An Oral Formulation of YK-4-279: Preclinical Efficacy and Acquired Resistance Patterns in Ewing Sarcoma

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Abstract

Ewing sarcoma is a transcription factor–mediated pediatric bone tumor caused by a chromosomal translocation of the EWSR1 gene and one of several genes in the ETS family of transcription factors, typically FLI1 or ERG. Full activity of the resulting oncogenic fusion protein occurs only after binding RNA helicase A (RHA), and novel biologically targeted small molecules designed to interfere with that interaction have shown early promise in the preclinical setting. Herein, we demonstrate marked preclinical antineoplastic activity of an orally bioavailable formulation of YK-4-279 and identify mechanisms of acquired chemotherapy resistance that may be exploited to induce collateral sensitivity. Daily enteral administration of YK-4-279 led to significant delay in Ewing sarcoma tumor growth within a murine model. In advance of anticipated early-phase human clinical trials, we investigated both de novo and acquired mechanism(s) by which Ewing sarcoma cells evade YK-4-279–mediated cell death. Drug-resistant clones, formed by chronic in vitro exposure to steadily increased levels of YK-4-279, overexpressed c-Kit, cyclin D1, pStat3(Y705), and PKC isoforms. Interestingly, cross-resistance to imatinib and enzastaurin (selective inhibitors of c-Kit and PKCβ, respectively), was observed and the use of YK-4-279 with enzastaurin in vitro led to marked drug synergy, suggesting a potential role for combination therapies in the future. By advancing an oral formulation of YK-4-279 and identifying prominent mechanisms of resistance, this preclinical research takes us one step closer to a shared goal of curing adolescents and young adults afflicted by Ewing sarcoma.

Introduction

Ewing sarcoma family of tumors, which encompass traditional Ewing sarcoma of bone (1), primitive neuroectodermal tumors (PNET; refs. 2), and Askin tumor of chest wall (3–5), have for more than a two decades been considered to represent clinical variants of the same molecularly distinct sarcoma subtype that bears a pathognomonic EWS–ETS oncogenic fusion protein (6). Heretofore, simply referred to as Ewing sarcoma, this sarcoma subtype is a highly aggressive malignancy of adolescents and young adults (7) that is rapidly fatal without effective multimodality treatment that includes surgery and/or radiation and extensive use of systemic chemotherapy. Although polychemotherapy has led to marked improvement in 5-year survival for patients diagnosed with localized Ewing sarcoma—approaching 80% with current generation clinical trials—those with metastatic disease or rapid tumor recurrence fare poorly and often quickly succumb to their disease (8–11).

Although cytotoxic chemotherapy will remain the mainstay for treating Ewing sarcoma in the near future given its well-trodden track record of antineoplastic activity, high-throughput “-omic” technologies and paired biomarkers are enabling an era of precision medicine that, as has occurred for more common carcinomas such as breast or lung cancer, will increasingly target the unique molecular complexity linked to each patient’s respective tumor. Therapies targeting the insulin-like growth factor 1 receptor (IGFIR), alone or in combination with inhibitors of mammalian target of rapamycin (mTOR), provide just one example of select biologically targeted therapies capable of inducing striking tumor regression among a subset of Ewing sarcoma patients (12, 13).

Although such therapies show tremendous potential for the treatment of Ewing sarcoma, and are likely to be even more effective once tissue-based biomarkers reliably predict a priori who will respond, recent evidence suggests that no single therapy will be universally effective. Rather, antineoplastic effects will rely upon a number of secondary mutation/amplification events or perturbed signaling cascades that maintain an aberrant cancer phenotype. Fortunately, though most nonleukemic malignancies lack a consistent primary oncogenic driver protein, Ewing sarcoma and nearly one third of the approximately 50 sarcoma

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subtypes result from near universal subtype-specific chromosomal translocations that occur at precise locations; this, of course, results in anomalous fusion proteins uniquely present in tumor cells and offers an attractive cancer-specific target that could be a candidate for therapeutic intervention.

For Ewing sarcoma, in particular, the chimeric fusion protein results from a double-strand break at one of two principal locations within N-terminal EWSR1 gene (22q12) and a balanced translocation with one of several ETS genes: most commonly FLI1 on chromosome 11 (85% prevalence; ref. 14), ERG (10%) on chromosome 21 (15), or other distinctly less common C-terminal ETS fusion partners, such as ETV1 (16), E1AF (ETV4; ref. 17), FEV1 (18), or ZSG (19), which jointly represent less than 5% of the cases (20, 21). Interestingly, FUS (an RNA-binding protein) (20, 21) demonstrated recently that EWS–FLI1 inhibited helicase activity of RHA and this inhibition was reversed by S-YK, suggesting a novel role for EWS–FLI1 in RNA processing. Herein, we test an effective enteral (37) formulation of YK-4-279 that could be advanced to human clinical trials if final preclinical examination in large-animal species proves safe and effective. Furthermore, as a prelude to those studies, we evaluate putative mechanisms of resistance (38) acquired by YK-4-279–selected cells lines grown in vitro.

Materials and Methods

Cell lines and antibodies

Ewing sarcoma cell lines (TC32 and TC71) were generously provided from the John Trent's laboratory (University of Miami, Cancer Center, Miami, FL). Cell lines were banked in multiple aliquots on receipt to reduce risk of phenotypic drift. Cell identity was confirmed by testing the occurrence of EWS–FLI1 translocation with RT-PCR, by checking a predominant cellular expression of CD99 biomarker with flow cytometry–based detection, and by validating the short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifiler Kit according to the manufacturer’s instructions (Applied Biosystems). The Ewing sarcoma cell lines-STR profiles were compared twice a year to known ATCC fingerprints (ATCC.org), and to the Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://biomicsformatics.itgclima/; ref. 39). Cell lines were maintained in RPMI-1640 medium (Mediatech) containing 10% (v/v) FBS (Gemini Bio-Products) and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin; Mediatech) in a humidified incubator at 37°C and in 5% CO₂ atmosphere. Antibodies used were: (i) Bcl-2, cyclin D1, phosphorylated PI3K-110 (Ser428/435) and (ii) Bcl-2, cyclin D1, phosphorylated PI3K-110 (Ser428/435) and RHA from Cell Signaling Technology, (ii) RHA and c-Kit from Abcam, and FLI1 from Santa Cruz Biotechnology.

Preparation of drugs stock solutions

For in vivo application, the primary stock solutions of YK-4-279 (5 mg/mL) were prepared in different intraperitoneal (i.p.; low hydrophilic vehicle: 10% ethanol, 40% PEG400, and 50% PBS) hydrophilic solvents. Regarding in vitro utilization, the primary stock solutions of YK-4-279 were prepared in DMSO and stored at −20°C. Gleevec drug was generously provided from the John Trent’s laboratory (University of Miami, Cancer Center) and prepared in DMSO (10 mmol/L) and stored at −20°C. Enzastaurin was purchased from Selleckchem, prepared in DMSO (10 mmol/L), and stored at −20°C.

Pharmacokinetic analysis

The pharmacokinetic study was performed using CF-1 mice. Thirty mice per group were assigned to receive either a single i.p. injection of YK-4-279 at a target dose of 45 mg/kg, or a single gavage administration (37) of small molecule at the target dose
of 90 mg/kg. Plasma was obtained from the mice and samples were processed by liquid extraction followed by analysis using liquid chromatography with mass spectrometry (LC/MS). Average plasma concentrations of YK-4-279 were measured at intervals from 0 to 12 hours after dosing.

**Ewing sarcoma acquired resistant clones generation**

TC32 and TC71 Ewing sarcoma YK-4-279 acquired resistant clones were obtained from the corresponding parental cell lines by maintaining them within an escalating small-molecule concentration (up to 3 μmol/L) for 7 months. All the parental and YK-4-279–resistant cell lines were tested twice a year for *Mycoplasma* contamination using the MycoAlert Detection Kit (Lonzza Group Ltd.) according to the manufacturer’s protocol.

**In vitro cell viability assay**

Cell viability was measured using a colorimetric assay in 96-well plates with 2-(4-isodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent (Roche). The sensitive and acquired YK-4-279–resistant Ewing sarcoma cell lines were seeded at 5,000 cells per well in triplicates with 10% FBS RPMI complete medium. The adherently growing Ewing sarcoma cells were cultivated for 24 hours before adding YK-4-279 in serial diluted concentrations (0.04–10 μmol/L) for up to 72 hours. WST-1 was added and cells were incubated for an additional 2 hours. Cell viability was measured at 450 nm in a microplate reader (DTX880; Beckman Coulter), and cytotoxicity of the YK-4-279 was expressed as percentage cell viability. IC50 values were calculated by sigmoidal dose–response curve fit using Prism GraphPad 6.0.

**Flow cytometry**

Ewing sarcoma cell resistance to YK-4-279 was also indirectly evaluated by enumerating positive cellular staining to Annexin v and propidium iodide (PI) apoptotic biomarkers, and Ki67 proliferative biomarker. After drug treatment, the cells were dissociated with PBS-based cell dissociation buffer (Life Technologies). Cells were stained with Annexin V–FITC and PI (BD Biosciences). Secondary antibodies were used as a starting point for amplification. Slides were probed with 183 validated primary antibodies, and 1,056 sample arrays were created on nitrocellulose-coated FAST slides (Schleicher & Schuell BioScience). Slides were probed with 183 validated primary antibodies, and signals were amplified using a DakoCytomation-catalyzed system (Dako). Secondary antibodies were used as a starting point for amplification. Slides were scanned, analyzed, and quantified using the ImageJ Gel Analysis tool (NIH, Bethesda, MD).

**RPPA and bioinformatics analysis**

RPPA analyses of either resistant and sensitive xenograft TC71 tumor samples to YK-4-279 or control vehicle-treated tumors were performed simultaneously using the same array. Lysates were normalized to 1 μg/μL and boiled in a solution containing sodium dodecyl sulfate (10%) and β-mercaptoethanol (10%). Supernatants were manually diluted in 5-fold serial dilution with lysis buffer. Using a 2470 Arrayer (Aushon Biosystems), 1,056 sample arrays were created on nitrocellulose-coated FAST slides (Schleicher & Schuell BioScience). Slides were probed with 183 validated primary antibodies, and signals were amplified using a DakoCytomation-catalyzed system (Dako). Secondary antibodies were used as a starting point for amplification. Slides were scanned, analyzed, and quantified using the MicroVigene software program (Vigenetech). This program provides automated spot identification, background correction, and individual spot-intensity determination (expressed in logarithmic units). RPPA experiments were performed as described previously (40). The resulting data were normalized for possible unequal protein loading, taking into account signal intensity for each sample for all antibodies tested. Log2 values were media-centered by protein to account for variability in signal intensity by time and using the formula log2 signal – log2 median. The data were analyzed using GeneSpring GX software program (version 12.1-GX; Agilent Technologies) by performing one-way ANOVA followed by the Tukey test to compare each pair of group in order to borrow
strength from more samples to estimate the between-group variance with minimum 2-fold change. We found 21 proteins significantly associated with difference between pair groups by hierarchically clustering the data with Euclidean correlation, Pearson centroid linkage, and Benjamini–Hochberg false discovery rate correction at 0.2.

Immunoprecipitation

Parental sensitive and acquired YK-4-279–resistant Ewing sarcoma cell lines were grown to 70% confluency and treated overnight with variable concentration of YK-4-279 (0–30 μmol/L). Nuclear lysate was collected using the Active Motif Magnetic Co-IP Kit (Active Motif). Protein quantification was determined using BCA protein assay kit (Thermo Fisher Scientific), and 500 μg lysate was bound to 1 μg of FLI1 antibody overnight at 4°C on a rotating axis with addition of same concentration of YK-4-279 as used for overnight treatment. Magnetic beads (Active Motif) were added to the lysates and tumbled for 2 hours at 4°C. Immunoprecipitated FLI1 proteins were resolved using 10% PAGE and transferred to PVDF membranes to analyze the proteins expression of FLI1 and RHA as described in "Western blotting".

Evaluation of oral YK-4-279 against Ewing sarcoma TC71 tumor xenografts

Male nonobese diabetic (NOD)-SCID-IL-2Rγnull mice (The Jackson Laboratory) were used to generate subcutaneous (10⁶ cells injected/animal) TC71 xenografts either with parental sensitive TC71 cells or in vitro acquired YK-4-279–resistant TC71#4A cell line. All mice were maintained under barrier conditions, and experiments were conducted using protocols and conditions approved by the University of Texas MD Anderson Cancer Center (MDACC; Houston, TX) Institutional Animal Care and Use Committee [eACUF Protocols (003-11-02531 and #03-11-02532)] and Institutional Biosafety Committee (eBC#HA0411-346-1). Mice bearing subcutaneous tumors were randomized into treatment and control groups, and received a weekly single/multiple i.p. or five times p.o. of YK-4-279 or vehicle control when their tumors reached a diameter of 6 mm. YK-4-279 was administered to mice in groups of five or more (50 mg/kg per dose). Tumor diameter was measured at the initiation of the study and two or three times a week for up to 30 days afterward. Tumor volumes were calculated using the formula (π/6) × D × d², in which D is the largest diameter and d is the smallest diameter.

Isobologram analysis

An IC₅₀ isobologram analysis was performed using in vitro cell-based assays to examine the dose-dependent interaction between YK-4-279 and enzastaurin drug combinations on Ewing sarcoma cell line sensitivity. Sensitive (parental TC71) and YK-4-279–resistant Ewing sarcoma (TC71#3) cell lines were seeded as described earlier and exposed to increasing concentrations of YK-4-279 (0–10 μmol/L) and a range of concentrations (0–20 μmol/L) of enzastaurin for 72 hours to determine the concentration of both agents necessary to achieve 50% inhibition (IC₅₀). Synergy between YK-4-279 and enzastaurin was determined using CalcuSyn software 2.1 (Biosoft), which analyzes the data from WST1 proliferation assays to determine the interaction between equipotent drug combinations as compared with the concentration of single drug. CalcuSyn express this interaction as combination index (CI) with values <1, =1, and >1 indicating synergy, additivity, and antagonism, respectively.

Biostatistical analyses

The biostatistical analyses in this study were performed using an unpaired t test with Gaussian distribution and followed with Welch correction. Differences were considered significant for P < 0.05.

Results

YK-4-279 pharmacokinetics

Purified YK-4-279 was synthesized, purified, and formulated as an oral solution to achieve a supratherapeutic serum drug concentration sufficient to kill Ewing sarcoma cells in vitro (IC₅₀ ≈1 μmol/L). Subsequently, the pharmacokinetic (41) profiles of i.p. (YKIP: 45 mg/kg) and enteral (YKPO: 90 mg/kg) formulations were determined within a murine preclinical model (Fig. 1). Maximal YK-4-279 concentrations (Cₘ₅ₐₓ) of 90 and 10 μmol/L, respectively, occurred 30 minutes after i.p. or p.o. dosing (Fig. 1). As expected, compared with i.p. dosing, oral gavage led to more subdued spike in YK-4-279 serum concentration and higher terminal half-life (Fig. 1). The pharmacokinetic parameters, shown in the Table 1, indicate statistically significant differences between i.p. and p.o., as measured by the area under the curve (AUC), drug clearance (CL), terminal half-life, terminal-phase volume of distribution (Vₜ), and volume of distribution of steady state (Vₘ). In addition, using three main pharmacokinetic variables (AUC, Cₘ₅ₐₓ and the time to reach maximum concentration), YKPO achieved an oral bioavailability of 61% to 73% and peak serum concentration...
YKPO and YKIP are effective in Ewing sarcoma xenografts

The antineoplastic activity of YKPO was compared with YKIP, in NOD-SCID-IL-2R null mice bearing heterotopic Ewing sarcoma tumors using a weight-adjusted daily dosing strategy (5 days on/2 days off), with short gaps in dosing during the weekends purely for practical limitations in personnel during that time rather than safety concerns. Briefly, six cohorts of at least 5 mice each were implanted subcutaneously (s.c.) with 1 × 10⁶ TC71 cells and monitored every other week until tumors measured 6 mm in diameter, at which time they were randomized to receive either placebo (i.p. or p.o. route) or YK-4-279 (i.p. or p.o. route), 1.2 mg/30 g body weight, for 1 month. As the route of placebo administration has no effect upon tumor growth, the five i.p. and five p.o. placebo-treated mice were merged into a single group of 10 mice for the subsequent statistical analyses.

Growth curves from individual xenografts and averaged tumor growth curves (Fig. 2A and B, respectively) indicated that daily i.p. dosing of YK-4-279 was required to achieve tumor regression, as once or three times weekly dosing was no better than the placebo control group. Tumor growth was persistent regardless of the route of YK-4-279 administration. To decipher the mechanism(s) by which YK-4-279 exposure led to induced drug resistance, two in vitro methods were used to generate resistance among TC32 and TC71 Ewing sarcoma cell lines grown in monolayer culture. The first method exposed parental cell lines to steadily increased drug concentration for 7 months, whereas the second exposed Ewing sarcoma cells to exceedingly high 3 μmol/L concentrations of YK-4-279 for a brief 72 hours period prior to selecting and expanding viable clones.

Both methods proved successful in establishing Ewing sarcoma cells resistant to YK-4-279, as selected YK-4-279-resistant cell lines (TC32-R and TC71-R) were eventually able to proliferate at a normal rate in the presence of YK-4-279 concentrations 20- to 50-fold higher than what had killed their more sensitive parental counterparts (Fig. 3A). In addition to conventional cell proliferation assays, acquired YK-4-279 resistance was confirmed by flow cytometry–based measures of apoptosis and proliferation, using Annexin and Ki67 immunostains, respectively (Fig. 3B). Furthermore, a colony formation assay was performed as a second in vitro measure of resistance; while the sensitive parental cells showed complete inhibition in higher concentrations of YK-4-279, significantly more

| Table 1. Pharmacokinetic parameters of YK-4-279 following i.p. and p.o. administration |
|-----------------------------------------------|-----------|-----------|
| Pharmacokinetic parameters | i.p. (n = 30) | p.o. (n = 30) |
| Dose, mg/kg | 45 | 90 |
| AUC, ng h/mL | 14,766 | 15,432 |
| CL, mL/h/kg | 3,047 | 16,566 |
| Terminal elimination rate, 1z (k)/h | 0.48 | 0.39 |
| Half-life | 1.43 | 1.75 |
| Vd, mL/kg | 2,025 | 35,077 |
| Vss, mL/kg | 2,003 | 34,932 |

Abbreviations: AUC, area under the plasma concentration–time curve; CL, clearance; Vd, volume of distribution; Vss, volume of distribution at steady state.

Tissue distribution of YK-4-279 provided either YKPO (90 mg/kg) or YKIP (45 mg/kg)

Upon completion of the previously described study, major organ systems were collected at necropsy to assess whether the enhanced antineoplastic activity was, in part, secondary to the delayed clearance and longer in vivo half-life of YKPO, which was hypothesized to result in greater tissue distribution than YKIP (Fig. 2D). Not unexpectedly, YKPO was associated with greater variability in plasma and organ concentrations than YKIP, and is likely attributable to minor variations in bioavailability. Because YKPO resulted in considerable tumor regression and necrosis, there was an insufficient tumor volume in all but one case to accurately assess intratumoral concentrations of YK-4-279. However, the YK-4-279 concentration achieved in the plasma and organs approximates the level required to induce half-maximal in vitro growth inhibition. Short-term experiments are planned to assess the intratumoral concentration of YK-4-279 before tumor regression occurs. Although YKPO led to consistently higher tissue levels than YKIP in this study, modulation of the i.p. dose (increasing dosing frequency or concentration) could theoretically be optimized to reach an equivalent or superior tissue concentration. As our principal goal was to determine an effective formulation of YK-4-279 feasible for the clinical setting, no attempts were made to achieve equipotent serum YK-4-279 concentrations using alternative i.p. dosing regimens. As single- and multiple-dose administration of YK-4-279 lacked meaningful toxicity (data not shown), more frequent dosing could be used to achieve an even higher tissue/plasma level sufficient to induce tumor regression in Ewing sarcoma tumors that have, or acquire, YK-4-279 resistance.

Generation and characterization of YK-4-279–resistant Ewing sarcoma cells

A near universal problem inherent in any biologically targeted therapy used to eradicate Ewing sarcoma—and many other cancers—is the emergence of primary or secondary drug resistance that enables tumor progression. Although the strong antineoplastic effect of YKPO persisted throughout the 1-month preclinical study duration, small residual tumors remained regardless of the route of YK-4-279 administration. To decipher the mechanism(s) by which YK-4-279 exposure led to induced drug resistance, two in vitro methods were used to generate resistance among TC32 and TC71 Ewing sarcoma cell lines grown in monolayer culture. The first method exposed parental cell lines to steadily increased drug concentration for 7 months, whereas the second exposed Ewing sarcoma cells to exceedingly high 3 μmol/L concentrations of YK-4-279 for a brief 72 hours period prior to selecting and expanding viable clones.

Both methods proved successful in establishing Ewing sarcoma cells resistant to YK-4-279, as selected YK-4-279-resistant cell lines (TC32-R and TC71-R) were eventually able to proliferate at a normal rate in the presence to YK-4-279 concentrations 20- to 50-fold higher than what had killed their more sensitive parental counterparts (Fig. 3A). In addition to conventional cell proliferation assays, acquired YK-4-279 resistance was confirmed by flow cytometry–based measures of apoptosis and proliferation, using Annexin and Ki67 immunostains, respectively (Fig. 3B). Furthermore, a colony formation assay was performed as a second in vitro measure of resistance; while the sensitive parental cells showed complete inhibition in higher concentrations of YK-4-279, significantly more
YK-4-279–resistant clones survived (Fig. 3C). Interestingly, the YK-4-279–resistant clones demonstrated an enhanced proliferative rate over their respective parental cell lines when cultured in drug-free media, suggesting they had adopted other phenotypic changes as well (Fig. 3C).

YK-4-279 mechanisms of resistance

YK-4-279–resistant TC71 Ewing sarcoma clone #3 was selected for the remaining experimental investigations rather than TC71#4A because the latter clone formed cell aggregates in monolayer culture that, theoretically, could have prevented uniform penetration of drugs and/or siRNAs into the innermost cells. Clone #3 was interrogated by immunoprecipitation to determine whether prolonged exposure to YK-4-279 affected RHA regulation and contributed to drug resistance. Sensitive and resistant cell lines were treated with YK-4-279 for 14 hours before immunoprecipitating FLI1 from the nuclear protein lysates followed by an immunoblot analysis for RHA complexes (Fig. 4A). In contrast to sensitive cell lines, which showed an expected loss of co-immunoprecipitated RHA following YK-4-279 treatment, resistant Ewing sarcoma

Table 2. Tumor growth delay and statistical analyses between the YK-4-279–treated and the control untreated groups of mice

<table>
<thead>
<tr>
<th>Growth delay (day)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-vehicle: i.p. + p.o. (n = 10)</td>
<td>NA</td>
</tr>
<tr>
<td>YK_{i.p.}: 1.2 mg ×1/W (n = 5)</td>
<td>3</td>
</tr>
<tr>
<td>YK_{i.p.}: 1.2 mg ×3/W (n = 5)</td>
<td>22</td>
</tr>
<tr>
<td>YK_{i.p.}: 1.2 mg ×5/W (n = 7)</td>
<td>&gt;34</td>
</tr>
</tbody>
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Figure 2.

Oral YK-4-279 dosing is effective in TC71 xenograft animal model of Ewing sarcoma. Individual tumor volumes (A) and grouped (averaged and smoothed) tumor volumes (B) are graphed for mice treated i.p. or p.o. either once, three, or five times per week: black (control pooled i.p.-p.o.-Vehicle), red (1.2 mg i.p. once per week); blue (1.2 mg i.p. three times per week); green (1.2 mg i.p. five times per week), and brown (1.2 mg p.o. five times per week). C, the Kaplan-Meier curves showing the survival percentage of i.p. or p.o. treated groups of mice with YK-4-279 and the control i.p./p.o. pooled untreated mice. D, tissue distribution of YK-4-279 in mice treated with i.p. or p.o. dosing (50 mg/kg/dose). Drug concentrations were averaged from at least 3 mice per condition.
maintained FLI1-RHA–binding affinity (Fig. 4B). DHX9 (which codes for RHA) was upregulated among YK-4-279–resistant Ewing sarcoma as assessed by microarray analyses (Supplementary Materials and Methods; GSE61042), suggesting that at least part of the acquired resistance could be explained by altered transcriptional machinery (Supplementary Fig. S1).
However, as nuclear, cytoplasmic, and cell lysate RHA levels were identical between both sensitive and YK-4-279–resistant cell lines (Fig. 4A and Supplementary Fig. S2), other resistance mechanisms are also clearly at play.

To assess whether the YK-4-279–resistant clones would maintain these phenotypic traits of YK-4-279 resistance and enhanced proliferation, two additional groups of mice were implanted with the YK-4-279–resistant TC71 #4A clone and treated with YKP, or YKPO. Tumor growth of the YK-4-279–resistant clone was compared with tumor growth from the sensitive parental TC71 cells that were treated five times a week either with vehicle or YK-4-279 drug (previously shown in Fig. 2). Despite daily i.p. or p.o. treatment with YK-4-279, the resistant clone (TC71 #4A) exhibited substantially faster tumor growth than the parental TC71 cell line and exhibited no sensitivity to YK-4-279 (Fig. 5A).

Proteomic analysis of resistant and sensitive TC71 xenograft implants was conducted using well-characterized reverse-phase protein lysate arrays that are enriched for 183 proteins and phospho-proteins commonly implicated in cancer (Fig. 5B). Analysis included three xenograft cohorts: (i) resistant Ewing sarcoma clone exhibited significant cross-resistance to imatinib as compared with the parental cell line (indicated by nonoverlapping of IC50 confidence intervals; Supplementary Fig. S3).

### Cotargeting of RHA and PKC-β is synergistic in vitro

In addition to YK-4-279–related upregulation of c-Kit, PKC isoforms were overexpressed in YK-4-279–treated xenografts and YK-4-279–resistant clones: PKC-pan-betaII-pS660 and PKC-delta-pS664 in xenografts (Fig. 5B) and PKC-α to a greater extent by both YK-4-279–resistant Ewing sarcoma clones (Supplementary Fig. S4) and YK-4-279–resistant xenografts (Fig 5C and D). These findings were especially intriguing in light of recent findings by Surdez and colleagues (42) that the EWS–FLI1 fusion can directly bind to the PRKCB promoter in vitro and upregulate PKC-β expression. Remarkably, this same group was able to demonstrate meaningful tumor regression in xenografts by single gene PRKCB knockdown. Through indirect mechanisms, the Ewing sarcoma fusion protein has also been shown to affect PKC-α, which operates downstream of CAV1, a protein of known importance for Ewing sarcoma oncogenesis (43) and drug sensitivity (44). Similar to previous experiments for c-Kit described above, transient siRNA knockdown of PKC-α (data not shown) partially reversed acquired resistance to YK-4-279. Because the α and β isoforms can independently phosphorylate histone H3, it is likely that classical PKC (α, β, and γ) antagonists, such as the enzastaurin, anti-PKC-β, will be required to fully reverse PKC-mediated resistance in our model.

To answer whether PKC upregulation would, in fact, lead to enzastaurin resistance, we conducted an in vitro proliferation assay. The YK-4-279–resistant Ewing sarcoma clone displayed significant cross-resistance to enzastaurin as compared with the parental cell line after 72 hours of treatment (Fig. 6A). To determine whether YK-4-279 and enzastaurin were synergistic, or simply additive in effect, an isobologram analysis was performed. As shown in Fig. 6B, dashed lines indicate the additive isoboles for the YK-4-279–resistant clone (red) and sensitive (blue) TC71 cell line at their respective IC50 values. As expected, the additive isobole of the resistant clone is shifted rightward compared with its drug-sensitive counterpart. Testing 49 individual concentrations of YK-4-279 (0–10 μmol/L) with enzastaurin (0–20μmol/L), with four different drug ratios (Fig. 6C...
and D), the CI was 0.6 in parental TC71 cells and 0.8 in the YK-4-279-resistant TC71#3 clone, indicative of drug synergy, particularly in the sensitive Ewing sarcoma cells that had not yet acquired collateral resistance to YK-4-279 and enzastaurin. Although it remains to be proven in vivo, these data suggest that the YK-4-279–enzastaurin combination should be provided...
together to maximize cell killing rather than waiting to add enzastaurin only after Ewing sarcoma cells acquired resistance to YK-4-279.

Discussion

Ewing sarcoma is the second most common pediatric bone malignancy and too often afflicts adolescents and young adults in the prime of their lives. Although traditional cytotoxic chemotherapies are extensively used for the treatment of Ewing sarcoma, given their substantial clinical activity, they nevertheless fail to cure most patients who suffer from recurrent or metastatic disease. To improve current therapies and limit their side effects, designer drugs that uniquely target specific genetic abnormalities present only in cancer cells (i.e., EWS–FLI1 or its variants) represent an optimal approach that could provide patients with the precision therapies needed to combat their respective cancers. Although the aberrant fusion proteins, such as EWS–FLI1, are particularly attractive targets given ubiquitous presence in Ewing sarcoma, they have remained stubbornly resistant to therapeutic intervention until recently with the development of YK-4-279.
YK-4-279 is the first precision-guided drug candidate to show preclinical activity in Ewing sarcoma, which was designed to adhere to FLI1 at a putative RHA-binding site and, in turn, downregulate transcriptional activity of the EWS–FLI1 fusion protein. Interestingly, because YK-4-279 has been reported to inhibit ERG and ETV1 (an ETS fusion partner less commonly present in Ewing sarcoma) in translocation-positive prostate cancer cell lines, one would predict that YK-4-279 would also be effective against the minority of Ewing sarcoma tumors bearing non-FLI1 associated translocations. This hypothesis remains to be tested but, if true, would suggest nearly all Ewing sarcoma patients could benefit from YK-4-279 without the need to pre-screen their respective tumors for the specific ETS fusion type. This provides a distinct advantage over less common therapeutic targets or ones with complex expression patterns and implies that even unselected Ewing sarcoma patients could benefit.

At the time of this writing, it remains to be determined whether the presence or absence of an ETS translocation is necessary for YK-4-279 to be effective or just overexpression of an ETS transcription factor. Experimental evidence by Riggi and colleagues in which the EWS–FLI1 was transfected in primary human MSC indicates that high expression of FLI1 or EWS is insufficient to induce malignant transformation, and ample evidence suggests that the EWS–FLI1 fusion is a more potent transcriptional activator than FLI1 by itself (25). Taken together, YK-4-279 may be more effective in ETS translocation-positive tumors. However, two facts support the counterargument: (i) the DNA-binding affinity of EWS–FLI1 and FLI1 are nearly identical, and (ii) high FLI1 expression confers a poor prognosis in translocation-negative leukemia patients (37, 45, 46). Clearly, if ETS dysregulation is sufficient to incite or maintain tumorigenicity, YK-4-279 may antagonize a much broader range of tumor types.

The EWSR1 gene is well known for its promiscuous binding to other ETS and non-ETS genes alike in a handful of other malignancies such as desmoplastic small round cell tumor (DSRCT), clear cell sarcoma, and extraskeletal myxoid chondrosarcoma that each exhibit widely divergent and often unique clinical presentations. As such, most of those cancer phenotypes must be ascribed to the dysregulated C-terminal side of the fusion protein. Given the apparent selectivity of YK-4-279 for several of the ETS proteins, no attempts were made to assess its activity in other EWSR1-related sarcomas. However, successful development of YK-4-279 beyond a preclinical proof-of-concept stage could provide a roadmap for developing other YK-4-279-like drugs customized to interfere with C-terminal partners of EWS in these other translocation-positive malignancies.

From a practical standpoint, a number of hurdles must be surpassed before YK-4-279 can be transitioned into the clinical setting, and our research has focused on the most pressing one—identification of an optimal formulation appropriate for eventual human testing. As we have confirmed in this study, others have reported an effective i.p. formulation used within a murine model but that route of administration obviously cannot be used in the clinical setting. Preclinical pharmacokinetic experiments were conducted using a wide range of diluents to identify several that enabled high YK-4-279 solubility (data not shown). Subsequently, each was formulated with YK-4-279 and the most promising one was assessed in vivo Ewing sarcoma xenograft animal model for its oral bioavailability. The peak serum concentration (C_{max}) of YKPO was considerably less than YKIP, as expected, but still 10-fold higher than what was required to kill Ewing sarcoma in vitro. As YK-4-279's affinity for in vivo plasma proteins remains to be determined, the amount of unbound YK-4-279 may be lower than expected. Therefore, additional experiments will be required to directly compare in vitro versus in vivo free drug levels.

The development of YK-4-279 is still in its infancy and it is too early to predict whether high peak serum concentrations or prolonged lower drug exposures are required to achieve maximum antineoplastic activity. Yet, our data provide a compelling argument to suggest that the latter is necessary. Although both YKPO and YKIP delayed Ewing sarcoma growth in vivo, tumor regression only occurred in mice treated with the YKPO formulation. The 17-fold higher V_{d}, longer serum half-life, and 3-fold higher tissue distribution associated with YKPO almost certainly contributed to that promising clinical endpoint (Table 1 and Fig. 2D). A direct assessment of the intratumoral concentration of YK-4-279 was attempted, but unsuccessful, because the YKPO-treated tumors were too small and necrotic to accurately measure. Importantly, though the Ewing sarcoma cell lines used for the in vivo experiments do not readily metastasize to the lung, which is the most common cause of Ewing sarcoma-induced patient mortality, enteral administration achieved intrapulmonary YK-4-279 concentrations above the level required to kill Ewing sarcoma cell lines.

It should be noted that a racemic mixture of YK-4-279, rather than a purified S-YK enantiomer, was used in our experiments. Although the S-enantiomer has been previously demonstrated to be considerably more potent than the R-enantiomer or the racemic mixture (34, 35, 47), a recent report by Hong and colleagues (34, 36) demonstrates little difference in antineoplastic and anti-helicase activities between the S-enantiomer and racemic mixture. In fact, only racemic YK-4-279 was capable of improving survival in their Ewing sarcoma xenograft model (34), an unexpected finding the authors ascribed to altered absorption or enhanced elimination of the S-enantiomer in the presence of the R-enantiomer. In arriving at a mature formulation ready for human clinical use, the decision to use the racemic S-enantiomer of YK-4-279 will have profound implications on the dose and route of drug administration, and potentially even affect drug toxicity or chemoresistance patterns.

Although no evidence of primary or acquired YK-4-279 resistance occurred within our preclinical murine model (albeit during a relatively short period of monitoring), we hypothesized that Ewing sarcoma tumors would eventually acquire drug resistance if exposed to YK-4-279 for a prolonged time period, as occurs for virtually any drug used to treat all but the most sensitive cancer types. Moreover, because YK-4-279 is directed at a specific protein target, one could hypothesize a number to putative de novo and acquired MOR. To identify such MOR, drug-resistant Ewing sarcoma cell lines were established from parental (i.e., sensitive) cells that were exposed to escalating concentrations of YK-4-279. Independently, drug-resistant clones were selected after exposing Ewing sarcoma cell lines to short, but that route of administration obviously cannot be used in the clinical setting.
YK-4-279 is unable to prevent RHA from binding to FLI1 in vitro by altering the molecular machinery required for Ewing sarcoma survival and growth. Intriguingly, co-immunoprecipitation assays suggest that YK-4-279 is unable to prevent RHA from binding to FLI1 in YK-4-279–resistant Ewing sarcoma cell lines (Fig. 4), and this remains an active area of future investigation. Possible explanations include mutations in the FLI1 or DHX9 gene (which encodes RHA), altered protein isoforms of FLI1 or RHA, enhanced migration of RHA into the cell nucleus, or changes to the EWS–FLI1 regulatory complex. In addition, though 72-hour exposure to YK-4-279 reduced DHX9 expression, prolonged exposure had an opposite effect—suggesting another possible MOR (Supplementary Fig. S1). Finally, even if the primary MOR relates directly to changes in FLI1 or RHA form, secondary factors almost certainly contribute to drug resistance.

To explore that hypothesis, protein lysates and extracted RNA from sensitive and YK-4-279–resistant Ewing sarcoma tumors were analyzed using a previously characterized high-throughput proteomic and genomic analyses. To determine key proteins and/or pathways responsible for YK-4-279 resistance (Fig. 5 and Supplementary Figs. S1 and S4), we assessed sensitive and resistant Ewing sarcoma cell lines by RPPA, and then validated the results using Western blotting and microarray gene expression analysis. Unsupervised double hierarchical clustering of the expression data perfectly separated the YK-4-279–resistant tumors from both the sensitive tumors and ones taken from mice treated with a placebo, which clustered together. To identify novel drug combinations likely to prevent or reverse acquired YK-4-279 resistance, the most highly upregulated proteins (c-Kit, Stat3-pY507, cyclin D1, and PKC isoforms) were chosen for additional scrutiny. A number of important proapoptotic proteins (Bim, Bax, Bid, and Bak) were downregulated in YK-4-279–resistant cell lines (Supplementary Fig. S4), suggesting that the apoptotic machinery was significantly altered during acquired YK-4-279 resistance.

Frequently observed in drug-resistant cancer cells, canonical activation of Stat3 occurs by constitutive phosphorylation at tyrosine p-705, which promotes its dimerization, nuclear translocation, and activation of other cancer genes (48). Therefore, we hypothesized that p-Y705 STAT3 upregulation in YK-4-279–resistant tumors may serve as biomarker predictive of YK-4-279 resistance. This remains, however, to be verified because nonphosphorylated STAT3 can also activate NF-kB subunits to induce oncogenes (49). Other sites of STAT3 phosphorylation, such as P-S727, have been shown to be important for chronic lymphocytic leukemia, but were not included within the 183 proteins validated for RPPA analysis (50). As STAT3 can promote transcription of cyclin D1 (51), their coexpression in YK-4-279–resistant Ewing sarcoma tumors represents a plausible MOR to be explored in future studies.

The finding that c-Kit and PKC upregulation is associated with YK-4-279 MOR is especially interesting because these proteins are both highly expressed in Ewing sarcoma and easily agonized by small molecules either already FDA-approved (imatinib) or in early-phase clinical trials (PKC inhibitors). Further studies are required to better understand why collateral resistance to imatinib and enzastaurin occurred in YK-4-279–resistant cell lines. Consistent with prior reports in human lung cancer and leukemia, one could postulate that p53 amplification or inactivation of alternative survival pathways (Bim, Bid, Bak, and Bax), as illustrated in Supplementary Fig. S4, protected the Ewing sarcoma cells from apoptosis (52, 53).

In addition to changes in Stat3 and c-Kit, Notch1 levels decreased, whereas Snail and PKC expression increased in YK-4-279–resistant cell lines, an effect we initially suspected would occur in the setting of YK-4-279–induced downregulation of caveolin (CAV1) as others have reported following YK-4-279 exposure (34). However, we saw no evidence that CAV1 was suppressed in cell lines or murine tumors exposed to YK-4-279, as assessed by Western blot analysis (data not shown). In addition, even if YK-4-279 had suppressed EWS–FLI1 transcriptional activity, and thereby CAV1, it would not have explained the marked upregulation of PKC observed in our YK-4-279–resistant xenograft tumors (Fig. 5) and cell lines (Supplementary Fig. S4). PKC-α has been reported to induce chemotherapy resistance to drugs commonly used in the treatment of Ewing sarcoma (such as doxorubicin; refs. 54–56) but has not yet been proven to induce drug resistance in sarcomas. Although the EWS–FLI1 fusion protein is only circumferentially tied to PKC-α expression, there is compelling evidence that it directly regulates PKC-β. Using chromatin immunoprecipitation (ChIP), others have shown that EWS–FLI1 binds the PRKCβ promoter, and siRNA-mediated knockdown of EWS–FLI1 leads to steep reductions in PKC-β transcription and protein expression (42). Although PKC-β targeting has been shown to induce Ewing sarcoma tumor regression, this study is the first to implicate PKC-β in acquired resistance to EWS–FLI1 targeted drugs such as YK-4-279. As one considers how this information could be used therapeutically, it would be tantalizing to use PKC-β inhibitors with YK-4-279, assuming the latter drug elicits single agent antineoplastic effects.

In summary, our data suggest a pivotal role for YK-4-279 in the treatment of Ewing sarcoma in the preclinical setting and provides a path to early-phase human clinical trials by identifying an oral formulation. Additional information such as drug toxicity data will certainly be required, and one should carefully test whether more frequent dosing regimens (twice daily, for example) would be even more effective than the approach presented herein. Many scientific questions remain to be answered: (i) should the racemic mixture or S-enantiomer be used prospectively, (ii) are other proteins (missing from the limited number present in our RPPA panel) even more important in inducing drug resistance, and (iii) can biologically targeted drug candidates, such as YK-4-279, be used safely with cytotoxic or biologic-targeted therapies currently used for the treatment of Ewing sarcoma or other types of sarcoma.

Disclosure of Potential Conflicts of Interest
R.A. Aiyer and J.E. O’Dorisio have ownership interest in a patent filed for the oral formulation used in the article—MDACC has shared intellectual property with Star Biotechnology, LLC relating to the oral formulation of YK-4-279. No potential conflicts of interest were disclosed by the other authors.

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