A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing’s sarcoma

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Many sarcomas and leukemias carry nonrandom chromosomal translocations encoding tumor-specific mutant fusion transcription factors that are essential to their molecular pathogenesis. Ewing’s sarcoma family tumors (ESFTs) contain a characteristic t(11;22) translocation leading to expression of the oncogenic fusion protein EWS-FLI1. EWS-FLI1 is a disordered protein that precludes standard structure-based small-molecule inhibitor design. EWS-FLI1 binding to RNA helicase A (RHA) is important for its oncogenic function. We therefore used surface plasmon resonance screening to identify compounds that bind EWS-FLI1 and might block its interaction with RHA. YK-4-279, a derivative of the lead compound from the screen, blocks RHA binding to EWS-FLI1, induces apoptosis in ESFT cells and reduces the growth of ESFT orthotopic xenografts. These findings provide proof of principle that inhibiting the interaction of mutant cancer-specific transcription factors with the normal cellular binding partners required for their oncogenic activity provides a promising strategy for the development of uniquely effective, tumor-specific anticancer agents.

There is a considerable need for new cancer therapies that enhance efficacy and reduce long-term morbidity. Protein products of tumor-specific chromosomal translocations provide unique targets for antitumor therapies1. These translocations span a broad range of malignancies, including carcinomas, hematopoietic malignancies and sarcomas2–4. In many cancers, these translocations lead to new fusion proteins that both initiate and maintain oncogenesis. Although some of these translocations, such as breakpoint cluster region–Abelson oncprotein (BCR-ABL)5, lead to constitutively activated kinases, the majority lead to fusion proteins that function as transcription factors and lack intrinsic enzymatic activity. These translocation-generated transcription factor fusion proteins are ideal targets of anticancer therapies, yet no specific pharmaceuticals have been developed to date.

The ESFTs are undifferentiated tumors that can occur anywhere in the body, most often in the second and third decades of life. ESFTs often respond well to initial chemotherapy, yet 40% of patients will develop lethal recurrent disease. Seventy-five to eighty percent of people who present with metastatic ESFTs will die within 5 years, despite high-dose chemotherapy6. ESFTs contain a well-characterized chromosomal translocation that fuses the amino half of EWS to the carboxy half of a ets (erythroblastosis virus E26 transforming sequence gene) family DNA binding protein7. The most common fusion protein is the oncogenic transcription factor EWS-FLI1. Elimination of EWS-FLI1 through antisense and small interfering RNA approaches results in the prolonged survival of ESFT xenograft–bearing mice8, but this approach currently lacks translation to clinical therapy9,10. As EWS-FLI1 lacks intrinsic enzymatic activity, small-molecule targeting would be directed toward the disruption of EWS-FLI1 from established transcriptional complexes. The EWS-FLI1 transcriptional complex includes: RNA polymerase II, cyclic AMP response element–binding protein and RHA11–13. Our previous investigations showed that RHA augments EWS-FLI1–modulated oncogenesis, suggesting that this protein–protein complex is particularly essential for tumor maintenance13. Small-molecule inhibitors that block RHA interaction by targeting the oncogenic fusion protein EWS-FLI1 would be the first in a new class of antitumor therapy directed at these proteins.

RHA has a crucial role in embryogenesis and thus might be a reasonable option as a partner for an oncoprotein in undifferentiated tumors and is indispensable for ectoderm survival in gastrulation of mammals14. RHA is also required beyond embryogenesis because RHA-null mouse fibroblast cells are not viable (C.-G. Lee (University of Medicine and Dentistry of New Jersey), personal communication). However, transient reduction of RHA protein expression in COS cells did not affect their viability15. RHA provides a transcriptional coactivator role in models of tumorigenesis, and in the nuclear factor-kB (NF-kB)16 and signal transducer and activator of transcription-6 (ref. 17) transcriptomes. RHA binds DNA in a sequence specific manner within the promoters of the genes encoding cyclin-dependent kinase inhibitor 2A (ref. 18) and multidrug resistance protein-1.

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(ref. 19). The amino-terminal region of RHA is most often the site for protein-protein interactions. cAMP-binding protein binds amino acids 1–250 of RHA20, RNA polymerase II and breast cancer protein-1 (ref. 21), and RNA-induced silencing complex components22 bind in the amino-terminal region. EWS-FLI1 binds RHA in a unique region that is not occupied by other transcriptional or RNA metabolism proteins13, thus increasing the attractiveness of this protein-protein interaction target.

Disruption of protein-protein interactions by small molecules is a rapidly evolving field. Proteins with more flexible structures, in some cases disordered proteins, have a greater potential for small-molecule binding than rigid proteins because of higher induced-fit sampling probabilities23. A disordered protein is defined, in part, by increased intrinsic movement and the inability to form rigid three-dimensional structures (reviewed in ref. 24). EWS-FLI1 is a disordered protein and requires the disorder for maximal transactivation of transcription25,26.

RESULTS

RHA is a validated target in ESFTs

A region of RHA that binds EWS-FLI1 was identified based upon phage-display epitope screening13 (Fig. 1a). To validate RHA as essential for the survival of ESFT cells, we lowered RHA levels with short hairpin RNA (shRNA), and ESFT cell viability was reduced by 90% (Fig. 1b,c). We stably transfected PANCl cells, a pancreatic cancer cell line that does not express EWS-FLI1, with the same shRNA vectors, yielding a similar reduction in RHA abundance (Supplementary Fig. 1a) but with no decrease in cell viability (Supplementary Fig. 1b). We further validated the RHA and EWS-FLI1 interaction with site-directed mutagenesis in the GST-RHA647–1075 fragment to identify mutants that don’t interact with EWS-FLI1. We expressed GST-RHA647–1075 mutants and immunoprecipitated them with full-length recombinant EWS-FLI1. Mutants P824A and D827A showed a significant decrease in binding to EWS-FLI1 compared to wild-type control RHA (P = 0.0129 and P = 0.0034, respectively; Fig. 1d). The full-length RHA mutant D827A maintained wild-type ATPase activity (Supplementary Fig. 2); therefore, we chose the D827A mutant to test whether RHA binding to EWS-FLI1 is required for neoplastic transformation.

We stably transfected mouse embryonic fibroblasts (W cells) that express low levels of endogenous RHA13 with EWS-FLI1 (WEF1 cells) and either full-length wild-type RHA or full-length RHA D827A. We observed a greater than additive effect of RHA and EWS-FLI1 expression on anchorage independent growth when comparing the colony numbers from W cells expressing RHA (227 ± 66 colonies) and WEF1 cells (115 ± 8 colonies) to those of WEF1 cells expressing RHA (582 ± 30 colonies) (Fig. 1c,f). The RHA D827A–expressing cells showed a 60% reduction in anchorage-independent growth (P = 0.0028) compared to cells expressing wild-type RHA (Fig. 1c,f). We quantified EWS-FLI1 expression by densitometry of the immunoblot (Fig. 1g,h). The markedly lower colony formation by the RHA D827A–expressing cells suggests a crucial role of RHA for transforming activity of EWS-FLI1 that is abrogated by RHA not binding to EWS-FLI1.

E9R peptide disrupts binding and inhibits growth

As RHA is necessary for optimal EWS-FLI1 activity, we developed reagents to block RHA binding to EWS-FLI1. The E9R peptide corresponds to amino acids 823–832 of RHA. With the immunoprecipitation assay, we assessed binding between bacterially expressed GST-RHA647–1075 and full-length purified recombinant EWS-FLI1 (Fig. 2a). Titration of E9R showed a dose-dependent reduction in the binding of GST-RHA647–1075 and full-length EWS-FLI1 with a decreased association to 50% with 0.1 μM E9R (Fig. 2a). We thus sought to determine whether disrupted EWS-FLI1–RHA binding inhibits cell growth.

Peptide delivery to growing cells is greatly facilitated by cell-permeable peptides (CPP)27. We fused the CPP antennapedia to the amino terminus of E9R or with the D827A mutation (E9R-P and

![Figure 1](https://example.com/figure1.png)

**Figure 1** RHA is necessary for optimal transformation by EWS-FLI1. (a) A schematic representation of RHA, including the region that binds EWS-FLI1. The E9R peptide corresponds to amino acids 823–832, located just proximal to the HA2 region of RHA. dsRBD, double-stranded RNA–binding domain, RGG box, arginine glycine glycine box; NTS, nuclear transport signal; aa, amino acid residue. (b) An shRNA expression vector was transfected into TC71 (ESFT) cells to reduce RHA levels. (c) TC71 viability after RHA knockdown, as measured by cell proliferation reagent water-soluble terazolium salt (WST) reduction. (d) Alanine mutagenesis within E9R sequence was followed by in vitro immunoprecipitation with EWS-FLI1. The density of the GST-RHA band was measured, and this graph is the average of three experiments. RHA P824A and D827A mutants have significantly lower binding to EWS-FLI1 (*P = 0.0129 and **P = 0.0034, respectively). (e) Mouse fibroblasts were placed in soft agar for anchorage-independent growth assays (empty vector (W), EWS-FLI1 alone (WEF1)). (f) The graph enumerates the colonies counted in three separate experiments. (g) Protein expression of fibroblasts, detected with antibody to Flag (top) or antibody to FLI1 (bottom). (h) Densitometry of the EWS-FLI1 blot, performed with MultiGauge software.
E9R(D5A)-P, respectively; Supplementary Table 1). We treated monolayer cultures of the EWS-FLI1–positive ESFT cell line, TC32, or a control EWS-FLI1–negative cell line, SKNAS (neuroblastoma), with fluorescein-conjugated peptides. Only the EWS-FLI1–containing TC32 cells showed reduced growth with E9R peptide, and the SKNAS cells showed mild stimulation from the E9R peptide via an unknown mechanism (Fig. 2b). Confocal microscopy showed uptake throughout the cell, including nuclei (as evidenced by DAPI overlay, Fig. 2c). E9R-P significantly reduced ESFT cell growth (P = 0.048), while neither the D5A mutant control nor antennapedia peptides alone reduced ESFT cell growth (Fig. 2d). Neuroblastoma cells treated with the same peptides did not have a statistically significant alteration in growth, although we observed a slight increase with E9R(D5A)-P–treated cells (P = 0.175; Fig. 2d). To determine the effect of E9R on anchorage-independent growth, we cloned the E9R-encoding sequence into an EGFP-expressing plasmid (pGE9R). We also expressed EGF-P-E9R peptide only in cytoplasm by adding a nuclear export sequence (LQLPPLERL TL) to the plasmid28. We stably expressed EGFP-E9R peptide only in cytoplasm by adding a nuclear anchorage-independent growth, we cloned the E9R-encoding

**Figure 2** E9R peptide prevents EWS-FLI1 binding to RHA with specific detrimental effects upon ESFT growth and transformation. (a) Immunoprecipitation of GST-RHA647–1075 using recombinant full-length EWS-FLI1 bound to a FLI1–specific antibody. (b) Growth reduction upon E9R-P (antennapedia-E9R) treatment (10 μM) in TC32 cells but not SKNAS cells. (c) E9R-P peptide uptake, tracked with FITC label (top images). DAPI nuclear counterstain (middle images) and merged (bottom images) are shown. Scale bar, 20 μm. (d) Graph showing growth response of TC32 and SKNAS cells to E9R–P, Antennapedia alone (Antp) or E9R-D5A-P. (e) TC71 and SKNAS cells expressing EGFP empty vector (pG), EGFP-E9R (pGE9R), EGF with nuclear export sequence (pGC) or EGFP-E9R with nuclear export sequence (pGCE9R). (f) Average colony numbers of three experiments in TC71 cells expressing E9R throughout the cell. Scale bar, 20 μm.

Expressing pGE9R did not show reduced anchorage-independent growth (data not shown). Only expression of EGFP-E9R in TC71 reduced anchorage-independent growth (Fig. 2e,f).

**Optimized small molecule binds to EWS-FLI1**

We screened a library of 3,000 small molecules (National Cancer Institute Drug Targeting Program) for EWS-FLI1 binding by using surface plasmon resonance (SPR). We selected compounds that bind monomeric EWS-FLI1. We evaluated the binding state of the compound to EWS-FLI1 by the ratio of actual binding resonance units (RUactual) to the theoretical binding resonance units (RUtheor). A ratio below 1.0 indicated monomeric binding of compound to EWS-FLI1. NSC635437 had an RUactual to RUtheor ratio of 0.9, signifying monomeric binding to EWS-FLI1. NSC635437 had a greater potential to chemical derivatization with favorable drug-like properties29. We synthesized 1.0 g of NSC635437 to complete our studies and for use as a standard during compound optimization (Fig. 3a).

In a cell-free assay, NSC635437 reduced the direct binding of GST-RHA647–1075 to full-length recombinant EWS-FLI1 (Fig. 3b). We used an aromatic optimization strategy to design analogs to improve the inhibition of RHA binding to EWS-FLI1 by NSC635437. One of these compounds (YK-4-279), substituted with a methoxy group at the para position (p-methoxy) of the aromatic ring (Fig. 3a), markedly reduced the protein-protein interaction of EWS-FLI1 with GST-RHA647–1075 in vitro (Fig. 3b). We calculated a K_{D} of 9.48 μM for the affinity of YK-4-279 with EWS-FLI1 by SPR (Fig. 3c). To support a model of YK-4-279 as having similar interaction qualities to E9R, we used an SPR displacement assay to show that 10 μM YK-4-279 reduces the binding of 64 μM E9R from 17 RU to 7 RU, and 32 μM E9R reduces the binding from 13 RU to 5 RU (Fig. 3d). YK-4-279 at 30 μM completely displaced E9R from EWS-FLI1 binding, as measured by fluorescence polarization assay (Fig. 3e).

**YK-4-279 functionally inhibits EWS-FLI1 and ESFT cells**

ESFT cells treated with YK-4-279 showed a dissociation of EWS-FLI1 from RHA by 10 μM, consistent with the K_{D} value (Fig. 4a). YK-4-279 did not directly affect EWS-FLI1 or RHA levels (Fig. 4a and Supplementary Fig. 3). To further support YK-4-279 as a functional inhibitor of EWS-FLI1, we transfected COS7 cells with EWS-FLI1 and NR0B1 reporter-luciferase plasmid (containing EWS-FLI1 regulatory GGAA elements30). The EWS-FLI1–transfected cells showed a dose-dependent decrease in promoter activity when treated for 18 h with 3 μM and 10 μM YK-4-279 (Fig. 4b,c). As an additional control for nonspecific promoter effects, we transfected an NF-kB–responsive reporter into COS7 cells and activated it with phorbol 12-myristate 13-acetate. YK-4-279 did not affect the NF-kB–responsive promoter (Supplementary Fig. 4a). In a recent publication, EWS-FLI1 was
Shown to modulate cyclin D1 protein abundance by altering a cyclin D1 splice site. Blocking the interaction of EWS-FLI1 with RHA using YK-4-279 nearly eliminated cyclin D1 in TC32 cells treated for 14 h. We established ESFT (orthotopic) with CHP-100 and TC71 or ESFT xenograft growth is inhibited by YK-4-279.

As an apoptotic indicator, caspase-3 activity rose in a dose-dependent (18-h) YK-4-279 treatment in COS7 cells. Together, these results support the specific toxicity of YK-4-279 in tumor cell lines containing EWS-FLI1 compared with other tumor and non-transformed cells.

To further support for the target specificity of YK-4-279 toxicity in ESFT cells, we reduced the levels of EWS-FLI1 and RHA proteins by using shRNA in A673 cells. Cells with knocked down RHA showed a YK-4-279 IC50 of >10 μM, whereas cells treated with the control shRNA (targeting luciferase) had a YK-4-279 IC50 of less than 1 μM (Supplementary Fig. 5c).

ESFT xenograft growth is inhibited by YK-4-279. We established ESFT (orthotopic) with CHP-100 and TC71 or prostate cancer with PC3 cell xenograft tumors in severe combined immunodeficient mice.

Figure 3 Small molecule binds EWS-FLI1 and displaces E9R from EWS-FLI1. (a) NSC635437, 3-hydroxy-3-(2-oxo-2-phenyl-ethyl)-1,3-dihydroindol-2-one, was synthesized with 100% yield. Aromatic optimization produced YK-4-279 a para-methoxy derivative of NSC635437. (b) EWS-FLI1 was incubated with NSC635437 (left) or YK-4-279 (right) followed by the addition of GST-RHA647-1075. EWS-FLI1 and GST-RHA647-1075 were precipitated from the solution with an FLI1-specific antibody. (c) YK-4-279 steady-state kinetics for binding to recombinant EWS-FLI1 that was immobilized on a CM5 Biacore chip. (d) SPR displacement assay of 64 μM E9R alone and with addition of YK-4-279; 32 μM E9R alone and with addition of YK-4-279. (e) Graph showing YK-4-279 displacement of E9R-P from EWS-FLI1, as measured by fluorescent polarization.

Figure 4 YK-4-279 reduces EWS-FLI1 functional activity. (a) TC32 cells were treated with YK-4-279, and resolved protein lysates were immunoblotted for co-precipitated RHA (top), EWS-FLI1 (middle) or total RHA (bottom). IP, immunoprecipitate; IB, immunoblot. (b) Luciferase reporter assay of the EWS-FLI1-responsive NR0B1 promoter upon dose-dependent (18-h) YK-4-279 treatment in COS7 cells. (c) Protein lysates from transfected cells showing expression of EWS-FLI1. (d) YK-4-279–treated TC32 cell lysates (treated for 14 h) were blotted for cyclin D1 and actin.
imunodeficient–beige mice. The tumor growth rate of YK-4-279–treated mice bearing CHP-100 (Fig. 6a) was lower than that in mice having PC3 prostate tumors (Fig. 6b). The cumulative data from five independent experiments with the ESFT xenografts (TC71 and CHP-100) show a marked overall tumor reduction ($P < 0.0001$) in the YK-4-279–treated mice (Fig. 6c). Pathological analysis of mice treated with YK-4-279 did not show any signs of toxicity, except for sterile inflammatory lesions in the abdominal cavities of mice, where

Figure 5 YK-4-279 is a potent and specific inhibitor of ESFTs. (a) TC32 cells were treated with a dose range of YK-4-279 and NSC635437. Cell growth, as measured by 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or WST reduction after 7 d in culture. (b) TC32 and HEK293 (nontransformed, lacking EWS-FLI1) cells were treated similarly to those in a. (c) Primary ESFT explant cell lines GUES1 and ES925 were treated for 3 d with YK-4-279. (d) Cell lines expressing EWS-FLI1 were compared to non–EWS-FLI1 malignant cell lines after 3 d in culture to establish the IC$_{50}$ using WST assay. (e) Caspase-3 activity of a panel of ESFT (TC32, TC71, A4573 and ES925), malignant non–EWS-FLI1–expressing (MCF-7, MDA-MB-231, PC3, ASPC1 and COLO-PL) and nontransformed (HEK-293, HFK and HEC) cells. Graph shows fluorescence in treated lysate divided by that of untreated lysate. (f) Arrows indicate apoptotic nuclear fragmentation after 50 µM YK-4-279 treatment of ESFT (TC32) cells and nontransformed cells (HEK-293, HFK and HEC). Scale bar, 200 µm.

Figure 6 YK-4-279 inhibits the growth of ESFT xenograft tumors. Xenografts were established with injection of either ESFT (CHP-100 or TC71) or prostate cancer (PC3) cells. (a) CHP-100 intramuscular xenografts (arrow indicates when tumors were palpable) received DMSO ($n = 4$) or 1.5 mg YK-4-279 ($n = 5$) ($P = 0.016$, by $t$ test comparison). The single-experiment growth curves depicted are representative of five independent experiments. (b) PC3 subcutaneous xenografts (arrow indicates when tumors were palpable) were treated as the CHP-100 cells were in a ($n = 5$ per group, representative of three independent experiments). (c) Overall response of ESFT xenografts (TC71 and CHP-100) to YK-4-279 (1.5 mg per dose). Tumor volumes at day 14 after treatment initiation compared across five experiments are shown (DMSO, $n = 19$; YK-4-279, $n = 25$; $P < 0.0001$, by Mann-Whitney test). (d) Tumors from the mice in a were analyzed by immunohistochemistry for activation of caspase-3 activity. (e) Caspase-3–positive cells were counted ($n > 500$ in three high-power fields) in four separately stained slides for each group ($P = 0.041$).
intrapерitoneal injections were applied. Tumors from mice treated with YK-4-279 were compared with those after DMSO treatment by immunohistochemistry to identify caspase-3 activity (Fig. 6d). The CHP-100 xenograft tumors from treated mice had a threefold increase in caspase-3 activity compared to control mice (Fig. 6e). These results show inhibition of tumor growth and concomitant increased apoptosis after YK-4-279 treatment in two models of ESFT.

**DISCUSSION**

EWS-FLI1 is a unique, cancer-specific molecule that is a potential therapeutic target in ESFT cells. RHA is essential for the function of EWS-FLI1. We showed that an E9R peptide that blocks RHA binding to EWS-FLI1 (E9R) specifically reduced the transformation activity of EWS-FLI1. We also identified a small-molecule lead compound that binds EWS-FLI1. The lead compound derivative, YK-4-279, along with E9R peptide, shows that the EWS-FLI1–RHA interaction can be blocked with a detrimental effect on ESFT cells both in vitro and in vivo. These findings validate a highly specific cancer target, the interaction of EWS-FLI1 with RHA.

These are to our knowledge the first experiments that evaluate a small-molecule inhibitor of EWS-FLI1 function. A series of xenograft experiments show that 60–75 mg per kg body weight YK-4-279 substantially decreases tumor growth. The small molecule not only inhibits RHA binding to EWS-FLI1 but also decreases EWS-FLI1 modulated transcription, on the basis of reporter assays. An additional putative function of EWS-FLI1 is splice-site modification34, which was recently supported by the EWS-FLI1–altered splicing of cyclin D1 (ref. 31). Treatment of ESFT cells with YK-4-279 led to decreased cyclin D1 levels. Additional investigations of the splicing complex are necessary to determine whether this effect is due to the disruption of an EWS-FLI1–RHA complex or allosteric interference with EWS-FLI1. Small-molecule inhibitors of protein–protein interactions have great therapeutic potential and will be immediately useful as functional probes.

EWS-FLI1 was recognized as a potential therapeutic target over 15 years ago, almost immediately after the protein was identified as a product of the breakpoint region t(11;22)35. We hypothesized that RHA is a functionally crucial partner of EWS-FLI1. We developed small-molecule protein–protein interaction inhibitors against EWS-FLI1 and RHA without benefit of a fixed structure of EWS-FLI1. The exact nature of the requirement of RHA by EWS-FLI1 is currently under investigation; however, we speculate that RHA could be involved in EWS-FLI1 function, synthesis or stability. Our data support multiple mechanisms and therefore require further enzymatic and structural studies of EWS-FLI1–bound RHA for resolution. The fact that YK-4-279 is still toxic to A673 cells with low EWS-FLI1 expression could be due to residual EWS-FLI1 or suggest broader action of the compound. In addition, although our data suggest that YK-4-279 has ESFT cell-specific toxic effects, we recognize that as additional cell and tumor models are tested, other protein interactions of YK-4-279 may be revealed.

Inhibitory peptides offer a higher likelihood of specificity than small molecules to validate protein–protein interaction targets and to evaluate protein-complex disruption; however, peptides are problematic for clinical development. Although small peptides are currently being developed as therapeutic agents36,37, 10–20–amino acid peptides present formidable pharmacokinetic stability and delivery challenges. Our investigations use peptides to compare the effects of disrupting protein–protein interactions with our small molecules. The E9R peptide may compete with full-length RHA binding to EWS-FLI1, and our data support a functional displacement of RHA by E9R. Small molecule YK-4-279 can ‘displace’ E9R peptide from EWS-FLI1, as shown by SPR and fluorescence polarization. Although our results support E9R and YK-4-279 binding to the same site on EWS-FLI1, allosteric interference cannot be excluded. Therefore, a structural model of EWS-FLI1 is required to fully prove both this interaction and the YK-4-279 binding site but is yet unavailable owing to the challenges of disordered proteins38.

The interaction of RHA with EWS-FLI1 presents an ideal opportunity for the development of small-molecule protein–protein interaction inhibitors. Both the evidence and the prevailing opinion support disordered proteins as potential targets of small molecule therapeutics38. Our data also support EWS-FLI1 protein interaction targeting to modulate oncogene function and potentially lead to new therapeutics. Additional experiments to evaluate multispecies specificity, toxicity and absorption, distribution, metabolism and excretion are required to advance a further optimized derivative of YK-4-279 into clinical trials. Small molecules that disable EWS-FLI1 function with minimal toxicity, in particular sparing hematopoetic stem cells, could potentially provide a valuable adjuvant therapy for patients with ESFT. In addition, this paradigm for drug discovery could be applied to many related sarcomas that share similar oncogenic fusion proteins.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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**AUTHOR CONTRIBUTIONS**

H.V.E., J.S.B.-R., M.M., L.Y., O.D.A., S.S., T.-h.C., A.U. and J.A.T. designed and carried out experiments. Y.K., S.D. and M.L.B. designed and synthesized chemical compounds. H.V.E. and J.A.T. wrote the manuscript. All authors reviewed, critiqued and offered comments to the text.

**COMPETING INTERESTS STATEMENT**

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ARTICLES


ONLINE METHODS

Materials. We obtained E9R peptide from Bio-synthesis. We obtained protein G beads (Invitrogen), antibody to GST, antibody to FLI1, antibody to cyclin D1 (all from Santa Cruz), fluorescent caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4- methylcoumarin) (Ac-DEVD-AMC), caspase-3 fluorescent substrate (BD Biosciences Pharmingen) and antibody to cleaved caspase 3 (Asp175) (Cell Signaling) from commercial sources.

Site-directed mutagenesis. We changed every nonalanine amino acid in the amino acids 823–832 region of RHA to alanine by site-directed mutagenesis with QuickChangeII XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s protocol.

Cell cultures. We established maintained TC32, TC71, A4573, CHP-100 and primary ES925 and GUES1 ESFT cell lines in RPMI (Invitrogen) medium supplemented with 10% FBS (Gemini Bioproducts). HEC and HFK cell lines, kind gifts from R. Schlegel, are previously described39. We tested subclones of these cells stably expressing EWS-FLI1 tested in an anchorage-independent growth assay as previously described13.

Protein immunoprecipitation assays. We made protein lysates and performed immunoprecipitations as previously published13. We prepared recombinant GST-RHA647–1075 from crude bacterial extracts without further purification.

Small molecule library screening and selection of lead compound. We established an SPR assay using the Biacore T100 with EWS-FLI1, prepared in our laboratory as previously published26. We used DNA oligonucleotides to quality-control the proper conformation of EWS-FLI1 on the surface of a CM5 chip. We prioritized small molecules from the Developmental Therapeutics Program of the National Cancer Institute, US National Institutes of Health on the basis of their molecular weight and solubility. We performed an initial screening of molecules at 1 μM or 10 μM compound, based on solubility. We used a model that compares the actual binding maximum (actual RU) with the theoretical binding maximum (RUtheor). If the RUactual to RUtheor ratio is 0.9–1.0, this suggests a binding, and such a compound is considered a ‘hit’. A team of medicinal chemists then reviewed hits, and those with structural potentials were selected for further study. We tested selected molecules in vitro in a solution co-immunoprecipitation assay with recombinant EWS-FLI1 and GST-RHA647–1075.

Synthesis and analysis of small molecule compounds. Details provided in the Supplementary Methods.

Fluorescence polarization assay. We added increasing concentrations of FITC- E9R to a fixed concentration of EWS-FLI1 (4.8 μM) to obtain a saturated binding curve. We performed the assay in 20 mM Tris, 500 mM NaCl and 0.67M imidazole, pH 7.4. We analyzed the fluorescence polarization in a QuantaMaster fluorimeter (Photon Technology International) equipped with polymer sheet polarizers at an excitation wavelength of 495 nm and emission wavelength of 517 nm. We added increasing concentrations of YK-4-279 to a fixed concentration of EWS-FLI1 and FITC-E9R (3.2 μM, as determined from saturated binding curve) with the same buffer and instrumental settings as described above.

Plasmids and reporter assay. We prepared EGF-P-E9R fusion constructs as previously published40. We transiently transfected the NR0B1 (ref. 31) luciferase reporter and full-length EWS-FLI1 into COS-7 cells with Fugene-6 (Roche) and performed the luciferase assay per the manufacturer’s protocol (Dual Luciferase Kit, Promega). Six hours after transfection, we treated cells with either 3 μM or 10 μM YK-4-279. We standardized cell lysate luciferase activity to Renilla activity from a nonaffected promoter and plotted as relative luciferase activity (RLA).

Caspase-3 activity measurement and nuclear fragmentation. We treated cells for 24 h with 10 μM YK-4-279. We incubated the Caspase-3 substrate DEVD-AMC with equal amounts of protein lysate and measured the fluorescence from cleaved substrate in a fluorimeter. We treated TC32 cells and nontransformed HEK-293, HFK and HEC cells for 6 h with high-dose (50 μM) YK-4-279. We photographed DAPI-stained cells at 600× magnification on an inverted fluorescence microscope.

Mouse strains and in vivo small-molecule testing. We orthotopically injected 1 million TC71 or CHP-100 cells in 100 μl HBSS into the gastrocnemius muscle of 4- to 8-week-old severe combined immunodeficient–beige mice (Taconic). We established prostate cancer xenografts by subcutaneous injection of 5 million PC3 cells into the flanks of 4- to 8-week-old nude mice (Taconic). We randomized mice to treatment groups receiving thrice weekly intraperitoneal injections of DMSO or YK-4-279 at 1.5 mg per dose when tumors were palpable. We began each of the mouse experiments with ten mice that were randomized into treatment and control groups when the tumors reached palpable size. In the control groups, some tumors exceeded the Institutional Animal Care and Use Committee maximal size (2 cm in any dimension) and were euthanized before day 14 and thus not included in the day 14 analysis (Fig. 6c). We measured tumor length and width every 2–4 d and calculated volume with the formula volume = D × d² × π/6, where D is the longest diameter and d is the shorter diameter. Xenograft studies were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Statistical analyses. We performed statistical analyses with GraphPad Prism.

Supplementary Figure Legends:

Supplementary Figure 1. Pancreatic cells survive when RHA levels are decreased. PANC1 cells were infected with virus containing either siRNA for RHA or control luciferase. a. Immunoblot showing protein levels following 6 days of selection. b. Viability of cells using WST reduction 6 days after selection and RHA reduction.

Supplementary Figure 2. RHA ATPase activity lost with P824A mutation but not D827A. a. ATPase assay was performed as previously published, except the Biomol Green was used to detect free phosphate. While the P824A mutant did show reduced ATPase activity, the D827A mutation did not affect RHA function. Phosphate standards were used to calibrate the assay and determine the rate of ATP hydrolysis. RHA(K417R) is a known NTPase-null mutant of RHA. Immunoglobulin control immunoprecipitations did not demonstrate ATPase activity (data not shown). b. Protein levels shown by immunoblot.

Supplementary Figure 3. RHA levels are not reduced by YK-4-279treatment. Immunoblot from log-phase cell lysates that were either treated with DMSO control or 10 µM YK-4-279 overnight.

Supplementary Figure 4. YK-4-279 does not reduce NF-kB activity nor cyclin D levels in non-ESFT cells. a. COS7 cells were transfected with an NFκB reporter construct followed by stimulation with PMA. Cells were treated with YK-4-279 following PMA treatment. Cell lysates were analyzed for NFκB induced luciferase activity as standardized to LTR activated renilla luciferase. b. Immunoblot from log-phase cell lysates that were either treated with DMSO control or 10 µM YK-4-279 overnight. c. Graph shows densitometry of treated/untreated with both standardized for β-tubulin expression.

Supplementary Figure 5. Additional studies evaluating specificity of YK-4-279. a. HEC and HFK, non-transformed endocervical cells and keratinocytes, were treated YK-4-279 for 72 hours and assayed for viability using WST reduction. b. TC71 cells were treated for 16 hours with YK-4-279 or doxorubicin. Lysates were assayed for cleavage of AMC-DEVD by induced caspase-3 and fluorescence was measured. c. RHA reduced TC71 cells were more resistant to YK-4-279 treatment than wild-type cells. d. An shRNA tet-inducible expression vector was stably transfected into A673 (ESFT) cells to reduce EWS-FLI1 levels. e. EWS-FLI1 reduced A673 cells were more resistant to YK-4-279 treatment than wild-type cells.
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Supplementary Figure 4

(a) Bar chart showing RLA values for different concentrations of YK-4-279 (µM) with PMA and NFκB reporter present or absent.

(b) Western blot analysis of Cyclin D1 and β-Tubulin for MCF7, MDA-MB-231, PC3, and ASPC1 cell lines with YK-4-279 (10 µM) treatment.

(c) Graph showing change in Cyclin D1 level (relative units) for different cell lines.