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Targeting the Apoptotic Pathway in Chondrosarcoma Using Recombinant Human Apo2L/TRAIL (Dulanermin), a Dual Proapoptotic Receptor (DR4/DR5) Agonist

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Abstract

Recombinant human Apo2L/TRAIL (dulanermin) is based on the ligand for death receptors (DR4 and DR5), which promotes apoptosis. We report a patient with refractory chondrosarcoma who showed a prolonged response to dulanermin and explore mechanisms of response and resistance. This heavily pretreated patient had progressive metastatic chondrosarcoma to the lung. On dulanermin (8 mg/kg i.v. on days 1–5 in a 21-day cycle), the patient achieved a sustained partial response with only subcentimeter nodules remaining. After 62 months of dulanermin treatment, progressive disease in the lungs was noted, and the patient underwent a resection that confirmed chondrosarcoma. DR4 was detected (immunohistochemistry) in the patient's tumor, which may have enabled the response. However, upregulation of prosurvival proteins, namely, phosphorylated (p)-NF- κ Bp65 (Ser 536), p-STAT3 (Tyr 705), p-ERK 1/2 (Thr 202/Tyr 204), p-mTOR (Ser 2448), FASN, and Bcl-2, were also detected, which may have provided the underlying mechanisms for acquired dulanermin resistance. The patient was restarted on dulanermin and has continued on this treatment for an additional 16 months since surgery (78 months since initiation of treatment), with his most recent computed tomography (CT) scans showing no evidence of disease. *Mol Cancer Ther*; 11(11); 2541–6. ©2012 AACR.

Introduction

Chondrosarcomas are the second most common primary bone tumors in adults, with histology ranging from low-grade to very high-grade tumors (1–3). Complete surgical resection remains the mainstay of treatment with a role for radiation in tumors with positive margins. This tumor is notorious for resistance to conventional types of chemotherapy that are effective against other bone sarcomas, and clinical outcomes have not changed during the past 30 years (2).

Apoptosis (type I programmed cell death) is a means by which excessive and unnecessary cells are eradicated by multicellular organisms. This mechanism could augment innate immunity against cancer. However, the process is aberrant in some tumor cells, and deregula-

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doi: 10.1158/1535-7163.MCT-12-0358

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tion of apoptosis is a key hallmark of cancer (4–6). Apoptosis can occur either through the intrinsic pathway (moderated by the Bcl-2 protein family) or the extrinsic pathway (controlled by cell surface proapoptotic death receptors). Advances in targeted drug development have led to the development of proapoptotic receptor agonists (PARA), which include the recombinant human protein apoptosis ligand 2/TNF-related apoptosis-inducing ligand (rhuApo2L/TRAIL or dulanermin); agonistic monoclonal antibodies directed against DR4, as well as the ligand-based molecule dulanermin trigger apoptosis via its cognate receptors DR4 and/or DR5 (4–6).

Binding of dulanermin to DR4 and DR5 has been shown to trigger programmed cell death by activating a highly conserved signaling cascade in various cancer cell lines (4–7). This preclinically significant activity has led to the experimental clinical development of several PARAs (4). These new proapoptotic agents may hold great promise in overcoming key resistance pathways, especially when combined with other targeted or chemotherapeutic agents (4). Herein, we report substantial and sustained tumor regression in response to dulanermin in a patient with refractory chondrosarcoma and explore the mechanisms of response and resistance.

Materials and Methods

We reviewed the medical record of a patient with chondrosarcoma who was seen in the Phase I Clinical

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Trials Program at the University of Texas MD Anderson Cancer Center (Houston, TX).

Patient selection, treatment, and clinical assessments

Treatment on investigational trials, data collection, and morphoproteomic analysis were conducted in accordance with the guidelines of the University of Texas MD Anderson Cancer Center Institutional Review Board and with the patient's consent. After initiation of an investigational therapy, the patient was evaluated clinically at approximately 3- to 4-week intervals. Tumor response was determined using response evaluation criteria in solid tumors (RECIST) by computed tomography (CT) scans or PET/ CT scans obtained about every 6 to 8 weeks. A section of recurrent tumor was available for analysis.

Immunohistochemical and morphoproteomic analysis

Immunohistochemical (IHC) probes were used to detect the following phosphorylated (p) antigens, as published previously (8-10): p-mTOR (Ser 2448); p-Akt (Ser 473); (p)-NF-κBp65-(Ser 536) and p-extracellular signalregulated kinase (ERK) 1/2 (Thr 202/Tyr204; Cell Signaling Technology); and TRAIL R1(DR4), p-STAT3 (Tyr 705; Santa Cruz Biotechnology]. Chromogenic signals were evaluated by brightfield microscopy and semiquantified with regard to percentage of cells stained (0%-100%) and the staining intensity (0: nonstaining, 1+: weak staining, 2+: moderate staining, and 3+: strong staining; refs. 8–10). Subcellular compartmentalizations were assessed as plasmalemmal, cytoplasmic, and/or nuclear. Concurrently run positive and negative IHC controls reacted appropriately. The methods have been published previously (10) and were conducted in a laboratory that is certified under the Clinical Laboratory Improvement Amendments of 1988 ("CLIA") as qualified to conduct high-complexity clinical testing.

Mutation analysis

Mutation analysis for *IDH1* and *IDH2* genes were conducted by PCR-based DNA primer extension analysis in the CLIA–certified Molecular Diagnostic Laboratory in the Division of Pathology and Laboratory Medicine at MD Anderson Cancer Center. The analysis was limited to codon 132 of the *IDH1* gene and codon 172 of the *IDH2* gene.

Results

Patient presentation and treatment

A Caucasian man (age: 58 years in 2005) developed left elbow lesions that were resected in the 1960s and were diagnosed as "synovial chondromas." In 1990, he developed a local recurrence and was referred to our institution where a repeat resection showed pathologic confirmation of synovial chondroma. In 1993, he again developed a localized recurrence and underwent arthrodesis of the elbow; pathology review revealed grade 1 chondrosarcoma in the synovium. After 2 years, an above-the-elbow amputation of the left upper extremity was conducted following malignant transformation in a site that would not permit limb-salvage surgery. The pathology review revealed sarcomatous transformation of chondromatosis. In 2000, the patient had a left axillary recurrence, underwent wide local excision of tumor with pathology showing metastatic chondrosarcoma. Radiotherapy (60 Gy) was given for microscopic residual disease. Chest X-ray and ultrasound follow-up identified recurrence in the left chest wall close to the left scapular tip that was recognized by needle biopsy as recurrent chondrosarcoma. In 2003, a chest CT revealed bilateral pulmonary metastases that were treated with 6 cycles of irinotecan, after which progression led to discontinuation of this agent. In 2004, the patient underwent a wide local excision of metastatic chondrosarcoma on the anterior left chest wall. Pathologic review of tissue at MD Anderson Cancer Center confirmed the diagnosis.

In 2005, progressive disease (Fig. 1A) and the absence of effective treatments led to the patient's referral to the Phase I Clinic at MD Anderson Cancer Center. The patient enrolled in the clinical study, "Phase I dose-escalation study of dulanermin, recombinant human Apo2L/TRAIL, a dual PARA, in patients with advanced cancer (11)." rhuApo2L/TRAIL (dulanermin; ref. 6) is the ligand for the death receptors DR4 and DR5, which upon ligation promote apoptotic cell death.

At the time of study initiation, he had multiple large lung nodules as well as a 4 cm axillary node. Dulanermin treatment was given as 8 mg/kg IV on days 1 through 5 in a 21-day cycle (11) with cycle 1, day 1 commencing in August 2005. The patient achieved a sustained partial response by RECIST, with only residual subcentimeter lung nodules remaining, which were not 2[18F]fluoro-2deoxy-D-glucose (FDG) avid on positron emission tomography (PET) scan (Fig. 1B; ref. 11). Moreover, he has tolerated the investigational therapy without significant side effects and maintained a performance status of 100%.

After 62 months (~5 years) of treatment, the patient was noted to have progressive lung disease (Fig. 1C and E) and underwent a resection that confirmed chondrosarcoma. He was restarted on dulanermin at the same dose and has continued on this treatment for an additional 16 months (78 months since initiation of treatment; Fig. 1D and F). At his restaging in October 2011, CT scans showed no evidence of disease (Fig. 1D and F).

Immunohistochemistry and morphoproteomic analysis on resistant tumor tissue

We conducted morphoproteomic analysis of the patient's resistant tumor (resected following disease progression after 65 months of dulanermin therapy) to elucidate mechanisms of response and resistance (9). Quantification by morphoproteomics allows for the following with respect to protein analytes in tumor and companionate cells: (i) their IHC detection and an assessment of their relative expression levels in the tumor cells *vis-a'-vis* the cells in the microenvironment of the tumor; (ii) their



Figure 1. CT scan of the chest of the patient with chondrosarcoma showing lung metastasis of chondrosarcoma. A, before Apo2L/TRAIL therapy. B, 3 years after Apo2L/TRAIL. The patient achieved a sustained partial response and ultimately a complete response by RECIST. C and E, image showing resistant tumor that emerged during Apo2L/TRAIL therapy before surgical resection. D and F, image showing scan after 75 months after Apo2L/TRAIL therapy showing a complete response

subcellular compartmentalization (nuclear vs. cytoplasmic vs. plasmalemmal), which has implications in signal transduction; and (iii) an assessment of their state of activation, including phosphorylation on putative sites of activation as determined by phosphospecific probes, compartmental translocation, and functional grouping with correlative expression of upstream transducers and downstream effectors in the signal transduction pathways (8, 9).

Bcl-2 is expressed in the resistant tissue

Hematoxylin and eosin-stained tumor sections revealed necrosis in more than 50% of the patient's residual tumor (Fig. 2A and C). Considered against the background of comparable digital images of the antiapoptotic protein Bcl-2 expressed in such regions, the relative overexpression of Bcl-2 in tumor cells that seem to be viable as compared with necrotic cells is noteworthy (Fig. 2B and D). The Bcl-2 family of antiapoptotic proteins has been implicated in resistance to Apo2L/TRAIL-mediated apoptosis (12–14), as has been inactivation of the proapoptotic Bcl-2 protein family member Bax (15). Therefore, strong Bcl-2 expression in residual, viable tumor cells was possibly a mechanism for resistance to dulanermin of this patient's tumor. As such, it may provide a target for further therapeutic intervention. DR4 is expressed in the plasmalemmal and cytoplasmic compartments of viable

tumor cells (Fig. 2K; *vide infra*). DR4 is a therapeutic target for dulanermin (11), and viable tumor cells undergo apoptosis unless antiapoptotic factors, such as Bcl-2, counteract the efficacy of the targeted treatment (Fig. 2B and C). Bcl-2, the antiapoptotic protein, plays a key role in chemotherapy resistance in chondrosarcomas and that this mechanism is a late event in central chondrosarcoma has been described (16–18).

Constitutive activation of prosurvival pathways in resistant tumor tissue

Prosurvival protein analytes that might also have contributed to resistance to dulanermin-mediated apoptosis in this patient's viable, residual chondrosarcoma include constitutively activated NF-κB and STAT3 pathways (19), the ERK pathway, the mTOR pathway (specifically, mTOR complex 2 signaling), and fatty acid synthase (FASN)-mediated signaling. The expression in viable tumor cells can be characterized as nuclear translocation of phosphorylated (p)-NF-ĸBp65-(Ser 536) and p-STAT3-(Tyr 705), of p-ERK-¹/₂-(Thr 202/Tyr 204), of p-mTOR-(Ser 2448) with nuclear translocation (mTORC2; ref. 20), and of cytoplasmic FASN (Fig. 2). IHC analysis of p-NFκBp65(Ser 536), p-STAT3(Tyr 705), p-ERK-1/2(Thr 202/ Tyr204), and p-mTOR (Ser 2448) revealed nuclear translocation of each of these analytes in viable tumor, consistent with their constitutive activation (Fig. 2E-H).

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Figure 2. IHC/morphoproteomic analysis of resistant tumor that recurred and was resected after 65 months of dulanermin therapy. A and C, largely viable and largely necrotic portions of tumor, respectively (hematoxylin and eosin; original magnification, ×600). B and D, corresponding expressions of antiapoptotoic, Bcl-2 protein in the largely viable and largely necrotic tumor, respectively (original magnification, ×600). Note relatively strong intensity in the viable tumor cells. Arrows in B indicate pale nuclear compartment devoid of Bcl-2. The Bcl-2 family of antiapoptotic proteins has been implicated in resistance to TRAIL-mediated apoptosis, as has inactivation of the Bcl-2 counteracting protein Bax. Therefore, strong Bcl-2 expression in residual, viable tumor cells was likely a mechanism for resistance to Apo2L/TRAIL of this patient's tumor. Phosphospecific IHC probes for the detection of activation sites of p-NF-xBp65 (Ser 536), p-STAT3 (Tyr 705), p-ERK 1/2 (Thr 202/Tyr204), and p-mTOR (Ser 2448) reveal nuclear translocation of each of these analytes in viable tumor, consistent with their constitutive activation (E–H, respectively). Cytoplasmic FASN expression is noted in viable appearing chondrosarcoma cells (I). The overnight negative control is provided as a reference for comparison (J). Original magnifications × 400 for E–H and J, and ×600 for I. IHC probes applied to viable tumor for the detection of TRAIL-DR4, PPAR-γ show strong chromogenic signal for TRAIL-DR4 on the plasmalemmal aspect of some tumor cells (K, arrows); nuclear signal for PPAR-γ (L); original magnifications, ×600 for K and ×400 for L.

Cytoplasmic FASN expression is seen in viable appearing chondrosarcoma cells (Fig. 2I) and negative control (without primary antibody; Fig. 2J). In addition to DR4 expression on the plasmalemmal and cytoplasmic compartments, as previously noted, peroxisome proliferatoractivated receptor (PPAR)- γ is expressed in the nuclei of the viable tumor cells in this patient's tumor (Fig. 2K and L). In preclinical studies, PPAR- γ ligands induce apoptosis in renal cell carcinoma cells and in chondrosarcoma by decreasing the levels of antiapoptotic proteins, Bcl-2 and Bcl-xL, and upregulating proapoptotic Bax (21–23). A review of morphoproteomic findings showed that the patient's tumor was relatively indolent, as shown by the mitotic index in the tissue of zero (0) mitotic figures per 10 (10) high-power fields. The mitotic index was calculated from the number of mitotic figures per 10 high-power fields (the field diameter of the high-power field is 0.5 mm and the area is 0.196 mm²).

Mutation analysis for IDH1 and IDH2

Recently, *IDH1* and *IDH2* mutations have been reported in central chondrosarcomas (24). We conducted *IDH1* mutation analysis by PCR-based DNA primer extension analysis and no mutation was detected in codon 132 of the *IDH1* gene. Similarly, there was no mutation of codon 172 of the *IDH2* gene. It would be interesting to

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assess *IDH1* and *IDH2* mutations in future patients with chondrosarcoma for any clinical and prognostic implications (24).

Discussion

The response of chondrosarcoma to dulanermin is noteworthy because this represents a targeted molecular therapy capable of inducing a near-complete remission in chondrosarcoma-a tumor that is notorious for its resistance to conventional types of chemotherapy (1–3). Our patient has continued treatment for a total of 78 months. Of interest, we resumed treatment with dulanermin in this patient after surgical resection of recurrent tumor noted at 62 months. We chose this strategy as chemoradiation is generally ineffective at eradicating this type of cancer, perhaps because of its low mitotic index. The patient continued on treatment with the drug as he was deriving clinical benefit from the drug. Moreover, he had no side effects and protocol allowed treatment after complete response. Of interest is the fact that the patient continued to respond even after initial progression, albeit with intervening surgery to remove some of his lung nodules. This phenomenon suggests that even patients with progressive disease may have remaining residual responsive clones. Similar results have been shown in colorectal cancer, in which retreatment after initial progression can occasionally reinduce response (25). Also, in breast cancer, continuation of trastuzumab (Herceptin) after progression may also result in superior outcome (26). This patient is now, at 78 months, in complete remission.

On the basis of clinical experience and biologic experiments, it seems that multiple pathways can drive cancer cell survival. Better success in cancer treatment will likely be achieved through combination treatment strategies targeted at key moieties in these diverse pathways, especially as few patients with any type of advanced malignancy are cured with single-agent therapy. Analysis of the resistant tumor tissue that emerged in our patient during dulanermin therapy has provided several potential insights into the biology of response and resistance mechanisms that might help inform the design of optimal combinations. We detected DR4 in the patient's tumor. It is conceivable that DR4 expression is important for the cognate ligand dulanermin to be effective. Indeed, epigenetic silencing of DR4 has been shown to contribute to Apo2L/TRAIL resistance (27). Although we were unable to analyze expression of DR5 (which also may mediate dulanermin activity), it is also plausible that deregulation

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of other oncogenic proteins contributes to resistance. For instance, in our patient's resistant tumor tissue assessed after treatment with dulanermin, we detected constitutive activation of prosurvival proteins [phosphorylated (p)-NF-kBp65 (Ser 536), p-STAT3 (Tyr 705), p-ERK 1/2 (Thr 202/Tyr 204)], and p-mTOR (Ser 2448) with nuclear translocation (mTORC2), and correlative expression of cytoplasmic FASN and antiapoptotic, Bcl-2, in the resistant tumor treated with dulanermin. These prosurvival and antiapoptotic signals may provide the underlying mechanisms for dulanermin resistance. Several agents targeting these pathways are now available for use in the clinical setting, and studies combining them with PARAs, such as dulanermin or DR4 and DR5 agonistic antibodies could be worthwhile. Finally, further exploration of PARAs in chondrosarcoma should be considered.

Disclosure of Potential Conflicts of Interest

R. Kurzrock has honoraria from the speakers bureaus of AACR and Amgen. R. Kurzrock also has commercial research grants from Genentech, Amgen, and Hoffman LaRoche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Writing, review, and/or revision of the manuscript: V. Subbiah, R.E. Brown, J. Trent, A. Ashkenazi, R. Herbst, R. Kurzrock

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Subbiah, J. Trent, R. Kurzrock Study supervision: V. Subbiah, J. Trent, R. Kurzrock

Development of the morphoproteomic concept and application to this study: R.E. Brown

Review and interpretation of the immunohistochemical staining reactions: R.E. Brown

Acknowledgments

Vivek Subbiah thanks The Connie and Jim Walter Fellowship in Sarcoma Research. The authors thank Ms. Joann Aaron for her scientific editing of the manuscript.

Grant Support

The University of Texas MD Anderson Cancer Center is supported in part by the NIH through Cancer Center Support Grant no. CA 016672. The patient studied here was enrolled into a phase I study of dulanermin that was sponsored by Genentech (South San Francisco, CA; Amgen, Thousand Oaks, CA).

Received May 7, 2012; revised July 9, 2012; accepted August 3, 2012; published OnlineFirst August 20, 2012.

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Mol Cancer Ther 2012;11:2541-2546. Published OnlineFirst August 20, 2012.

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