Open-Label, Multicenter, Phase I/II, First-in-Human Trial of TK216: A First-Generation EWS::FLI1 Fusion Protein Antagonist in Ewing Sarcoma

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ABSTRACT

PURPOSE Ewing Sarcoma (ES), a rare cancer with a pathognomonic translocation resulting in the Ewing sarcoma gene (EWS)::FLI1 oncoprotein, has a poor prognosis in the relapsed/refractory (R/R) setting. Tokalas (TK)216 was designed to bind EWS::FLI1 proteins directly, disrupt protein-protein interactions, and inhibit transcription factor function. TK216 plus vincristine showed synergistic activity in preclinical tumor models. To our knowledge, we report the results of a first-in-class, first-in-human phase I/II trial of TK216 in R/R ES.

PATIENTS AND METHODS TK216 was administered intravenously as a continuous infusion to patients with R/R ES in 11 cohorts. The dosing duration of 7 days was later extended to 10, 14, and 28 days. Vincristine could be added on day 1 after cycle 2, per investigators' choice. The trial used a 3 + 3 design with an expansion cohort at the recommended phase II dose (RP2D).

RESULTS A total of 85 patients with a median age of 27 years (range, 11-77) were enrolled. The maximum tolerated dose for the 14-day infusion of TK216, 200 mg/m^2 once daily, was determined in cohort 9 and selected as the RP2D. The median previous number of systemic therapies regimens was three (range, 1-10). The most frequent-related adverse events in patients treated at the RP2D included neutropenia (44.7%), anemia (29.4%), leukopenia (29.4%), febrile neutropenia (15.3%), thrombocytopenia (11.8%), and infections (17.6%). In cohorts 9 and 10, two patients had a complete response, one had a partial response, and 14 had stable disease; the 6-month progression-free survival was 11.9%. There were no responses among the eight patients in cohort 11.

CONCLUSION TK216 administered as 14-day continuous infusion with or without vincristine was well tolerated and showed limited activity at the RP2D in R/R ES.

ACCOMPANYING CONTENT

Appendix

Data Sharing Statement

Protocol

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INTRODUCTION

Ewing sarcoma (ES) is a rare aggressive bone cancer.[1] Advances in the care of patients with ES have included optimization of multidrug cytotoxic chemotherapy regimens and dose-dense chemotherapy made possible with the introduction of cardioprotective agents and hematologic growth factors.[2-4] While those advances have led to improved 5-year survival rates of approximately 75% for those with localized disease, chemoresistance occurs and <30% of those diagnosed with relapsed/refractory (R/R) or metastatic disease survive.[5-7] Newer investigational approaches are not yet translated into improved survival.[8-10] There remains a significant unmet need for novel agents.

Our understanding of ES has advanced, with the identification of the pathognomonic balanced t(11;22) (q24;q12) translocation, followed by research characterizing how the resulting Ewing sarcoma gene (EWS)::FLI1 fusion protein (FP) and its less common *ETS* variants (eg, ERG, ETV1 and ETV4) drive oncogenesis and tumor growth.[11,12] The FP has epigenetic effects that are elicited by binding GGAA microsatellite repeats at enhancer and super-enhancer sites, indirect repressive effects at transcriptional start sites

CONTEXT

Key Objective

To investigate the possibility of directly targeting the fusion protein (FP) coded by a pathognomonic translocation between *EWSR1* and a member of the *ETS* family genes that drives Ewing Sarcoma.

Knowledge Generated

A novel small molecule that disrupts the binding of the FP to protein partners was identified, including RNA helicase. We performed, to our knowledge, a first-in-human, first-in-class study to investigate the ability of this compound to achieve clinical responses in relapsed/recurrent ES. We observed three extraordinary responses, demonstrating proof of concept.

Relevance *(S. Bhatia)*

This trial provides proof of concept that fusion oncoproteins in ES can be targeted in the setting of acceptable side effects.*

*Relevance section written by *JCO* Associate Editor Smita Bhatia, MD, MPH, FASCO.

mediated by disrupting transcription factor co-occupancy, and alternative pre-mRNA splicing that results in novel protein isoforms.[13-18] The chimeric EWS::FLI1 FP can exhibit neomorphic activity, activating novel ES-specific transcripts at previously silent genomic sites.[19]

The FP plays a predominant role in ES oncogenesis and tumor growth. Infrequent mutations of p53 or STAG2, or deletions in CDKN2A, also occur but have been considered untargetable since they repress gene function.[20-22] The most logical anticancer approach to ES targets the FP. An indirect approach has yielded some success, for example, by using IGF-1R antibodies[23-28] or mTOR inhibitors[29-31] to suppress EWS::FLI1-mediated activation of the IGF/PI3K/mTOR pathway. A more direct approach aimed at the pathognomonic FP has major appeal since FP antagonists could be cancer-specific.

Transcription factors were generally considered untargetable because of significant amino acid sequences that occur as intrinsically disordered regions.[32] The ES FP was reported to be intrinsically disordered on the basis of its hydrophobic and hydrophilic amino acid balance in 2004.[33] No EWS::FLI1 crystal structure exists, precluding rational structure-based drug design. Since ES chromosomal translocations are in-frame, the EWS::FLI1 FP chimera bears normal EWS and FLI1 proteins joined at a fusion boundary that is non-immunogenic or currently targetable.[34] Unable to target the intrinsically disordered EWS or EWS::FLI1 breakpoint, attempts have been made to block fusion protein-deoxyribonucleic acid binding[35] or prevent critical protein partners from binding FLI1.[36]

One such protein partner, RNA helicase A (RHA), is required for the transforming activity of EWS::FLI1.[37] The small molecule YK-4-279 was discovered through a screen of small molecules that bind to EWS::FLI1 and blocks interactions with other proteins, including RHA. Blocking the interaction with RHA leads to ES cell apoptosis and xenograft tumor regression.[38,39] By blocking specific proteins, YK-4-279 alters the splicing profile of ES cells.[40] Tokalas (TK)216 is a small-molecule analog of (S)-YK-4-279 that directly binds to EWS::FLI1 and inhibits its function by blocking its binding to RHA in an enantiospecific fashion (Fig 1).[41] TK216 disrupts additional protein-protein interactions (PPI) essential for the oncogenic function of EWS::FLI1.[41] TK216 showed better potency and efficacy than the original compound in cell-based and in vivo tumor models that led to IND-enabling investigations by Oncternal Therapeutics, Inc.[41] A rat model of ES treated with (S)-YK-4-279 showed rapid elimination of the drug, indicating a requirement for prolonged dosing, such as continuous infusion.[41]

We conducted, to our knowledge, a first-in-class, first-in-human open-label phase I/II trial of TK216 in patients with R/R ES. The study objectives were to define the toxicity of TK216 and to identify the recommended phase II dose (RP2D). The study enrolled additional patients in an expansion cohort to estimate the objective response rate and progression-free survival of patients with ES to TK216. An oral formulation of TK216 was not available, the binding of TK216 is reversible, and the half-life was short in animal models. Therefore, administration by continuous IV infusion was used.

PATIENTS AND METHODS

Eligibility

Eligibility requirements included a confirmed diagnosis of ES in patients with R/R disease. All patients had received conventional cytotoxic chemotherapy for ES, including doxorubicin. Demonstration of an ES-associated translocation was not required. Patients had to have measurable disease by

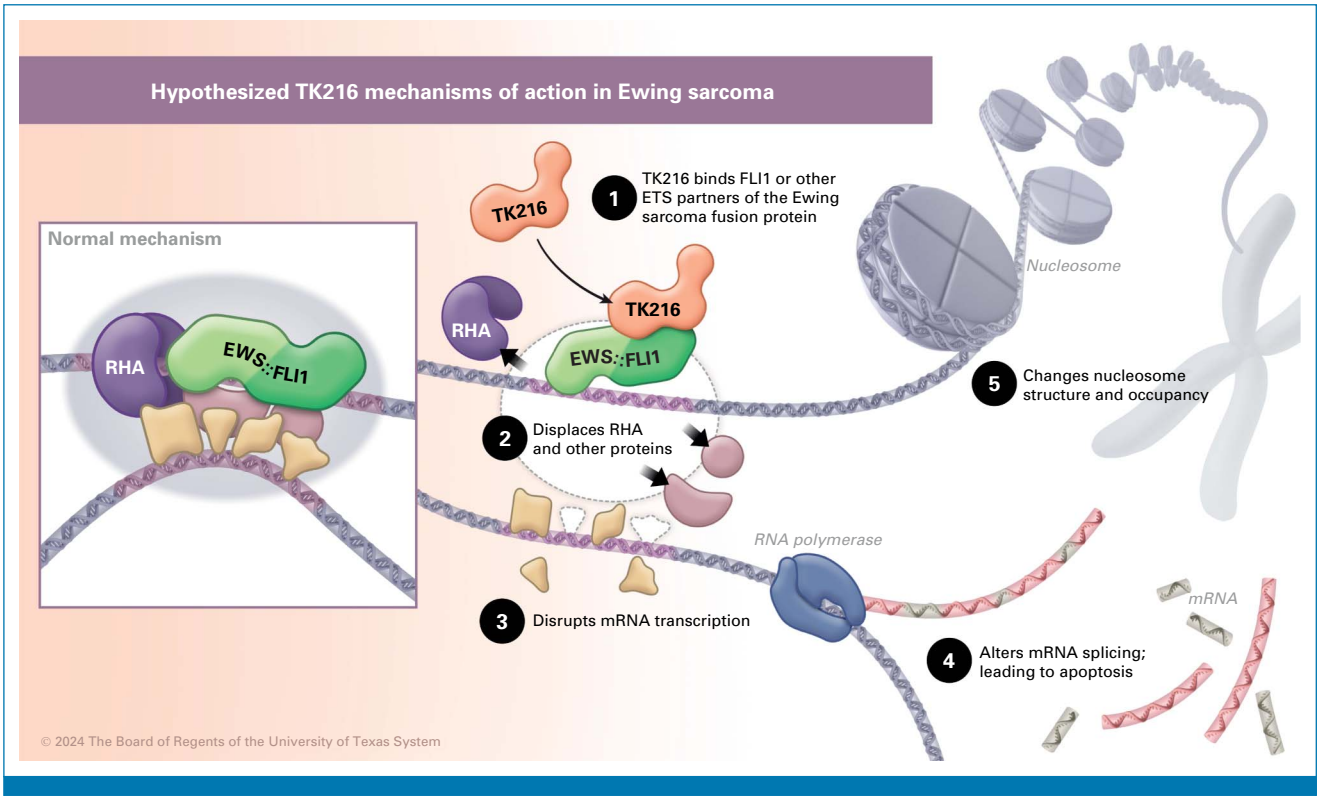

FIG 1. Hypothesized mechanism for TK216 leading to apoptosis. (1) TK216 binds to EWS::FLI1 which is believed to include parts of the C-terminal intrinsically disordered region. (2) RHA and other proteins are disrupted and displaced, so they no longer bind to EWS::FLI1. (3) Transcription of mRNA is altered, putatively because of displaced coregulators (both activators and suppressors). (4) Post-transcriptional splicing of mRNA is altered because of the disruption of EWS::FLI1 from splicing regulators. (5) EWS::FLI1 interaction with chromatin regulators in the BAF complex is disrupted altering the nucleosome structure and promoter occupancy. The specific region of EWS::FLI1 and its biophysical characterization that interacts with other proteins is an area of active research. Data suggest that alteration of a biomolecular condensate accounts for the protein displacements, shown in the figure by a purple cloud in the normal mechanism panel and disrupted by TK216. RHA, RNA Helicase A.

RECIST criteria. Patients had to have recovered from previous chemotherapy and radiation therapy, have adequate organ function, and have no symptomatic brain metastases. Adequate hematologic function (absolute neutrophil count 1,000/mm^3, platelets 100,000/mm^3, and hemoglobin 9 g/dL) was required. Brain imaging was not required if patients were asymptomatic. Pulmonary function testing was not required. The protocol specified that patients should have a life expectancy of 3 months. The minimum age for cohort 1 was 18 years; for cohorts 2-5, the age was 12 years; for cohorts 6-10, the age was 10 years; and for cohort 11, the age was 8 years. The US Food and Drug Administration required that the first patients treated with, to our knowledge, this first-in-human use of an investigational agent be adults; subsequent cohorts were permitted to enroll younger patients. The protocol permitted any number of previous lines of therapy for cohorts 1-10. Previous therapy for cohort 11 was limited to less than five previous lines of therapy. Previous lines of therapy for the patients in the RP2D cohort are shown in the swimmers plot. The study was approved by local institutional review boards according to institutional policy. All participants or their legally authorized guardians provided consent, and children provided assent as appropriate.

Study Design

The starting dose for TK216 was chosen at 10% of the maximum tolerated dose (MTD) in preclinical studies in dogs (18 mg/m^2 once daily x 7 days). The duration of infusion for the first six cohorts was 7 days, followed by 14 days without treatment, also on the basis of preclinical studies. If no DLTs were observed in three patients, the subsequent cohort was administered with a doubling of the dose of TK216. Beginning with cohort 7, the infusion duration was prolonged, first to 10 and then to 14 days. The dose of 200 mg/m^2 once daily of the investigational agent was chosen for cohort 9 to reduce the incidence of neutropenia observed in cohorts 5-8. The initial sample size for the RP2D cohort was calculated using a promising overall response rate (ORR)/standard deviation of 30% and an uninteresting ORR/standard deviation of 10%, with a one-sided α-level of .10 and a power of 80%. After an initial objective response, the cohort size was increased, and when two additional responses were observed, the sample was expanded to delineate tolerability, efficacy, and safety to 44 patients. An additional cohort (cohort 11) tested the tolerability of a 28-day 175 mg/m^2 once daily continuous infusion.

The RP2D regimen allowed for vincristine, a standard therapy for ES, once a patient completed cycle 2 of any scheduled escalation cohort. Vincristine was administered on day 1 of subsequent cycles at the discretion of the treating investigator. The initial dose of vincristine was 0.75 mg/m² once daily given via intravenous infusion before initiating the TK216 infusion. Subsequent doses were 1.5 mg/m² once daily up to a maximum dose of 2 mg once daily.

Dose-Limiting Toxicity

Dose-limiting toxicity (DLT) was defined as possibly, probably, or definitely drug-related grade 3 and above for nonhematologic toxicity or grade 4 and above for hematologic toxicity in the first cycle. The MTD is the dose level at which zero of six or one of six patients experience first-cycle DLT and at least two of three or two of six patients experience first-cycle DLT at the next higher dose level. Not more than one intrapatient dose reduction was allowed. Dose re-escalation was not permitted after dose reductions for drug-related toxicity, even when there was minimal or no toxicity with the reduced dose. Patients experiencing ≥grade 2 neutropenia were offered granulocyte colony stimulating factor after the first cycle.

Evaluation of Safety

Adverse events (AEs) were recorded for patients who received at least one dose of TK216. All patients were followed for a month after stopping treatment. Severity was assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI Common Terminology Criteria for Adverse Events [CTCAE]), version 4.03. Treatment-emergent adverse events (TEAEs) were summarized and defined as any event that began or worsened on or after the date of first dose of study treatment. All AEs were mapped to preferred terms and system organ classes using the Medical Dictionary for Regulatory Activities (MedDRAv23.1). At each level of summarization, if a patient experienced the same AE more than once, then that patient was counted only once for the summary of that AE, using the highest grade. All toxicities, including grades 1-5, were recorded by research nurses after each cycle of therapy.

Temperature, blood pressure, and pulse were measured before each infusion. Hematology, blood chemistry, urinalysis, and physical examinations were monitored regularly.

Evaluation of Efficacy

The evaluation plan required follow-up imaging by computed tomography or magnetic resonance imaging after every two cycles of therapy. Treatment response was evaluated by RECIST v1.1. A response had to last for at least 4 weeks to be considered a partial response (PR) or complete response (CR). Duration of response (DOR) was determined per RECIST criteria. Progression-free survival (PFS) was defined as the time from the start of study treatment to objective disease progression per RECIST v1.1 by the investigator or death from any cause, whichever occurred first. Patients were censored when they withdrew consent, when they started a new antineoplastic therapy, or at the last follow-up visit.

RESULTS

Patient Characteristics and Patient Enrollment

Eighty-five patients were enrolled in this study. Pathologic diagnoses were confirmed at each enrolling center. Patients' demographic and clinical characteristics at study entry are summarized in Table 1. Molecular characterization was not required. However, tissue from eight patients was analyzed by multiplex morphometric fluorescence in situ hybridization. EWS::FLI1 fusion was found in five, EWS::ERG was found in two, and one patient demonstrated a 16p11 FUS rearrangement. Most patients were heavily pretreated, with the median number of previous systemic therapies being three (range, 1-10). Nonsystemic previous therapy for metastatic disease included surgery (77.6%) and radiotherapy (80.0%).

Using 3 + 3 dose escalation, the MTD of a 7-day infusion regimen of TK216 was 220 mg/m² once daily in cohort 6. Because preclinical data suggested that longer exposure resulted in greater antitumor activity, longer infusion times were explored. A 10-day infusion of TK216 at 220 mg/m² once daily in cohort 7 was not tolerated. For both cohort 8 and cohort 9, there were no AE observed in the first two participants. The next two participants were enrolled simultaneously, leading to four patients in each cohort. The RP2D was established in cohort 9 as 200 mg/m² once daily for 14 days as a continuous infusion followed by 14 days off treatment; cohort 10 used the same dose schedule for the phase II expansion cohort. A total of 48 patients (cohorts 9 and 10) received treatment with the RP2D schedule. To explore even longer infusion times, an additional eight patients were enrolled in cohort 11, which administered TK216 at 175 mg/m² once daily as a 28-day continuous infusion.

Safety

Toxicity was graded using CTCAE-v4. DLTs were observed in cohorts 5 and 7 (Table 2). The most frequent TEAEs included neutropenia (44.7%), anemia (29.4%), leukopenia (29.4%), febrile neutropenia (15.3%), thrombocytopenia (11.8%), and infections (17.6%; Appendix Table A1, online only). Most TEAEs were grade 1 or 2 in severity, with neutropenia, leukopenia, and anemia reporting mostly grade ≥3. Cohorts 9 and 10 included 48 patients who received the RP2D of TK216 200 mg/m² once daily for 14 days per 28-day cycle. Of those, 46 patients received TK216 in combination with vincristine and two patients received TK216 alone. All 48 patients

TABLE 1. Patient Demographics

Demographic	All Patients (N = 85)	Cohorts 1-8 (n = 29)	Cohort 9 and Expansion Cohort (RP2D; n = 48)	Cohort 11 (n = 8)
Age, years				
No.	85	29	48	8
Mean (standard deviation)	28.9 (13.1)	28.9 (12.2)	29.2 (14.1)	27.0 (10.0)
Median (Q1, Q3)	27.0 (19.0, 34.0)	27.0 (21.0, 34.0)	27.5 (18.0, 36.0)	27.0 (19.0, 31.5)
Min, max	11.0, 77.0	14.0, 71.0	11.0, 77.0	15.0, 46.0
Male	56/85 (65.9%)	18/29 (62.1%)	32/48 (66.7%)	6/8 (75.0%)

	All Patients (N = 85)	Cohorts 1-8 (n = 29)	Cohort 9 and Expansion Cohort (RP2D; n = 48)	Cohort 11 (n = 8)
Race				
Asian	7/85 (8.2%)	6/29 (20.7%)	1/48 (2.1%)	0/8 (0.0%)
White	69/85 (81.2%)	19/29 (65.5%)	45/48 (93.8%)	5/8 (62.5%)
Black or African American	0/85 (0.0%)	0/29 (0.0%)	0/48 (0.0%)	0/8 (0.0%)
Native Hawaiian or Other Pacific Islander	2/85 (2.4%)	0/29 (0.0%)	1/48 (2.1%)	1/8 (12.5%)
American Indian or Alaska Native	0/85 (0.0%)	0/29 (0.0%)	0/48 (0.0%)	0/8 (0.0%)
Other	7/85 (8.2%)	4/29 (13.8%)	1/48 (2.1%)	2/8 (25.0%)
Missing	0/85 (0.0%)	0/29 (0.0%)	0/48 (0.0%)	0/8 (0.0%)
Ethnicity				
Hispanic or Latino	12/85 (14.1%)	4/29 (13.8%)	7/48 (14.6%)	1/8 (12.5%)
Not Hispanic or Latino	72/85 (84.7%)	25/29 (86.2%)	40/48 (83.3%)	7/8 (87.5%)
Not reported	0/85 (0.0%)	0/29 (0.0%)	0/48 (0.0%)	0/8 (0.0%)
Unknown	1/85 (1.2%)	0/29 (0.0%)	1/48 (2.1%)	0/8 (0.0%)

	All Patients (N = 85)	Cohorts 1-8 (n = 29)	Cohort 9 and Expansion Cohort (RP2D; n = 48)	Cohort 11 (n = 8)
Baseline disease characteristics				
Time from diagnosis to enrollment, years				
No.	85	29	48	8
Mean (standard deviation)	4.6 (4.0)	4.5 (3.4)	4.7 (4.5)	3.7 (2.4)
Median (Q1, Q3)	3.4 (2.0, 5.6)	3.6 (2.0, 5.7)	3.2 (1.9, 5.5)	3.6 (1.5, 5.7)
Min, Max	0.4, 19.9	1.1, 15.7	0.4, 19.9	0.7, 7.0
No. of previous systemic treatment regimens[a]				
No.	85	29	48	8
Mean (standard deviation)	3.6 (2.0)	3.6 (1.9)	3.7 (2.2)	3.3 (1.8)
Median (Q1, Q3)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)	4.0 (2.0, 4.5)
Min, Max	0.0, 10.0	1.0, 9.0	1.0, 10.0	0.0, 5.0

	All Patients (N = 85)	Cohorts 1-8 (n = 29)	Cohort 9 and Expansion Cohort (RP2D; n= 48)	Cohort 11 (n = 8)
No. of previous systemic treatment regimens for recurrent/metastatic disease[a]				
No.	85	29	48	8
Mean (standard deviation)	2.5 (2.0)	2.4 (1.7)	2.6 (2.2)	2.1 (1.6)
Median (Q1, Q3)	2.0 (1.0, 3.0)	2.0 (2.0, 3.0)	2.0 (1.0, 3.5)	2.0 (1.0, 3.5)
Min, Max	0.0, 9.0	0.0, 8.0	0.0, 9.0	0.0, 4.0

Abbreviations: Q1, first quartile; Q3, third quartile; RP2D, recommended phase II dose.
[a]The protocol permitted any number of previous lines of therapy for cohorts 1-10. Previous therapy for cohort 11 was limited to less than five previous lines of therapy.

TABLE 2. Patient Treatment Cohorts

Cohort	Phase	TK216, mg/m² once daily	Infusion Duration days	Patients, No.	DLT, No.	Adverse Events Grade ≥3
1	I—dose escalation	18	7	3	0	
2	I—dose escalation	36	7	3	0	
3	I—dose escalation	72	7	3	0	
4	I—dose escalation	144	7	3	0	
5	I—dose escalation	288	7	7	3[a]	
6	I—dose escalation	220	7	3	0	
7	I—schedule escalation	220	10	3	3[b]	
8	I—schedule escalation	220	14	4	0	
9	I—RP2D reached	200 (RP2D)	14	4	0	3
10	II—RP2D expansion	200 (RP2D)	14	44	NA	
11	II—exploratory	175	28	8	NA	7

Abbreviations: DLT, dose-limiting toxicity; RP2D, recommended phase II dose.
[a]Febrile neutropenia.
[b]Two patients with febrile neutropenia, one patient with thrombocytopenia.

experienced TEAEs as shown in Appendix Table A1. TEAEs in ≥30% of patients, regardless of relationship to TK216, included neutropenia (58.3%), anemia (45.8%), leukopenia (31.3%), fatigue (41.7%), alopecia (39.6%), pyrexia (35.4%), nausea (35.4%), and headache (31.3%). Most of the TEAEs were grade 1 or 2 in severity.

In cohort 11, a total of eight patients received a 175 mg/m² once daily TK216 dose for 28 days per 28-day cycle. All eight patients experienced TEAEs as shown in Appendix Table A1, with TEAEs in ≥25% of patients, regardless of relationship, including neutropenia (75.0%), anemia (25%), thrombocytopenia (25%), alopecia (37.5%), hypoalbuminemia (37.5%), and pain in extremity (37.5%). Most of the TEAEs were grade 1 or 2 in severity, with neutropenia reporting mostly grade ≥3.

Throughout the study, 53 treated patients died, predominantly from disease progression (n = 50). Death attribution for the remaining three patients was unknown; however, no deaths were specifically attributed to TK216 toxicity.

Pharmacokinetics

We evaluated the pharmacokinetics of TK216 in patients treated in cohorts 1-7. For patients treated in cohort 7 at the dose of 220 mg/m² once daily as a continuous infusion, we measured plasma concentration of TK216 after 10 days of continuous infusion. Mean plasma concentrations of TK216 exceeded 1,000 ng/ml before the dose administered on day 10 and remained above that level for 8 hours after the administration of the investigational agent.

Efficacy

Antitumor activity of TK216 alone and when combined with vincristine was measured by ORR, DOR, and PFS and overall survival. The follow-up time was calculated using time to disease progression, death, or censoring. The ORR for all patients was 3.5% (3 of 85 patients). CRs were observed in two patients (2.3%), both in the RP2D group (cohort 9 and expansion cohort 10). A PR was seen in one patient (1.2%) in the RP2D group, stable disease (SD) in 18 patients (21.2%), mainly in the RP2D group, progressive disease (PD) in 54 patients (63.5%), mostly in cohorts 1-8 and cohort 11, with 10 patients (11.8%) with no postbaseline tumor assessment because of rapid clinical progression. The overall disease control rate (DCR defined as CR, PR, or SD) was 24.7%, with a median DOR of 25.5 months (95% CI, 1.1 to 35.2). Among patients with available data, the median duration of stable disease was 2.3 months (95% CI, 1.5 to 3.9).

For dose escalation/expansion cohorts 1-8, the ORR, CR, and PR were 0.0% (Fig 2). SD was observed in four patients (13.8%), and the median duration of SD was 2.5 months (95% CI, 1.1 to 5.8). Patients treated with the RP2D in cohort 9 and the expansion cohort reported ORR in three patients (6.3%), with a CR in two patients (4.1%), a PR in one patient (2.1%), SD in 14 (29.2%), and PD in 25 patients (52.1%). The median DOR was 25.5 months (95% CI, 1.1 to 35.2), with a median duration of SD of 2.3 months (95% CI, 1.4 to 3.9). For patients who received monotherapy with a TK216 dose of 175-200 mg/m² once daily in cohort 11, the ORR, CR, PR, and SD were 0.0% (Fig 3), with PD in all six evaluable patients (75%). The median PFS for cohort 9 and the expansion cohort (RP2D) was estimated to be 1.8 months (95% CI, 1.5 to 2.8; Fig 4). The 6-month PFS for this group of patients is estimated to be 11.9% (95% CI, 4.4 to 23.6).

Three patients treated with TK216 at the RP2D had confirmed extraordinary responses, two with objective CR and one with objective PR. One patient achieved a CR after five relapses of ES. The patient continued treatment with TK216 for 28 months. After discontinuation of TK216, the patient

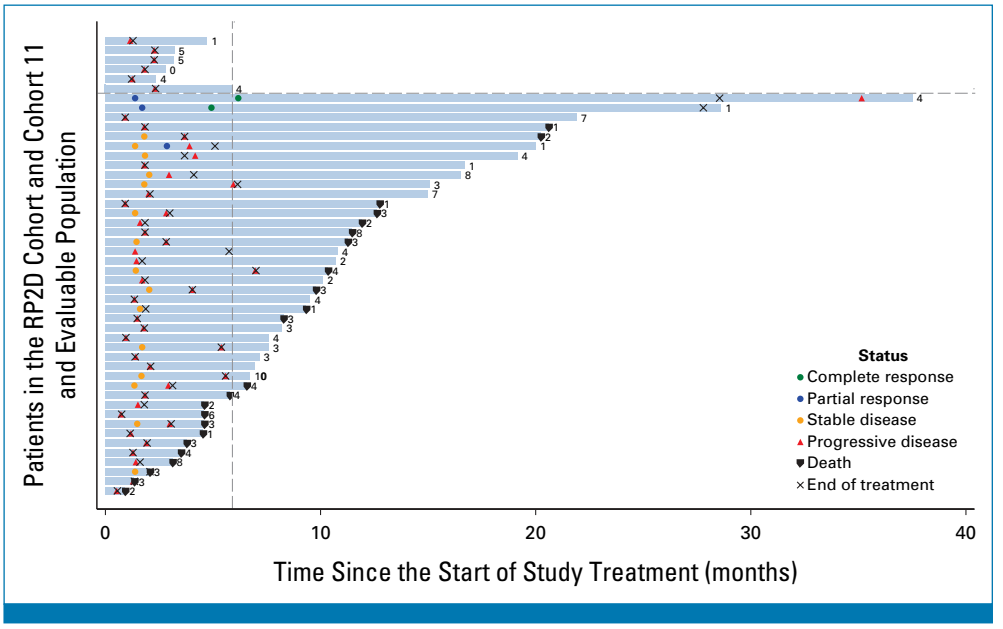

FIG 2. Swimmer's plot. Each bar represents one patient in the study, with months from the start of study treatment on the *x* axis. The time at which stable disease, response, or progressive disease was first documented is indicated with the symbols in the Status box. The number to the right of each bar is the number of previous treatment regimens for Ewing sarcoma before entering this study. Patients in cohort 11 are presented on/above the horizontal dashed line. All lines below the horizontal dashed line are part of the RP2D cohorts. RP2D, recommended phase II dose.

had a new metastatic recurrence. Another patient achieved a CR in first relapse of ES with 10 pulmonary nodules within 1 year of diagnosis. The patient continued TK216 for 46 months and then developed a confirmed relapse in the lungs, liver, and brain while on treatment. Treatment with TK216 was discontinued because of progression. The patient with a PR was enrolled after four relapses of ES. He had a single persistent nodule after the disappearance of all other measurable lesions. That nodule was resected and shown to be viable ES, with the same EWS::FLI1 translocation found at diagnosis. After resection of the residual nodule, the patient resumed TK216 treatment for 29 additional months before electively stopping treatment and remains disease-free for 55+ months from study initiation.

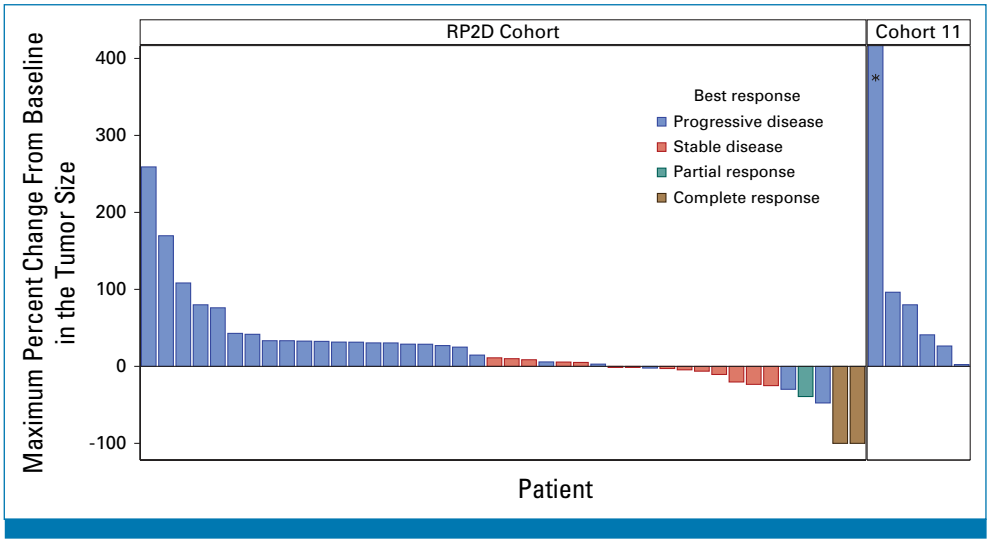

FIG 3. Waterfall plot. Each bar represents one patient in the study, with maximum percent change in tumor size from baseline on the *y* axis, sorted from largest positive to most negative change from baseline. The color of the bars indicates the best objective tumor response. RP2D, recommended phase II dose.

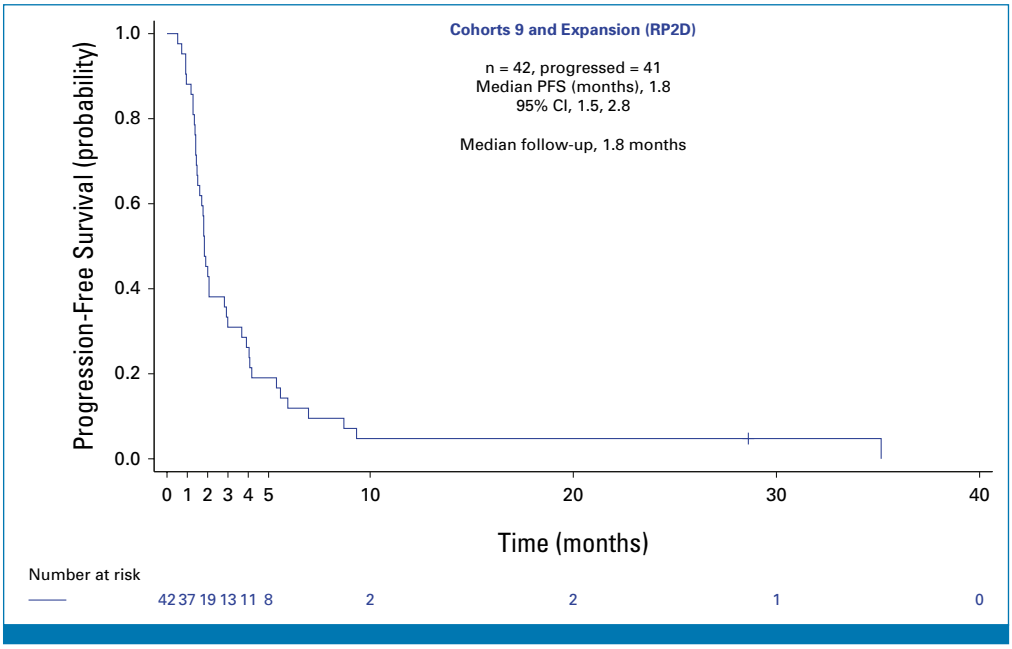

FIG 4. PFS. Kaplan-Meier curve showing progression-free survival in the cohort 9 and RP2D evaluable population treated with TK216. PFS, progression-free survival; RP2D, recommended phase II dose.

Response Correlates

We cannot identify any characteristics of the three patients who responded to TK216 that differentiate them from the nonresponders. There was no correlation with the number of previous lines of systemic therapy nor numbers of relapses before study entry. We did not require tumor or germline DNA for study participation and cannot evaluate any possible correlation of specific ES-associated translocation, secondary tumor mutations, or germline mutations with a response. One patient, who had a PR at first response evaluation, had a persistent nodule resected for genomic profiling that had the identical breakpoint translocation as the tumor at study entry and no additional mutations were identified.

DISCUSSION

To our knowledge, this was the first attempt to target the pathognomonic FP in ES using a first-generation small molecule designed to inhibit EWS::FLI1 PPI. This project was conceived in an era when most investigators deemed transcription factors to be undruggable.[42-45] This label of undruggable was applied EWS::FLI1 as an intrinsically disordered protein.[33] Toretsky and Üren identified small molecules that bound to full-length recombinant protein, leading to YK-4-279.[38] While lacking a specific binding site, YK-4-279 shows enantiospecificity in multiple cell-free, in vitro, and in vivo assays.[46-48] Emerging theory and data support EWS::FLI1 participating in biomolecular condensates leading to novel protein interactions,[49,50] and therefore, condensate alteration by TK216 is a putative mechanism. This suggests that EWS::FLI1 can serve as a viable target if second-generation antagonists emerge in ES or other potential ETS malignancies.

The objective response rate of the 48 patients treated at the RP2D was 3 of 48 (6%). Two of the responses were in complete remission in either a heavily treated patient (four previous cytotoxic regimens) or a patient with multiple pulmonary nodules. Neither of these patients would have been expected to survive 6 months, yet they remained disease-free 4 years after study entry.[51] In a sarcoma type where spontaneous tumor regressions do not occur, the two complete and one PR provide strong proof of concept that antitumor activity can occur. Study investigators concluded that TK216 lacked sufficient clinical activity to justify further testing of this agent with this dose schedule and formulation. The lack of clinical correlates precludes the kind of in-depth molecular characterization to identify a predictive biomarker used to enrich for responders. Efforts were made to obtain tumor tissue a posteriori to determine if pharmacodynamic effects varied between responders and nonresponders. However, besides the nodule described above, other samples were unavailable.

TK216 was advanced as a novel inhibitor of EWS::FLI1 despite pharmacologic challenges to creating an oral formulation. In vivo studies showed that continuous exposure to TK216 was required for optimal efficacy.[47] This continuous infusion was a logistical challenge and negatively affected study enrollment rates. Reformulating TK216 as an orally available compound would make study participation far easier for patients.

Another limitation of this study was the lack of a pharmacodynamic readout of TK216 activity. Although the downstream proteins and signaling cascades linked to EWS::FLI1 FP are well known (eg, NROB1, CAV1, IGF1, PRKCB), repeat

biopsy samples from patients were not a requirement of this study. We recommend that all future trials of investigational agents for the treatment of ES require submission of both tumor and germline DNA to permit analysis of possible genetic contributions to response or resistance to novel therapies. We did not require confirmation of an ES-associated translocation. In retrospect, this is an additional limitation of the study. The present study confirms that hematologic toxicity can be managed effectively with neutrophil-stimulating agents. Routine use of neutrophil- and platelet-stimulating agents for the treatment of recurrent/refractory ES should be implemented to avoid study drug dose modifications. To avoid treating so many patients at subtherapeutic doses, particularly for highly aggressive diseases like ES, we should reexamine the starting doses for investigational agents and consider using a higher fraction of the preclinical MTD. Novel trial designs that move beyond the traditional 3 + 3 dose escalation paradigm, such as Bayesian Optimal Interval Design or intracohort dose escalations, should be considered.[52,53]

While synergy studies are not yet published for TK216, YK-4-279 does demonstrate synergy with multiple agents, including vinca alkaloids.[54] The vinca alkaloid synergy demonstrated disruption of microtubules as a possible mechanism of cell death.[54] A recent study confirmed that TK216 can bind to microtubules, which may indicate a secondary mechanism of cell death.[55] We modified the study to investigate the potential clinical benefits of synergy with vincristine; however, there were not enough responses to conclude the potential clinical benefit of the combination.

This phase I/II clinical trial provides proof of concept that fusion oncoproteins can be targeted with acceptable side effects. The challenges in biomarker development and pharmacologic delivery are surmountable, which would optimally use an oral dose formulation or less frequent IV dosing that is easier for patients to receive. While we opted to not require on-study biopsies and repeat sampling during treatment, we acknowledge that these samples would have strengthened this study and aided future efforts. Maximizing research benefits while minimizing the burden and risk for study participants is a delicate balance. Nevertheless, our study provided abundant information and a potential path forward to investigate prospective FP-targeted drug candidates in ES and other sarcoma subtypes reliant on their unique fusion oncoproteins.

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APPENDIX

TABLE A1. Treatment-Emergent AEs Grade 3-5

System Organ Class–Preferred Term	All Patients (N = 85), No. (%)	Cohorts 9 and 10 (expansion cohort; both at RP2D; n = 48), No. (%)	Cohort 11 (n = 8), No. (%)
No. of patients with an event	62 (72.9)	37 (77.1)	7 (87.5)
Blood and lymphatic system disorders	49 (57.6)	28 (58.3)	6 (75.0)
Neutropenia	38 (44.7)	22 (45.8)	6 (75.0)
Anemia	25 (29.4)	15 (31.3)	2 (25.0)
Leukopenia	25 (29.4)	15 (31.3)	2 (25.0)
Febrile neutropenia	13 (15.3)	4 (8.3)	2 (25.0)
Thrombocytopenia	10 (11.8)	2 (4.2)	2 (25.0)
Lymphopenia	1 (1.2)	1 (2.1)	0 (0.0)
Pancytopenia	1 (1.2)	1 (2.1)	0 (0.0)
Infections and infestations	15 (17.6)	10 (20.8)	1 (12.5)
Bacteremia	4 (4.7)	3 (6.3)	1 (12.5)
Device-related infection	3 (3.5)	2 (4.2)	0 (0.0)

NOTE. At each level of summarization (system organ class or preferred term), patients who experienced more than one AE were counted only once. Denominators are the total number of patients overall and within the RP2D cohorts and cohort 11. All AEs were coded using the Medical Dictionary for Regulatory Activities (MedDRA) version 23.1.
Abbreviations: AE, adverse event; RP2D, recommended phase II dose.