hypothesis that the activity observed with afatinib against chordoma cell lines could be linked to its ability to ablate two proteins relevant for chordoma cell growth, and not merely to its potency or covalent mechanism of EGFR inhibition.

To investigate whether this particular mechanism occurred also in the other afatinib-responsive chordoma cell lines, MUG-Chor1 and Chor-IN-1 were treated with the compound and analyzed by immunoblot. Figure 3 shows that downmodulation of EGFR and brachyury occurred in the same dose-response manner within each cell line, which correlated with the measured IC50s. Afatinib therefore turned out to be the only EGFR inhibitor displaying activity, although with different potency, across the chordoma cell line panel and the only inhibitor inducing a downmodulation of two biomarkers known to play a major role in chordoma cell growth.

To explore potential correlations between EGFR and brachyury, their reciprocal impact upon siRNA was analyzed (Supplementary Methods). Transfection of U-CH1 cells with specific siRNA oligonucleotides induced a complete ablation of the corresponding target protein (EGFR or brachyury), with no effect on the other protein. In both cases, a strong impairment of cell viability was observed, therefore confirming that brachyury and EGFR play a crucial role in chordoma cell growth (Supplementary Fig. S3A and S3B). We ruled out that this downregulation occurred transcriptionally, because treatment of U-CH1 cells with 0.1 and 1 μmol/L afatinib for 24 or 48 hours did not affect brachyury mRNA and only slightly decreased EGFR mRNA (Supplementary Fig. S4A).

Instead, treatment of U-CH1 cells with afatinib in the presence of MG-132 (proteasome inhibitor) or bafilomycin (autophagy inhibitor) prevented afatinib-induced EGFR and brachyury protein ablation (Supplementary Fig. S4B), therefore showing that the downmodulation of these proteins involves pathways of protein degradation.

We then assessed the activity of afatinib in vivo in U-CH1 xenografts and SF8894, CF322, and CF365 PDX (29) mouse models, generated as described in the Materials and Methods section. Daily oral treatment of mice with 20 mg/kg afatinib for the indicated times induced potent tumor growth inhibition in all
the analyzed models, with no apparent signs of toxicity (Fig. 4). These data confirm that the activity observed in vitro with afatinib translates into impressive in vivo efficacy in four out of four models, providing a strong rationale for testing this drug in the clinic.

Finally, we exploited the cell lines with different levels of response to afatinib to start exploring potential biomarkers of sensitivity to this drug. We investigated whether the differences in the IC_{50}s among these cell lines could be related to the total EGFR levels, to the extent of receptor activation or to a differential response to the inhibitor. Total and membrane-bound EGFR levels were found to be comparable among the different chordoma cell lines, as detected by flow cytometry analysis with cetuximab (Supplementary Methods; Supplementary Fig. S5A).

The level of EGFR phosphorylation was analyzed, both in basal conditions and after treatment with afatinib, by immunoprecipitation of the receptor from total cell lysates followed by immunoblot with anti–P-Tyr antibody. Figure 5A shows that EGFR is efficiently immunoprecipitated from all cell lysates (bottom) and that sensitive cell lines harbor phosphorylated EGFR (top), whereas JHC7 has a minimal level of basal phosphorylation. Interestingly, treatment with afatinib induced complete EGFR dephosphorylation in U-CH1 cell line, but did not decrease the weak P-Tyr band detected in JHC7 (Fig. 5A, top). Because treatment with afatinib completely abolished EGFR phosphorylation also in MUG-Chor1, Chor-IN-1, and U-CH2 cell lines (Supplementary Fig. S5B), the band observed in JHC7 might be due to recognition of a different phosphorylation site, not linked to receptor activation. Therefore, the sensitivity of chordoma cell lines to afatinib seems to generally correlate with EGFR phosphorylation, and the lack of EGFR activation in JHC7 cell line likely accounts for the measured higher IC_{50}.

To identify other potential determinants of differential sensitivity to afatinib, we analyzed the expression of AXL, reported to mediate resistance to anti-EGFR therapies in different tumor types (49). Figure 5B shows that AXL expression inversely correlates with sensitivity to afatinib, with a weak expression in U-CH1 and UM-Chor1 cell lines, and higher levels in the less sensitive MUG-Chor1, U-CH2, and Chor-IN-1 cell lines. No expression of AXL was observed in JHC7, where the EGFR pathway is not activated.

Interestingly, in a whole kinome-targeted sequencing, evaluating in parallel the differential expression of approximately 500 kinases in U-CH1 versus the other sacral chordoma cell lines, STK33 emerged as the only kinase with undetectable expression in U-CH1 and higher expression in the other cell lines (27). We then evaluated STK33 expression in all the cell lines by RT-qPCR and found no expression in both afatinib highly responsive U-CH1 and UM-Chor1 cell lines, while confirming relevant expression in the others.

**Figure 4.** 
In vivo efficacy of afatinib on U-CH1 xenograft and different PDX models. Nude mice bearing U-CH1 or SF8894, CF322, and CF365 tumors were dosed daily with afatinib 20 mg/kg po for 28 days (bar indicates the length of treatment) or to the end of treatment, as indicated.

20 mg/kg; po; qdx28
Finally, we performed a pharmacodynamics study evaluating EGFR signaling as well as AXL and STK33 levels after 1 and 2 hours of treatment of U-CH1, MUG-Chor1, and Chor-IN-1 cell lines with afatinib. No relevant alterations of AXL and STK33 mRNA or total protein levels were detected (Supplementary Fig. S6A and S6B). Interestingly, although EGFR phosphorylation was abrogated in all cell lines at 0.1 μmol/L afatinib, P-AKT was shut down in U-CH1 cell line, but displayed a slight increasing trend in MUG-Chor1 and Chor-IN-1 (Supplementary Fig. S6B and S6C), highlighting that the activity of afatinib in these cell lines is contributed by cellular mechanisms that go beyond EGFR kinase inhibition.

Discussion

Only a few clinical trials have been conducted so far to assess the efficacy of targeted therapeutic agents in chordoma (20–24), certainly limited by the exiguous number of preclinical models of this indication available so far. We recently analyzed in depth at the genomic level a panel of chordoma cell lines of sacral origin that included also the novel cell line Chor-IN-1, established and characterized in our laboratories (27). With the goal of identifying new drugs that might have an immediate therapeutic application for chordoma patients, we evaluated the in vitro antiproliferative activity of inhibitors of MET, PDGFRβ, and EGFR tyrosine kinases, reported to be frequently expressed in chordomas. MET inhibitors did not display significant activity and were not further investigated. PDGFR inhibitors were also not active, even in the cell lines with strong expression of PDGFRβ such as U-CH2 and UM-Chor1. Considering that a phase II clinical trial with imatinib demonstrated some clinical benefit for chordoma patients, PDGFRβ inhibition in the tumor microenvironment might result in effects that go beyond mere antiproliferative activity on tumor cells in vitro, or PDGFRβ in tumors might be
activated by paracrine mechanisms that are lost in vitro. Interestingly, responses to imatinib observed in the clinic are primarily related to changes in tumor features rather than dimension (17). Multiple reports in the literature highlight the activity of different EGFR inhibitors in chordoma cell lines, in animal models, and also sporadically in patients (reviewed in refs. 24, 50), providing evidence of a potential clinical relevance of EGFR inhibitors in chordoma, but also raising questions about the most suitable agent.

We focused our studies on clinically approved EGFR inhibitors and observed that all of them displayed some activity, although with different potencies, against U-CH1 and UM-Chor1 cell lines. Afatinib was the most potent inhibitor in these cell lines, whereas lapatinib was the least active in addition to being inactive on the remaining cell lines, which might have predicted the poor activity observed for lapatinib in the clinic. Afatinib was also the only drug with activity across the chordoma cell lines panel, with the exception of JHC7, that despite expressing significant levels of EGFR is not driven by EGFR signaling. The activity of afatinib in chordoma cell lines is particularly relevant because this drug is very selective biochemically and its cellular activity is dependent upon EGFR pathway activation (41, 42). The activity of afatinib in U-CH1 and UM-Chor1 chordoma cell lines is equivalent to that reported for mutant EGFR-dependent lung tumors in vitro (42, 48), suggesting that chordoma might also represent a potential indication for this drug. These cellular data were also corroborated by impressive in vivo efficacy, with tumor growth inhibition in one chordoma xenograft and in three different PDX models.

Schiopel and colleagues (51) published a focused compound screening describing the activity of EGFR inhibitors in a subset of chordoma cell lines, reporting, in line with our data, that U-CH1 and UM-Chor1 cell lines are sensitive to EGFR inhibitors, but unexpectedly they found afatinib active on UM-Chor1 but not in U-CH1 cell line. Considering the potent activity we repeatedly observed for afatinib on U-CH1 both in vitro, with modulation of downstream pathways, and particularly in vivo, the lack of activity they observed in U-CH1 might be related to different screening conditions or compound handling.

Differently from erlotinib, gefitinib, and lapatinib that are reversible inhibitors, afatinib contains an electrophilic group capable of Michael addition to conserved cysteines within the catalytic domains of EGFR (Cys797), HER2 (Cys805), and HER4 (Cys803), that induces complete and long-lasting inhibition of receptor phosphorylation (42). However, the activity of afatinib in chordoma cell lines appears to be related not only to its higher potency and covalent mechanism, as neratinib and dacomitinib, sharing the same mechanism, were active only in U-CH1 and UM-Chor1 cell lines. This activity appears to be strongly contributed by its unique ability to downmodulate the total level of EGFR and brachyury proteins, a feature not shared by the other inhibitors. Interestingly, afatinib was recently shown to induce a downmodulation of the total level both of HER2 and EGFR in tumors harvested from NCI-N87 xenografts treated with afatinib (52).

Brachyury is a distinctive marker of sporadic chordomas, duplication of the T gene locus is typical of familial chordomas (6), and a DNA polymorphism in the T gene is associated with chordoma predisposition in the general population (53). Accordingly, silencing of brachyury in chordoma cell lines was widely shown to impair tumor growth both in vitro and in vivo (7, 8; Supplementary Fig. S3B). Brachyury would represent an obvious target for drug development in chordomas, but direct inhibition of transcription factors with small molecules has thus far been extremely challenging.

The identification of a small molecule that, in addition to inhibiting EGFR signaling, induces brachyury degradation represents a new approach indirectly targeting this important transcription factor with a kinase inhibitor.

Overall, testing EGFR inhibitors across different cell lines and in vivo models suggests that a subset of chordomas are driven by the EGFR signaling pathway and are strikingly sensitive to EGFR inhibition. Preclinically, this sensitivity correlates with the level of EGFR phosphorylation and it will be important to assess if this holds true in the clinical study. Interestingly, Mug-Chor1, U-CH2, and Chor-IN-1 chordoma cell lines display a strong expression of AXL receptor, which deserves further investigation, because it represents a mechanism of resistance in lung cancer (49). Should this occur also in chordoma, a combination therapy with AXL inhibitors, which are already in the clinic, might be beneficial to these types of patients. STK33, which is absent in the cell lines most sensitive to afatinib, also represents a candidate biomarker for afatinib sensitivity which deserves further investigation.

These data provide a strong rationale for formal evaluation of afatinib in the treatment of chordoma patients. Accordingly, a European phase II study on afatinib in advanced chordoma is about to start patient recruitment.

Disclosure of Potential Conflicts of Interest

S. Stacchiotti is a consultant/advisory board (Advisory Board) member of Bayer. No potential conflicts of interest were disclosed by the other authors.

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