Combination of PI3K/mTOR Inhibition Demonstrates Efficacy in Human Chordoma

JOSEPH SCHWAB1, CRISTINA ANTONESCU2, PATRICK BOLAND3, JOHN HEALEY3, ANDREW ROSENBERG4, PETUR NIELSEN4, JOHN IAFRATE4, THOMAS DELANEY5, SAM YOON6, EDWIN CHOI7, DAVID HARMON7, KEVIN RASKIN1, CAO YANG1, HENRY MANKIN1, DEMPSEY SPRINGFIELD1, FRANCIS HORNICEK4 and ZHENFENG DUAN1

1Department of Orthopedic Surgery, Section of Orthopedic Oncology, Sarcoma Research Laboratory, 4Department of Pathology, 5Department of Radiation Oncology, 6Department of Surgery, Section of Surgical Oncology and 7Department of Medical Oncology, Massachusetts General Hospital, Boston, MA; 2Department of Pathology, and 3Department of Surgery, Section of Orthopedic Oncology, Memorial Sloan Kettering Cancer Center, New York, NY, U.S.A.

Abstract. Background: Chordomas are rare tumors of the axial skeleton for which surgical resection remains the most reliable means of cure. PI-103 is a inhibitor of PI3K/AKT and mTOR activation. This study aims to determine whether the PI3K/mTOR pathway was active in chordomas and whether their inhibition could lead to decreased proliferation and increased apoptosis. Materials and Methods: Thirteen human chordoma were tested for activation of the PI3K/mTOR pathway. The human chordoma cell line UCH-1 was treated with increasing doses of PI-103. Inhibition of AKT and mTOR was examined and assays assessing proliferation and apoptosis were performed. Results: The chordoma specimen demonstrated activation of the PI3K/mTOR pathway. The human chordoma cell line UCH-1 was treated with increasing doses of PI-103. Inhibition of AKT and mTOR was examined and assays assessing proliferation and apoptosis were performed. Results: The chordoma specimen demonstrated activation of the PI3K/mTOR pathway. PI-103 inhibited the AKT and mTOR activation in the UCH-1 cell line. PI-103 inhibited proliferation and induced apoptosis in UCH-1. Conclusion: The PI3K/AKT and mTOR signaling pathway is constitutively activated in chordoma. PI-103 decreases proliferation and induces apoptosis in the UCH-1 via inhibition of the PI3K/mTOR pathway.

The advent of targeted molecular therapy has fostered hope for new systemic therapies against historically chemoresistant tumors such as chordomas. Unfortunately, the molecular pathogenesis of chordomas is poorly understood. While radiation therapy has been shown to be a useful adjuvant in chordoma (1), complete surgical resection remains the most reliable means of obtaining a cure (2, 3). However, complete surgical excision is difficult and local recurrence rates of over 40% are common (2). Furthermore, there are no proven systemic agents available in cases of distant metastasis, which occurs in 1 out of every 3 patients (2-5). Improved systemic therapies are needed to treat this relatively chemoresistant tumor.

There is increasing interest in using targeted therapies for treating chordomas. One study reported an objective radiological response in six cases of recurrent chordoma treated with imatinib mesylate (Gleevec, Novartis) (6). The authors attributed the response to inhibition of the platelet derived growth factor-β receptor (PDGFR-β) which they demonstrated to be phosphorylated. PDGFR-β transmits its message through phosphatidylinositol-3,4,5-trisphosphate (PIP3) via AKT and mTOR phosphorylation. The PI3K/AKT/mTOR pathway suppresses apoptosis, promotes cell growth and drives cellular proliferation.

Abnormalities in the PI3K/AKT/mTOR pathway have been implicated in many forms of cancer (7, 8). Inhibition of the mTOR signaling pathway with rapamycin has been shown to be an effective means of treating many tumors in vitro and in vivo (9). Problems with the solubility and stability of rapamycin have lead some to pursue analogs of this agent (9). While many of these mTOR inhibitors will soon be available clinically, it remains to be seen whether highly selective inhibitors will have clinical efficacy. Several highly selective molecular inhibitors have had disappointing clinical results in spite of impressive pre-clinical results (10). In addition, some authors have attributed the success of other targeted therapies to their ability to inhibit more than one target (11). Furthermore, selective inhibition of mTOR has been shown to have the paradoxical
effect of activating AKT via the dis-inhibition of upstream mechanisms (12). For these reasons targeted therapy is moving toward utilizing more than one selective inhibitor or inhibitors with more than one target.

PI-103 is an agent that has been shown to inhibit the growth of glioma cells and leukemia cells through its inhibition of both PIP3 and mTOR (12). The goals of this study were to determine whether the PI3K/mTOR pathway was active in human chordoma, and also to discern whether inhibition of PI3K/mTOR by PI-103 could affect proliferation and apoptosis in a chordoma cell line.

Materials and Methods

Human chordoma tissue. Thirteen surgically treated patients diagnosed with chordoma were identified and utilized for the study under an IRB approved protocol. Tissue specimens obtained for protein analysis were maintained at –70˚C by the tissue procurement service. All diagnoses were confirmed by histological review of Hematoxylin and Eosin stained sections. The histological diagnosis of chordoma is based on sheets and cords of cells in a myxoid stroma. The cells often have a vacuolated, so-called physiliferous, appearance. Specimens with chondroid differentiation were excluded.

Cell line. UCH-1 cell line is an established chordoma cell line generously supplied by Dr. Bruderlein of Ulm, Germany (13). The cell line was cultured in RPMI-1740 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin and 100 μg/mL of streptomycin (All purchased from Invitrogen, Carlsbad, CA, USA). The cells were grown on collagen-coated (Collagen Type I Rat Tail, BD Biosciences, Bedford, MA, USA) flasks, and incubated at 37˚C with 5% CO2.

PI-103 (3-(4-(4-morpholinyl)pyrido[3',2':4,5]furo[3,2-d]pyrimidin-2-yl)phenol) was purchased from EMD chemicals, Inc. (Gibbstown, NJ, USA) and dissolved in DMSO.

Chordoma tissue Western blotting. Western blotting was carried out on 13 chordoma samples. Whole cell lysate of the tumor was prepared by grinding 1 g of snap frozen tumor tissue, using a PowerGen 700 Homogenizer (Omni International, Marietta, GA, USA). The ground tissue was resuspended in Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing a cocktail of protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA), sodium fluoride, sodium orthovanadate and phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using the Bio-Rad protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis and immunoblotting was performed on the protein extracts using the standard protocol, using 50 μg of protein per sample. Antibodies tested on the immunoblots included: mouse anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-phospho-AKT (Th 308), anti-AKT, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-PDK1(Ser241), anti-PDK1, anti-phospho-S6 ribosomal protein (Ser235/236) and anti-S6 ribosomal protein (Cell Signaling Technology, Inc., Danvers, MA, USA). The secondary antibodies used included Donkey-anti-mouse secondary (Santa Cruz) and anti-rabbit (Calbiochem, La Jolla, CA, USA). Following hybridization with the secondary antibody, the blots...
were incubated with Immun-Star horseradish peroxidase luminal/enhancer (Bio-Rad) and exposed onto Kodak Biomax MR Film (Eastman Kodak Company, Rochester, NY, USA).

UCH-1 cell line Western blot. The rabbit polyclonal antibodies to mTOR, p-mTOR, AKT, p-AKT and p-Stat3 were purchased from Cell Signaling Technologies (Cambridge, MA, USA). The mouse monoclonal antibody to actin was from Sigma-Aldrich. The Brachyury rabbit antibody was from Santa Cruz Biotechnology Inc. Goat anti-rabbit-HRP and goat anti-mouse-HRP were purchased from Bio-Rad. SuperSignal® West Pico Chemiluminescent Substrate was purchased from PIERCE (Rockford, IL, USA). Total UCH-1 cell lysates were prepared, and Western blot analysis was performed as previously described (14). Briefly, the UCH-1 cells were lysed in 1X RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA, USA) and protein concentration was determined by the DC Protein Assay (Bio-Rad). Twenty-five micrograms of total protein were resolved on NuPage™ 4-12% Bis-Tris Gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween-20 with gentle agitation overnight at 4˚C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were incubated in TBS (pH 7.4) with 5% nonfat milk (Bio-Rad) and 0.1% Tween-20, at a 1:2000 dilution for one hour at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSingal® West Pico Chemiluminescent Substrate.

Cytotoxicity assay. PI-103 and chemotherapy drug cytotoxicity was assessed in vitro via CellTiter 96® AQueous One Solution Cell Cytotoxicity Assay (Promega, Madison, WI, USA). Briefly, 2x10^3 cells per well were plated in 96-well plates in culture medium (RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/ streptomycin) containing increasing concentrations of PI-103 chemotherapy drug such as cisplatin or doxorubicin. After 6 days of culture, 20 μL of CellTiter 96®AQeuous One Solution Cell reagent were added to each well and the plates were incubated for 4 h at 37˚C. The resulting absorbance at a wavelength of 490 nm (A490) was read on a SPECTRAMax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Dose-response curves were fitted with use of GraphPad PRISM® 4 software (GraphPad Software, San Diego, CA, USA).

Apoptosis assay. Quantification of apoptosis in UCH-1 was also evaluated using the M30-Apoptose ELISA assay kit, as per manufacturer's instructions (Peviva AB, Bromma, Sweden) (15). UCH-1 cells were seeded at 8,000 cells/per well in a 96-well plate for 24 hours before treatment with 1 μM PI-103, 1 μM doxorubicin, 10 μM cisplatin or a combination of either of the cytotoxics and PI-103 for an additional 24 hours. The cells were then lysed by adding 10 μL 10% NP-40 per well, and the manufacturer’s instructions for the apoptosis assay were then followed.

Results

PI3K/AKT/mTOR pathway activation in human chordoma. Western blot analysis of 13 samples obtained from surgically resected human chordomas demonstrated positive staining for key components of the PI3K/AKT/mTOR signaling cascade (Figure 1). Both phosphorylated and total PDK, AKT, mTOR and S6 were expressed in the majority of specimens. Figure 1 displays the results of 13 chordoma samples with one human KIT sample serving as a control.

AKT and mTOR in human chordoma cell line inhibition by PI-103. Western blot analysis of human chordoma cell line UCH-1 demonstrated that PI-103 inhibited the phosphorylation of both AKT and mTOR in a dose-dependent manner (Figure 2). In addition, the anti-apoptosis protein Bcl-XL was also inhibited by PI-103. Stat 3 was not inhibited by PI-103 at the doses given. Brachyury is used as a surrogate marker for chordomas and it was positive in the UCH-1 cell line.

PI-103 inhibits proliferation of the UCH-1 human chordoma cell line. UCH-1 proliferation was diminished after exposure to PI-103 (Figure 3). Cell proliferation was determined by CellTiter (96 AQeuous) One Solution Proliferation Assay. The absorbance was read at 490 nm using a SPECTRAMax Microplate Spectrophotometer reader.

PI-103-induced apoptosis in UCH-1. PI-103 induced apoptosis in the UCH-1 (Figure 4). After exposure to 5 μM PI-103, over 50% of the UCH-1 cells underwent apoptosis. The level of apoptosis continued to increase with 10 μM PI-103.

PI-103-induced apoptosis with doxorubicin and cisplatin. To further confirm the role of AKT/mTOR in chordoma cells, the effect PI-103 had on apoptosis in cells treated with doxorubicin and cisplatin was evaluated. The addition of PI-103 to UCH-1 cells exposed to either doxorubicin or cisplatin resulted in at
least additive apoptosis. Cytotoxicity assay demonstrated that PI-103 had at least an additive effect on cisplatin- and doxorubicin-induced cell death (Figure 5).

Discussion

The data support the PI-103 targeting of the PI3K/AKT/mTOR signaling pathway in chordoma. Key downstream proteins within the PI3K/AKT/mTOR pathway were shown to be phosphorylated in the human samples tested including PDK, AKT, mTOR and S6. In addition, it was shown that inhibiting key aspects of the PI3K/AKT/mTOR signaling pathway, specifically PI3K and mTOR, with PI-103 inhibited the proliferation of a human-derived chordoma cell line (UCH-1). Furthermore, PI-103 induced apoptosis in UCH-1. The induction of apoptosis was increased with the addition of both doxorubicin and cisplatin.

Inhibition of both the PI3K and mTOR pathways has been shown to be important in human glioma cells with regard to cell growth where selective inhibition of the PI3K moiety did not affect a change in proliferation (12). Fan et al. tested multiple agents with the ability to selectively inhibit class IA PI3Ks including PI10α, PI10β and PI10δ. None of the selective PI3K inhibitors were able to slow proliferation. PI-103 was shown to inhibit both PI10α and mTOR, and it was the only compound in their study capable of decreasing proliferation and increasing apoptosis. The mTOR protein is known to be activated by growth factor stimulation via class 1A PI3Ks. Another means by which mTOR activation occurs is via nutrient stimulation by class 3 PI3Ks (16, 17). Therefore, inhibition of only the class 1A PI3Ks do not prevent mTOR activation via the nutrient pathway.

Furthermore, inhibition of the mTOR pathway alone has been shown to lead to PI3K activation (18). Therefore, inhibition of mTOR alone may paradoxically lead to the activation of mTOR via the disinhibition of PI3K. For this reason, it is important to consider inhibition of mTOR and PI3K when targeting the PI3KAKT/mTOR pathway. The compound PI-103 effectively limits proliferation of a human chordoma cell line likely through its dual inhibitory effect on the PI10α PI3K and mTOR.

PI-103 induced apoptosis in the UCH-1 cell line at least in an additive fashion with doxorubicin and cisplatin. Neither doxorubicin nor cisplatin have been shown to be effective in human chordomas. The effect of PI-103/doxorubicin and PI-103/cisplatin in human chordoma deserves further study.

In conclusion, the PI3K/AKT/mTOR pathway is active within human chordomas based on Western blot analysis of 13 surgical specimens. PI-103 decreased proliferation and induced apoptosis in the UCH-1 human chordoma cell line. The apoptotic effects of PI-103 were augmented by doxorubicin and cisplatin. PI-103 deserves further study as a systemic therapy in human chordoma.

References


<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Doxorubicin 1 μM</th>
<th>Cisplatin 10 μM</th>
<th>PI-103 1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 5. PI-103 induces apoptosis in a synergistic manner with doxorubicin and cisplatin. UCH-1 Human chordoma cell lines were exposed to doxorubicin 1 μM, cisplatin 10 μM and PI-103 1 μM as single agents. In addition, UCH-1 was exposed to combinations of doxorubicin/PI-103 as well as cisplatin/PI-103. The combination caused a synergistic increase in the level of apoptosis.