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Inhibition of the oncogenic fusion protein EWS-FLI1 causes G₂-M cell cycle arrest and enhanced vincristine sensitivity in Ewing's sarcoma

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Abstract

Ewing's sarcoma (ES) is a rare and highly malignant cancer that grows in the bones or surrounding tissues mostly affecting adolescents and young adults. A chimeric fusion between the RNA binding protein EWS and the ETS family transcription factor FLI1 (EWS-FLI1), which is generated from a chromosomal translocation, is implicated in driving most ES cases by modulation of transcription and alternative splicing. The small-molecule YK-4-279 inhibits EWS-FLI1 function and induces apoptosis in ES cells. We aimed to identify both the underlying mechanism of the drug and potential combination therapies that might enhance its antitumor activity. We tested 69 anticancer drugs in combination with YK-4-279 and found that vinca alkaloids exhibited synergy with YK-4-279 in five ES cell lines. The combination of YK-4-279 and vincristine reduced tumor burden and increased survival in mice bearing ES xenografts. We determined that independent drug-induced events converged to cause this synergistic therapeutic effect. YK-4-279 rapidly induced G₂-M arrest, increased the abundance of cyclin B1, and decreased EWS-FLI1-mediated generation of microtubule-associated proteins, which rendered cells more susceptible to microtubule depolymerization by vincristine. YK-4-279 reduced the

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Competing interests: United States Patent and Trademark Office awarded for YK-4-279 to Georgetown University, inventors include A.Ü. and J.A.T. Georgetown University executed license with Oncternal Therapeutics Inc., in which J.A.T. and A.Ü. are founding shareholders, and J.A.T. is a scientific consultant. The other authors declare that they have no competing interests.

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expression of the EWS-FLI1 target gene encoding the ubiquitin ligase UBE2C, which, in part, contributed to the increase in cyclin B1. YK-4-279 also increased the abundance of proapoptotic isoforms of MCL1 and BCL2, presumably through inhibition of alternative splicing by EWS-FLI1, thus promoting cell death in response to vincristine. Thus, a combination of vincristine and YK-4-279 might be therapeutically effective in ES patients.

Introduction:

Ninety-five percent of Ewing's sarcoma (ES) cases are driven by a fusion protein involving the RNA-binding protein EWS and an erythroblastosis virus E26 transforming sequence (ETS) family transcription factor, most frequently FLI1 (EWS-FLI1) (1). In patients with ES, the goal is to eradicate micrometastatic disease and facilitate effective local control because the outcome for most patients who relapse is poor (2). EWS-FLI1 functions, in part, as an aberrant transcription factor that deregulates gene expression and has different protein-protein interactions than the wild-type proteins that constitute the fusion (3). The small-molecule YK-4-279 inhibits EWS-FLI1 activity; YK-4-279 induces apoptosis in both cultured cells and animal models of ES (4, 5), at least in part, by disrupting its interactions with RNA helicase A (4) and p68 DDX5 (3). An analog of YK-4-279, TK216, is currently in a phase 1 clinical trial ([NCT02657005](#)).

Vincristine (VCR) is a cytotoxic drug commonly used in ES therapy that inhibits cell proliferation by altering the dynamics of mitotic spindle microtubules (2). Cells are particularly sensitive to VCR during the transition into G₂-M, which is modulated by a rise and fall of cyclin B1 (6, 7). In normal cell cycle progression, ubiquitin-conjugating enzyme E2C (UBE2C) contributes to the decrease in cyclin B1 abundance that enables release through the G₂-M checkpoint (8); a decrease in UBE2C leads to increased cyclin B1 abundance, causing significant arrest at the S and G₂-M phases of the cell cycle, and decreased cell proliferation (9, 10). *UBE2C* gene expression is increased by EWS-FLI1, which could have an impact on cell cycle regulation (11). Inhibiting UBE2C might repress cell cycling in ES. However, cell cycle arrest does not always lead to cell death. For example, high abundance of prosurvival isoforms of the B cell lymphoma 2 (Bcl-2) family inhibits apoptosis. The balance of proand antiapoptotic isoforms determines the effect of gene expression on cell survival (12). *BCLX*, *MCL1*, and other *BCL2* family mRNAs undergo alternative splicing that switches this balance from antiapoptotic, long isoforms to proapoptotic, short isoforms (13). In many cancers, Bcl-2 family proteins contribute to resistance to various chemotherapeutic agents, including VCR (14). Current strategies to inhibit Bcl-2 family proteins include small-molecule inhibitors to the Bcl-2 homology region 3 (BH3)-binding pocket, which is essential for the antiapoptotic function of Bcl-2 after its heterodimerization with proapoptotic members such as Bim and Bid; targeting the BH3-binding site disarms the antiapoptotic function of Bcl-2 and induces apoptosis (15, 16).

Here, we sought greater detail in the mechanism of action of YK-4-279 to identify synergistic drug interactions that might be used to more effectively treat ES. We found that YK-4-279 induced a potent G₂-M cell cycle arrest that was facilitated through increased amounts of cyclin B1. This cell cycle arrest enhanced VCR toxicity in ES. Although the G₂-

M arrest alone may explain the synergistic activity, our analysis of ES alternative splicing also identified an isoform switch of Bcl-2 family members. Both mechanisms provide support for enhanced apoptosis through a combination of two non-cross-reacting pathways. This novel combination of synergistic agents remains to be more thoroughly and clinically evaluated but may be a viable strategy to enhance current chemotherapeutic regimen in ES patients.

Results:

YK-4-279 shows synergistic cytotoxicity in combination with vinca alkaloids in ES cell lines

We assessed cell viability (inferred from relative metabolic activity through WST-1 color change) in cultures of ES cell line TC71 exposed to each of 69 established anticancer (some antisarcoma) drugs, either as single agents or in combination with the small-molecule YK-4-279. Synergistic cytotoxicity was assessed by the combination index (CI) (17, 18). Overall, YK-4-279 showed synergistic cytotoxic activity with 19 (28%) of the anticancer drugs tested (table S1). The highest percentage of synergistic candidates per pharmacological group [U.S. Food and Drug Administration (FDA) and Medical Subject Headings (MeSH)] was seen when YK-4-279 was combined with antimetabolites (40%), nucleic acid synthesis inhibitors or nucleoside metabolic inhibitors (46%), immunosuppressive or immunomodulating agents (50%), and tubulin modulators/microtubule inhibitors (50%); the latter group was composed of both taxanes and vinca alkaloids. Vinca alkaloids inhibit microtubule polymerization, whereas taxanes stabilize microtubules (19). Synergy with YK-4-279 was observed in combination with all tested vinca alkaloids but did not occur when combined with taxanes (table S1). Overall, YK-4-279 induced synergy with two (VCR and busulfan) of six tested anticancer drugs currently used for ES treatment (table S1) (2).

Combinatorial treatment of YK-4-279 and VCR significantly enhances apoptosis, leading to increased survival of ES xenografts

We pursued the combination of YK-4-279 and vinca alkaloids because of the central role VCR has in current chemotherapeutic regimens for ES (2). Fraction affected (Fa)–CI plots for treatment with vinblastine, VCR, or vinorelbine in combination with YK-4-279 in TC71 cells showed a significant reduction in cell viability with CIs of 0.7 to 0.8 (fig. S1, A to C). The combination of YK-4-279 and VCR exhibited synergy with CIs of 0.6 to 0.9 in five of eight ES cell lines (table S2). In addition to TC71, we show three ES cell lines with left shifts in VCR dose-response curves from half-maximal inhibitory concentration (IC₅₀) of 4.85 to 2.38 nM (A4573), 0.68 to 0.03 nM (SKES), and 25.14 to 13.24 nM (TC32) when treatment included YK-4-279 (Fig. 1A and fig. S1, D to F). Overall, YK-4-279 increased in cytotoxicity when combined with VCR (fig. S1G). In our panel of eight ES cell lines, VCR in combination with YK-4-279 led to a decrease in VCR dose by as much as 96% while maintaining equivalent cytotoxicity (table S3). Among the three cell lines that displayed only additive cell growth inhibition, two (COG-E-352 and CHLA25) still showed significant VCR dose reductions for equivalent cytotoxicity (table S3).

Single and combinatorial treatments induced apoptosis in a time-dependent manner, as evidenced by both increased fluorescence of single-positive fluorescein isothiocyanate–conjugated annexin V (FITC–annexin V) cells and caspase-3 activity (Fig. 1, B and C, and fig. S1H). Caspase-3 activity was significantly higher in the combinatorially treated cells for 18 hours (Fig. 1B). After 12 hours of treatment with YK-4-279 and VCR, the amount of single-positive FITC–annexin V cells doubled when compared to dimethyl sulfoxide (DMSO) treatment, reaching a total of ~10% of the total number of cells after 18 hours of treatment in A4573 cells (Fig. 1C).

The effects of YK-4-279 and VCR combinatorial treatment were evaluated in ES xenograft mouse models. The animals started treatment when primary tumors were well established (250 to 300 mm³) with single or combinatorial regimens of VCR and various doses of YK-4-279. Combinatorial treatment showed dose-dependent reduction in tumor growth rate in two different xenograft models (Fig. 1D and fig. S1I). The combination of YK-4-279 (50mg/kg) and VCR (1mg/kg) displayed significantly increased survival in A4573 xenograft-bearing animals as compared to those treated with VCR alone (Fig. 1E). At higher doses of YK-4-279 (>150 mg/kg) in combination with VCR, weight loss led to early euthanasia of some animals (Fig. 1D and fig. S1I). TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining of xenografts confirmed that the number of apoptotic cells increased significantly in combinatorial treatment compared to VCR alone–treated xenografts (Fig. 1, F and G).

YK-4-279 causes more G₂-M cell cycle arrest than VCR

Cells in culture treated with both YK-4-279 and VCR showed significantly increased numbers of detached rounded cells as compared to VCR alone–treated cells (fig. S2, A and B). After treatment with YK-4-279, the chromosomes in both cultured cells and xenograft tumors exhibited incomplete alignment at the metaphase plate, with residual chromosomes unevenly distributed at one or both spindle poles (fig. S2, C and D); these observations suggested an arrest at a metaphase-like stage of the cell cycle. To resolve mechanistic aspects of combinatorial treatment, xenografted animals of two different ES cell lines were treated in separate experiments for 3 days to provide uniform treatment and necropsy times. Singly treated xenografts displayed wide, clear cytoplasmic brims enclosing a center of condensed chromatin (Fig. 2A); this was consistent with either cell cycle arrest or early apoptosis before nuclear fragmentation (20–22). The number of A4573 cells with this morphology significantly increased in combinatorially treated xenografts compared to those treated with a single agent (Fig. 2, A and B). Not all cells with condensed chromatin were apoptotic as assessed by TUNEL staining (Fig. 1F), which prompted further investigation of this phenotype.

These findings suggested a cell cycle arrest as an initiating mechanism for synergy between VCR and YK-4-279. YK-4-279 led to a G₂-M block; 53% of ES cells were arrested at G₂-M by 4 hours, and 90% of cells were arrested at G₂-M by 8 hours when treated with YK-4-279 (Fig. 2C). Combinatorial treatment with VCR and YK-4-279 did not achieve a more rapid cell cycle arrest compared to YK-4-279 treatment alone in TC32 cells (Fig. 2C). Two different mitotic phase–specific antibodies were used to cross-validate our results in vitro

and in vivo. Mitotic phase-specific phosphorylation of histone H3 occurs at threonine 11 (p-H3Thr¹¹) (23), which starts at early prophase; dephosphorylation is required to advance to late anaphase (24). Amounts of p-H3Thr¹¹ in A4573 cells increased by 4 hours and continued to rise over time, without decreasing, thus, confirming G₂-M-arrested cells after treatment with YK-4-279 (Fig. 2D). To confirm the cell cycle observations in vivo, tumor tissues of singly and combinatorially treated A4573 xenografts were evaluated for another specific mitotic marker: phosphorylation of histone H3 at serine 10 (p-H3Ser¹⁰) (Fig. 2, E and F). H3Ser¹⁰ is phosphorylated in association with mitotic chromatin condensation in late G₂-M phase of the cell cycle (25). In control tumors, as expected, p-H3Ser¹⁰-stained nuclei were found only in cells undergoing mitosis (Fig. 2E). The p-H3Ser¹⁰ staining increased significantly for xenograft tumors treated with either YK-4-279 or VCR compared to those that received DMSO treatment (Fig. 2, E and F). Combinatorial treatment significantly further increased p-H3Ser¹⁰ compared to single VCR treatment, which was consistent with amitotic arrest (Fig. 2, E and F). These marker data corroborated G₂-M as the cell cycle stage corresponding to the observed chromosomal misalignment at the metaphase plate.

G₂-M arrest by YK-4-279 is driven by increased abundance of cyclin B1 and decreased expression of the EWS-FLI1 target gene UBE2C

We sought cell cycle regulators that might explain the connection between YK-4-279 and the G₂-M arrest. The primary kinase that regulates G₂-M transition during the cell cycle is cyclin-dependent kinase 1 (CDK1), whose activity requires cyclin B1 (26). Cyclin B1 forms a stable complex with CDK1, activating CDK1 and allowing the cell to enter mitosis. At the end of the G₂-M transition, cyclin B1 is degraded by the ubiquitin-proteasome pathway (27, 28). Nondegradation of cyclin B1 prevents cell cycle progression through the G₂-M checkpoint (29). TC32 cells in culture treated with YK-4-279 showed markedly high cyclin B1 abundance in mitotic-arrested cells (Fig. 3A). Immunoblotting confirmed the increased cyclin B1 in both single and combinatorial treatments with YK-4-279 and VCR in A4573 cells (Fig. 3B). Cyclin B1 accumulation was also confirmed in xenograft tumors where combinatorial treatment significantly increased cyclin B1 positively stained cells compared to singly treated xenografts (Fig. 3, C and D).

Additional data support the activation of the cyclin B1/CDK1 complex in YK-4-279-treated cells. CDK1 activation requires dephosphorylation of an inhibitory residue, Tyr¹⁵ (30, 31). Although overall CDK1 protein abundance remained stable, CDK1 phosphorylation at Tyr¹⁵ decreased after single and combinatorial treatments in a time-dependent manner, suggesting persistent CDK1 activation (Fig. 3B). Given that the inactivation of CDK1 requires degradation of cyclin B1, we sought YK-4-279-modulated pathways that could increase cyclin B1 abundance. UBE2C is an E2 ubiquitin ligase known to participate in cyclin B1 degradation (32). Analysis of public databases revealed that *UBE2C* is highly expressed in both ES cell lines and patients (fig. S3, A and B). YK-4-279 also reduced the amount of *UBE2C* RNA (Fig. 3E) and UBE2C protein compared to DMSO-treated controls, whereas the combination of YK-4-279 and VCR further reduced UBE2C protein abundance after 24 hours of treatment (Fig. 3B).

To further study the role of UBE2C in the synergy between YK-4-279 and VCR, we established both a knockdown of *UBE2C* using short hairpin RNA (shRNA) in A4573 and CHLA9 cell lines (fig. S3, C to E) and *UBE2C* overexpression models (Fig. 3, F and G). CHLA9 was used as a control in knockdown studies because VCR sensitivity was minimally altered in combinatorial treatments (table S3). Reduction of UBE2C protein led to increased cyclin B1 protein abundance in A4573 cells but mixed results in CHLA9 cells (fig. S3C). Reduction of UBE2C induced a left shift in the dose response for VCR from an IC₅₀ of 1.6 to 0.6 nM in A4573 cells (fig. S3D) and a 6% increase in cells in G₂-M (fig. S3E). Reduction of UBE2C did not alter the sensitivity of CHLA9 cells to VCR (fig. S3D) nor did it change the number of cells in G₂-M (fig. S3E). In a follow-up experiment, A4573 cells were infected with a vector containing DDK-tagged UBE2C for overexpression (Fig. 3, F and G) with which we measured a threefold increase in the YK-4-279 IC₅₀ from 12.8 to 36.8 μ M (Fig. 3G). Although YK-4-279 treatment reduced the endogenous UBE2C protein, there was no effect on the exogenously expressed protein (Fig. 3F).

To further decipher the connection between YK-4-279 sensitivity and the UBE2C–cyclin B1 axis, we reduced cyclin B1 abundance using shRNA in A4573 cells (Fig. 3, H and I, and fig. S3F). Cells with cyclin B1 reduction showed a decrease in p-H3Ser¹⁰, which was partly rescued by YK-4-279 treatment in A4573 cells (Fig. 3H). The cyclin B1 reduction also increased resistance to YK-4-279, shifting the IC₅₀ from 5.4 to 6.4 μ M (fig. S3F). In addition, cyclin B1–reduced cells also showed a 7% decrease in G₂-M arrest (Fig. 3I).

Combinatorial treatment with YK-4-279 and VCR enhances disruption of spindle microtubules and centrosomes compared with either single-drug treatment

To investigate the degree of spindle perturbation in mitotic-arrested cells receiving combinatorial treatment, we expanded and validated a previously published scoring system (fig. S4A) (6) to analyze the phenotypic changes in spindle architecture, chromosome alignment, and centrosome number (Fig. 4A and fig. S4B). VCR-deformed spindles displayed longer astral microtubules, shortened pole-to-pole distance, multiple pole structures, and, in later stages, a punctate pattern of β -tubulin aggregates (Fig. 4A and fig. S4B). This effect of VCR was time-dependent (fig. S4C). Combinatorial treatment led to β -tubulin staining in a punctate pattern (Fig. 4A and fig. S4B). This pattern was caused by a redistribution of protein localization rather than changes in abundance (Fig. 4B). Centrosomes were disrupted by VCR alone, showing an abnormal number and distribution, which was increased with combinatorial treatment (Fig. 4A and fig. S4B). Cells treated with both drugs showed marked punctate bodies positive for both β - and γ -tubulin (Fig. 4A and fig. S4B). A semiquantitative analysis of the microtubule and centrosome changes showed significant damage to the mitotic apparatus in cells that were combinatorially treated (fig. S4C). Despite minimal β -tubulin alteration from YK-4-279 alone, the chromosomes in both cultured cells and xenograft tumors were unevenly distributed at one or both spindle poles (Fig. 4, A to C, and fig. S4B). In cells treated with YK-4-279 alone, the spindle apparatus itself remained intact, with longer and more prominent astral microtubules (Fig. 4, A and C, and fig. S4B). Although the incubation time of YK-4-279 increased the number of mitotic-arrested cells, the spindle architecture of these cells did not deteriorate further (fig. S4C).

Overall, these results suggested an alternative metaphase-timed mechanism for YK-4-279 that causes a cell cycle arrest, which then augments VCR toxicity.

Three microtubule-regulating proteins, previously unrecognized as important EWS-FLI1 targets, were identified from our database of EWS-FLI1-regulated genes (3). The centrosome-associated protein E (CENPE) is a kinesin-related microtubule motor protein that is essential for chromosome alignment during prometaphase; its reduction slows the metaphase-to-anaphase transition (33, 34). Kinesin family member 22 (KIF22) is required for proper chromosome alignment at the metaphase plate and depletion of KIF22 results in substantial alignment defects (35, 36). Kinesin family member 2C (KIF2C) is required for proper establishment and maintenance of the spindle. Depletion of KIF2C perturbs spindle microtubules, leading to long astral microtubules (37, 38). Treatment with the EWS-FLI1 inhibitor YK-4-279 reduced the expression of *CENPE*, *KIF22*, and *KIF2C*, which may explain how microtubule architecture would be altered without reducing total tubulin amounts (Fig. 4D).

To further characterize the synergistic mechanism, in which G₂-M cell cycle arrest by YK-4-279 sensitizes cells to VCR-induced spindle perturbation, we performed a series of experiments where treatments were temporally sequenced. ES cells were assayed for caspase-3 activity to evaluate apoptosis after treatment with different regimens of YK-4-279 and VCR. Pretreatment with YK-4-279, delivered 4 hours before the addition of VCR, significantly increased apoptosis compared to a nonsequential combinatorial regimen with VCR in A4573 cells (Fig. 4E). In contrast, pretreatment with VCR did not cause significant differences in apoptosis from nonsequential treatment (Fig. 4E). These results suggest a temporally ordered synergistic cascade of G₂-M cell cycle arrest both preceding and enabling microtubule perturbation by VCR.

YK-4-279 inhibition of EWS-FLI1 alters splicing of MCL1 and BCL2, leading to isoform ratios that favor apoptosis

G₂-M cell cycle arrest does not always lead to apoptosis, so we investigated other mechanistic pathways that could be altered by YK-4-279 that would lead to apoptosis in arrested cells. Because YK-4-279 also inhibits the alternative splicing function of EWS-FLI1, we evaluated genes that regulate cell survival through variant isoforms, linking mitotic spindle disruption and apoptosis. RNA sequencing (RNA-seq) data from ES cells expressing wild-type *EWS-FLI1*, ES cells in which *EWS-FLI1* was knocked down (EF) (fig. S5A), and ES wild-type cells treated with YK-4-279; ES wild-type cells showed alternative splicing in *MCL1*, *BCL2*, and *BCLX* (*BCL2L1*). Our RNA-seq data sets were consistent with published EWS-FLI1-regulated gene targets using gene set enrichment analysis (GSEA) (fig. S5B) (39).

RNA-seq and genome-assisted de novo isoform reconstruction revealed five different mRNA transcripts for myeloid cell leukemia 1 (*MCL1*) gene, two of which (*MCL1S* and *MCL1ES*) are proapoptotic, and the others of which are antiapoptotic (*MCL1L*) (Fig. 5A). One isoform lacks exon 2, which translates to a proapoptotic Mcl-1S protein (40, 41). On the basis of the Mcl-1 protein-coding region, and given that *MCL1ES* uses an alternate inframe splice site in the 5' coding region compared to the other variants (40), two of the remaining four

transcripts align to each *MCL1L* and *MCL1ES* (Fig. 5A and table S4). Analysis of transcript expression showed that YK-4-279-treated cells exhibit low expression of antiapoptotic (*MCL1L*) and high expression of proapoptotic (*MCL1S* and *MCL1ES*) *MCL1* isoforms (Fig. 5B and fig. S6A). This “shift” toward *MCL1S* was further validated by quantitative polymerase chain reaction using specific primers spanning exons 1 and 3 in TC32 cells treated with YK-4-279, VCR, or a combination of both drugs (Fig. 5C). In comparison to single VCR treatment, treatment with YK-4-279 alone and combinatorial treatment significantly affected *MCL1* splicing, measured by an increase in shorter, two-exon transcripts with respect to DMSO (Fig. 5D). As a cell type specificity control, single and combinatorial treatments of human embryonic kidney (HEK) 293 cells did not show the same effect on alternative splicing of the *MCL1* isoforms (Fig. 5, C and D).

The splicing changes in *MCL1* were reflected by an increase in Mcl-1S protein at 16 hours of single and combinatorial treatments compared to DMSO (Fig. 5E). Corresponding to known proapoptotic effects, a measurable, time-dependent decrease in Mcl-1L protein was observed after both single and combinatorial treatments (fig. S6B). Combinatorial treatment sustained the decreased Mcl-1L more prominently than either single YK-4-279 or VCR treatment (Fig. 5E). The interplay between the increase in Mcl-1S, followed by the decrease in Mcl-1L, is a well-described phenomenon leading to apoptosis (40); our data support this through an observed increase in Mcl-1S/Mcl-1L ratio over time (Fig. 5F).

In addition, our splicing analysis showed three *BCL2* gene transcripts in wild-type, EF, or YK-4-279-treated TC32 cells (Fig. 6A). When compared to untreated ES cells, YK-4-279 induced a distinct proapoptotic *BCL2* alternative splicing pattern toward the short, proapoptotic *BCL2 beta* transcript (Fig. 6B and fig. S6C). This splicing pattern was validated by quantitative real-time polymerase chain reaction (qRT-PCR) using specific primers spanning exons 1 and 2 in TC32 cells treated with YK-4-279, VCR, or a combination of both drugs (Fig. 6C and fig. S6D). In HEK293 cells, both *BCL2* isoforms are constitutively expressed with relatively small changes in *BCL2 alpha*, but virtually no change in *BCL2 beta* expression upon drug treatment (Fig. 6C and fig. S6D). YK-4-279 increased the ratio of *BCL2 beta/BCL2 alpha* expression in ES but not in HEK293 cells. In YK-4-279-treated ES cells, this ratio remained significantly increased in combination with VCR compared to VCR alone by densitometric analysis (Fig. 6D). Immunoblotting for Bcl-2 alpha throughout a time course of YK-4-279 treatment revealed a second band, which we presume to be the Bcl-2 beta isoform, which is 34 amino acids shorter (Fig. 6E). In contrast to *MCL1* and *BCL2*, splicing changes affecting the ratio of pro- and antiapoptotic transcripts after treatment with YK-4-279 were not observed for *BCLX*, another *BCL2* family member (fig. S7).

To distinguish the specific protein dependence of mitochondrial apoptosis in ES cell lines, we used the BH3 agonist ABT-737. ABT-737 enhances the effects of apoptotic signaling through inhibition of antiapoptotic Bcl-2 family proteins Bcl-xL, Bcl-w, and Bcl-2. Mcl-1 has been reported to mediate resistance to ABT-737 (42). ES cell lines A4573, TC32, and TC71 cells exhibited IC₅₀ values for ABT-737 in excess of 10 mM (fig. S8), similar to cell lines previously being reported ABT-737-resistant (43). As expected, the Bcl-2-dependent neuroblastoma cell line NB1643 displayed significantly increased apoptosis after ABT-737

treatment compared to TC32 cells (at 0.5 μ M; Fig. 6F). These results suggest that Mcl-1 is an important Bcl-2 family mediator of ES survival.

Discussion

Our study revealed synergy between YK-4-279 and VCR in ES cells in culture and tumors in vivo, mediated by a convergent, proapoptotic mechanism triggered by inhibition of EWS-FLI1-specific activity. Growth in tumors was blocked by a checkpoint arrest at metaphase caused, at least in part, by increased cyclin B1 abundance, followed by YK-4-279-induced decreased expression of EWS-FLI1 target gene *UBE2C*. YK-4-279 concomitantly reduced the alternative splicing of Bcl-2 family proteins, presumably by EWS-FLI1, such that proapoptotic isoforms were more abundant. These effects of YK-4-279 augmented microtubule and spindle disruption by VCR and primed ES cells for apoptosis (Fig. 7).

In contrast to treatment with either single agent, combinatorial treatment with YK-4-279 and VCR augmented spindle perturbation. Microtubule detachment from spindle poles, rather than a change in polymer content, accounts for inhibition of mitosis by vinca alkaloids (44). The mechanism of mitotic arrest differs between taxanes (which were not synergistic with YK-4-279) and vinca alkaloids, reflecting opposing actions of microtubule-stabilizing drugs versus microtubule-destabilizing drugs (45). Our data show that the disruption of spindle architecture occurred at low YK-4-279 concentrations, which might be important to consider for future treatment schema in case of potentiated toxicity for both drugs. Overall, our findings are consistent with other studies indicating that the progression from prometaphase to anaphase is the most vinca alkaloid-sensitive phase of the cell cycle (6, 46).

YK-4-279 does not reduce tubulin or actin protein abundance, yet it impairs microtubule dynamics through alteration of microtubule-interacting proteins. Chromosomes need to establish connections with the chromosomal segregation machinery to undergo cell division (47). Our immunofluorescence studies showed a time-dependent deterioration of spindle architecture, chromosome alignment, and centrosome formation upon VCR treatment of ES cells, an effect that was less remarkable with YK-4-279 treatment alone. However, YK-4-279 induced chromosome compaction into metaphase plates, with residual chromosomes unevenly scattered at the spindle poles. We also saw reduced EWS-FLI1-driven expression of kinetochore members *CENPE*, *KIF22*, and *KIF2C* by YK-4-279. Decreases in *CENPE*, *KIF22*, and *KIF2C* expression could explain the observed chromosomal alignment defects and prominent astral microtubules after YK-4-279 treatment (33-37). Further studies that delve into the relationship of microtubule-binding proteins with EWS-FLI1 in ES may reveal additional target opportunities.

Our results suggest that YK-4-279 causes G₂-M arrest through impaired proteasomal degradation of cyclin B1. The E2 ubiquitin ligase UBE2C is putatively regulated, in part, by EWS-FLI1. However, our data also show that overexpressing UBE2C only transiently reduces cyclin B1, which suggests that UBE2C alone is not responsible for cyclin B1 regulation; an E3 ligase is required for efficient degradation (48). The necessity of tuning cyclin B1 amounts for entry into mitosis is well known (49). High cyclin B1 abundance can even directly cause cytotoxicity (50). Together, these results support our claim that

YK-4-279 reduction of UBE2C can contribute to increased cyclin B1 abundance in ES, causing G₂-M arrest and supplying the mechanistic basis for synergy with VCR.

However, G₂-M arrest alone does not lead to apoptosis. Rather, we found a switch induced by YK-4-279 that appears to prime ES cells for death upon further stress with VCR. EWS-FLI1 alters splicing by directly binding to known splicing factors; splicing activity is also altered in ES cells by treatment with YK-4-279 (3). Our results demonstrate that YK-4-279-treated cells shift splicing outcomes from longer antiapoptotic transcripts to shorter proapoptotic gene products of *BCL2* and *MCL1*, thereby promoting apoptosis (51). Our findings are consistent with previous studies where inhibition of Mcl-1L is necessary and sufficient to trigger massive cell death in cancer (52). Wild-type ES cells do not express *MCL1S*, whereas HEK293 cells express both *MCL1S* and *MCL1L* transcripts, and in contrast to ES cells, drug treatment does not affect this ratio in HEK293 cells. The increase in *MCL1L* transcripts upon combinatorial treatment in our qRT-PCR validation experiments could be due to inclusion of *MCL1ES* transcripts, thereby obscuring a greater difference of the *MCL1S/MCL1L* ratio by qRT-PCR. The Mcl-1S/Mcl-1L ratio is determined in the alternative pre-mRNA splicing step that is regulated by splicing factor 3B1 (51), which we have previously identified to exist in a complex with EWS-FLI1 (3). Mcl-1 protein abundance decreased upon treatment with VCR, which was consistent with previous findings showing degradation of Mcl-1 during mitotic arrest caused by microtubule-targeted agents (14). With this in mind, it may be informative to elucidate the contribution, if any, of the tumor-suppressor protein FBW7 in ubiquitination and subsequent proteasomal degradation of Mcl-1, which sensitizes cells for apoptosis in response to microtubule-interfering agents (14).

Altered cell survival proteins appear to enhance the apoptotic effects of dysfunctional mitosis as a result of the G₂-M blockade in ES. The sequence of tubulin polymerization, CDK1 activation, mitotic arrest, and the engagement of the intrinsic mitochondrial pathway leads to apoptosis by microtubule-interfering agents (53-55). Together with alteration of Bcl-2 family proteins, the cyclin B1/CDK1 complex appears crucial in switching cells from mitotic arrest to apoptosis (56, 57). YK-4-279 may further induce apoptosis through the maintained activation of CDK1, suggested by our study by an observed time-dependent dephosphorylation of CDK1 at the known inhibitory site Tyr¹⁵ (30, 31). ES cells show resistance to Bcl-2 inhibitor ABT-737, supporting our conclusion that Mcl-1 is a key Bcl-2 family survival protein in ES after VCR treatment. Future studies will investigate combinatorial strategies with recently reported Mcl-1 inhibitors, such as S63845 (58).

In conclusion, combinatorial treatment with YK-4-279 and VCR shows enhanced cytotoxicity in ES, compared to treatment with single agents. VCR, as part of a multiagent chemotherapeutic regimen, is standard of care for ES patients but can cause neurotoxicity (59). We found that synergy between VCR and YK-4-279 in different ES cell lines led to an average decrease in VCR dosage of 58% when combined with YK-4-279; notably, tumor cell cytotoxicity equivalent to that of single-agent VCR was still obtained despite this significant dose reduction. Clinical efficacy of YK-4-279 is seen in many animal models (5, 60, 61); this led to the development of an analog, TK216, now in phase 1 human clinical trials that has better pharmacologic properties and identical effects on ES cells (62). Thus,

our results lay a foundation for an effective combinatorial regimen using VCR in combination with TK216 to reduce side effects through dose reduction.

Materials and Methods

Cell culture, cell survival, and apoptosis assay

ES cell lines TC32, TC71, A4573, and SKES were grown in RPMI 1640 with 10% fetal bovine serum (FBS). COG-E-352, CHLA9, CHLA10, and CHLA25 cells were grown in Iscove's modified Dulbecco's medium with 10% FBS and 1% insulin-transferrin-selenium (Sigma-Aldrich). NB1643 cells were cultured in RPMI 1640 with 10% FBS, 1% Hepes, 1% penicillin-streptomycin, and 1% L-glutamine. All cell lines were maintained at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. To support the rigor of this manuscript, we used eight cell lines to show the combinatorial effects of YK-4-279 with vinca alkaloids. Specific cell lines are reported on the basis of their validation in initial toxicity screen (TC71), animal assays (A4573 and SKES), RNA-seq (TC32), cell imaging screen (A4573 and TC32), and differential VCR effect (CHLA9). For continuity, A4573 is used throughout to show consistency across assays. Cell line integrity was confirmed by short tandem repeat fingerprinting of TC32, TC71, SKES, and A4573. Cell lines COG-E-352, CHLA9, CHLA10, and CHLA25 were directly obtained from a Children's Oncology Group (COG) cell culture and xenograft repository and used within limited passages. Cell lines were tested mycoplasma-negative in domo.

With the exception of YK-4-279 (structure nuclear magnetic resonance verified and high-performance liquid chromatography >98% purity; Albany Molecular Research Inc.) and ABT-737 (Selleck Chemicals), all drugs were obtained from the Developmental Therapeutics Program at the National Cancer Institute/National Institutes of Health (NIH). The Approved Oncology Drugs Set comprised anticancer drugs frequently used in sarcoma treatment. Drugs were first tested individually in TC71 cells. For the purpose of interpretation, anticancer compounds were categorized by their mechanism(s) of action using two different pharmacological classifications (MeSH and FDA).

Cellular toxicity for test agents or vehicle alone (DMSO) was assessed by triplicate plating at a density of 5000 to 15,000 cells per well, depending on cell line, in a 96-well plate. Cell viability was evaluated using WST-1 (11644807001, Roche Diagnostics) assay according to the manufacturer's protocol after 72 hours. IC₅₀ were calculated using GraphPad Prism 4.0.

Synergy analysis

On the basis of the CI theorem of Chou-Talalay, a plot of CI values at different effect doses, referred to as Fa, can be determined by computer simulation (18). After absorbance measurements from cell viability experiments were taken, Fa-CI plots were generated using CompuSyn software (Biosoft), and its common categorization to define synergy or antagonism ($0 < CI < 1$ indicates synergy, $CI = 1$ indicates additive effect, and $CI > 1$ indicates antagonism) was further divided into mild and strong synergy or antagonism. CI values for synergistically tested compounds with YK-4-279 in different ES cell lines after treatment for 72 hours were generated from at least three different experiments; minimum of

four adjacent CI values per experiment was included to average. Single experiments were carried out in triplicates.

Apoptosis assays

FITC–annexin V and caspase-3 activity represent well-established probes for measuring apoptosis in vitro. For apoptotic assays, A4573, TC32, and NB1643 cells were grown overnight at a density of 1×10^5 cells per well and submitted to different regimens of single and/or combinatorial treatment of YK-4-279 and VCR. For the FITC–annexin V assay, harvested cells were washed with phosphate-buffered saline (PBS), centrifuged, resuspended in $1 \times$ binding buffer [10 mM Hepes (pH 7.4), 140 mM NaOH, and 2.5 mM CaCl_2], stained with FITC–annexin V and propidium iodide, and evaluated for apoptosis by FACS analysis. Cells undergoing apoptotic cell death were identified as annexin V-positive and propidium iodide-negative. The caspase-3 assay was performed as previously described (63). Briefly, the caspase-3 substrate DEVD-AMC (BD Biosciences Pharmingen) was combined with equal amounts of protein lysate, and the fluorescence from cleaved substrate was measured in a fluorimeter (Synergy H4 Hybrid Microplate Reader; BioTek).

Orthotopic mouse xenograft model

Two million A4573 or SKES cells in 0.1 ml were injected into an orthotopic paraosseous location, adjacent to the left proximal tibia, in 5-week-old female severe combined immunodeficient/beige (SCID/bg) mice (Harlan Laboratories Inc.). After primary tumors reached 250 to 300 mm³, mice were randomized and received intraperitoneal injection with vehicle control of DMSO in 20 ml of saline once daily, VCR at a dose of 1 mg/kg once weekly, racemic YK-4-279 at different concentrations (10 to 150 mg/kg in A4573 and 75 to 400 mg/kg in SKES) once daily for 5 days on/2 days off or 7 days, or combined treatment with VCR injected 1 hour before racemic YK-4-279 at the indicated concentrations once daily for 5 days on/2 days off or 7 days. The tumor volume was determined by the formula $(D \times d^2/6) \times \pi$, where D is the longer diameter and d is the shorter diameter. Tumor volume was monitored every day by caliper until the tumor size reached 1 cm³. Mice were euthanized, and primary tumors were collected. An Institutional Animal Care and Use Committee of the Georgetown University approved the animal studies.

Histology, immunohistochemistry, and slide evaluation

All tumor tissues were fixed for a minimum of 24 hours in 10% neutral buffered formalin, dehydrated through a graded series of alcohols, cleared in xylenes, infiltrated with paraffin wax, and embedded in wax molds. Tissue sections were cut at 5 μm and placed onto Superfrost Plus charged slides (Thermo Fisher Scientific). Hematoxylin and eosin (Leica Microsystems Inc.) staining was performed on a Leica Autostainer XL.

Five-micrometer sections from formalin-fixed, paraffin-embedded tissues were deparaffinized with xylenes and rehydrated through a graded alcohol series. Heat-induced epitope retrieval was performed by immersing the tissue sections at 98°C for 20 min in 10 mM citrate buffer (pH 6.0) with 0.05% Tween 20. Immunohistochemical staining was performed using the Vectastain kit from Vector Laboratories according to the manufacturer's instructions.

Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxidase for 10 min. To prevent nonspecific stainings, we performed several blocking steps with avidin (A9275, Sigma-Aldrich), biotin (B4501, Sigma-Aldrich), superbblock (IDSTM003, ID Labs), and mouse block (IDSTM003, ID Labs). The sections were incubated with specific antibodies against p-H3Ser¹⁰ (ab5176, Abcam) and cyclinB1 (GNS1SC-245, Santa Cruz Biotechnology) at 4°C overnight. The next day, the sections were incubated with a biotinylated secondary antibody (IDSTM003, ID Labs) and horseradish peroxidase (HRP) (IDSTM003, ID Labs) for 10 min. Specific signals were amplified using 3-amino-9-ethylcarbazole (BP1108, ID Labs) under visual control, followed by a counterstaining with hematoxylin (1.092.491.000, Merck). The sections were mounted using Aquatex (1.08562.0050, Merck). All antibodies were incubated overnight at 4°C and diluted in PBS + 1% bovine serum albumin (BSA). Slides were visualized on a Nikon Ti Eclipse microscope. Qualitative and quantitative analyses of sections were evaluated by two different individuals in a blinded fashion.

Cell cycle analysis

Fixed single-cell suspensions were analyzed for their DNA content by FACS to determine cell cycle status. ModFit software was used to evaluate cell cycle status of analyzed cells.

Western blots

Samples were lysed in radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific) containing complete protease inhibitor cocktail (Roche Diagnostics), and protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific). Western blots were performed with 50 µl of cell lysate from each sample electrophoresed through 6 to 12% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% nonfat dry milk in TBST (20 mM tris-HCl, 150 mM NaCl, and 0.5% Tween 20), incubated with the designated primary antibodies for p-H3Thr¹¹ (9764, Cell Signaling), p-H3Ser¹⁰ (ab5176, Abcam), cyclin B1 (GNS1 SC-245, Santa Cruz Biotechnology), CDK1 (ab32094, Abcam), p-CDK1 (Tyr15) (47594, Abcam), UBE2C (ab187181, Abcam), β-tubulin (T4026, Sigma-Aldrich), Mcl-1 (4572, Cell Signaling), Bcl-2 (clone 100/D5;MS123P, Thermo Fisher Scientific), FLI1 (C-19 SC365, Santa Cruz Biotechnology) following the manufacturer's instructions, and HRP-conjugated secondary antibody (GE Healthcare) at 1:2000 dilution. HRP anti-actin (I-19, Santa Cruz Biotechnology) antibody was added in 1:5000 dilution to secondary antibody or separately incubated after membrane stripping. Detection was carried out using Millipore Immobilon Western Chemiluminescent HRP Substrate per the manufacturer's instructions (Millipore Corp.) using a Fujifilm LAS-3000 imaging system. Densitometric analysis of protein bands was carried out with ImageJ software.

Immunofluorescence microscopy

Cells were grown on culture slides (BD Biosciences) in complete media, and designated drug treatments of test agents or vehicle alone were added after 24 hours. Cells were fixed (ice-cold methanol), rehydrated with PBS, and blocked with 10% normal goat serum and 1% BSA, followed by incubation with primary antibodies for β-tubulin (T4026, Sigma-Aldrich), γ-tubulin (T3320, Sigma-Aldrich), or cyclin B1 (GNS1 SC-245, Santa Cruz Biotechnology)

in concentration of 1:1000 and staining with phalloidin-conjugated goat anti-mouse (Alexa Fluor 488, Invitrogen) and anti-rabbit (Alexa Fluor 594, Invitrogen) immunoglobulin G in concentration of 1:300. Cells were counterstained with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) and visualized on a Nikon Ti Eclipse microscope. Images were acquired and merged with NIS-Elements software. Additionally, an Olympus FV300 confocal microscope and 60×/1.4–numerical aperture oil lens were used for imaging. Images were acquired and merged with FluoView 300 software. Immunofluorescence images were analyzed for morphological changes of spindle apparatus, chromatin organization, and centrosome number by β -tubulin, DAPI, and γ -tubulin staining, respectively. Abnormalities for microtubules and chromatin were characterized on the basis of the categories implemented in previous publications studying the effect of vinca alkaloids (fig. S4A) (6). To assign the change on centrosomes stained by γ -tubulin to the microtubule and chromatin alteration, we categorized one or two centrosomes into type I, three centrosomes into type II, and more than three centrosomes into type III. The amount of centrosomes varies between one and two centrosomes throughout the abnormal spindle types I, II, and III, whereas the distance between centrosomes decreases progressively (fig. S4A).

Reagents used for knockdown and overexpression experiments

A nonsilencing shRNA construct empty vector control and both *UBE2C* and *CCNB1* shRNA constructs were purchased from GE Healthcare Dharmacon Inc. *EWS-FLI1* shRNA was a gift from C. T. Denny (University of California, Los Angeles). Lentiviral vector delivery of shRNA encoding *EWS-FLI1*, *UBE2C*, and *CCNB1* in different ES cell lines was performed as previously established (3). Briefly, lentiviral stocks were made by transiently transfecting 3 μ g of expression vector, 675 ng of vesicular stomatitis virus glycoprotein–expressing plasmid pCMV, and 2 μ g of packaging plasmid pCMVHR8.2 deltaR. Viral stocks were collected 2 days after transfection, filtered, and frozen. Reduction of *EWS-FLI1* using shRNA in TC32 cells is shown in fig. S5A. After lentiviral production, 40% of confluent A4573, TC32, and CHLA9 cells were infected using a 1:500 ratio of polybrene (Sigma-Aldrich) to virus. After incubating for 4 to 6 hours, virus was diluted 1:2 in serum-free media and incubated for 18 hours. Virus was removed, and fresh media were added for 4 to 6 hours. To select for infected cells, puromycin (2 μ g/ml; Invitrogen) was added for 24 hours. Medium containing puromycin was removed, and virus, polybrene, and serum-free media were repeatedly added for 24 hours to double-infect. Puromycin selection was performed for at least 5 days after infection.

RNA-seq analysis

RNA isolation and sequencing were carried out as previously described (3). Briefly, 100 million paired-end reads were aligned to hg19 using TopHat2/Bowtie2 with default parameters for Cufflinks-based genome-guided transcriptome reconstruction. Normalization and differential expression were computed as part of the standard TopHat2 pipeline. The R language and Bioconductor packages ggplot, cummeRbund, Gviz, GenomicRanges, and GenomicFeatures were used for visualization. RNA-seq data were publicly available through Gene Expression Omnibus (GEO) (GSE103837).

For each gene, the known mRNA transcripts and de novo isoforms that were constructed via Cufflinks were aligned to the reference sequence (RefSeq) hg19 database to identify the corresponding protein isoforms. For mRNA transcripts that did not have identical spliced isoforms to existing transcripts in the RefSeq database, we suggest their biological activity based on similarities in protein-coding reading frames between newly identified transcripts and RefSeq-established transcripts.

RNA-seq validation by qRT-PCR

Validation of alternative splicing was performed using isoform-specific primers that target an area that can be used to differentiate between isoforms. Transcript expression validation used 18S as the internal normalizergene with the primer set for the transcript of interest. PCR products were separated on 3% agarose gel, and quantification of bands was performed using densitometry. Primers are listed in table S5.

Gene set enrichment analysis

Chromatin immunoprecipitation sequencing (ChIPseq) peaks from A673i doxycycline-inducible cell line (39) were annotated using ChIPpeakAnno R/Bioconductor package in conjunction with Ensembl annotation version 75. ChIPseq peaks were filtered for only those with a score greater than 10. ChIPseq targets were used to generate a set of EWS-FLI1 (EF) target genes, defined as having a peak passing filter criteria between -2 kb and $+500$ base pairs of the transcription start site for a given gene. These genes were used to generate a gene set for enrichment analysis by GSEA (implemented in the fgsea R/Bioconductor package). Gene-wise expression values were generated as described in the “RNA-seq analysis” section. Genes were ranked on fold-change difference from wild-type. Because only one gene set was tested, P value correction was not necessary. Visualization of GSEA enrichment was created in R using the ggplot2 framework (fig. S5B).

Statistics

Statistical analysis of most data was performed by unpaired t tests with two-tailed P values at a 95% confidence interval. Comparison of survival curves was performed by a log-rank test. All statistics were acquired with GraphPad Prism 4.0.

Supplementary Materials

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. YK-4-279 synergizes with VCR, significantly increasing apoptosis and leading to improved survival of ES-xenografted mice.

(A) Cell viability assessed by WST-1 staining in A4573 cells treated with different concentrations of VCR and YK-4-279. Dose-response curves through nonlinear regression analysis are shown. (B and C) Apoptosis assessed by (B) caspase-3 activity and (C) flow cytometry for annexin V (AV)/propidium iodide (PI) staining in A4573 cells treated with YK-4-279 (3 μ M), VCR (10 nM), both, or DMSO. (D and E) Change in tumor volume (D) and percent survival (E) assessed in A4573 xenograft mice intraperitoneally injected with YK-4-279 (YK; 10, 50, 100, and 150 mg/kg), VCR (1 mg/kg), or DMSO. Figure key shows number (*n*) of mice per group. (F and G) TUNEL staining (white arrows) (F) and quantification (G) to assess apoptosis in A4573 xenografts from mice intraperitoneally injected with YK-4-279 (150 mg/kg), VCR (1 mg/kg), or DMSO for 3 days. Magnification, $\times 800$; (scale bars, 50 μ m). Data are means $\pm$ SEM of greater than or equal to seven xenografts. All other data are means $\pm$ SEM of greater than or equal to three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, and **** $P < 0.0001$ by unpaired, two-tailed *t* test (B, D, and G) or log-rank test (E). hpf, high-power field.

Fig. 2. YK-4-279 leads to a G2-M cell cycle arrest, which is enhanced upon combination with VCR.

(A and B) Abnormal chromatin condensation (ACC; white arrows) assessed by hematoxylin and eosin (H&E) staining (A) and quantified (B) in A4573 xenografts from mice after treatment as described for Fig. 1F. Magnification for large images, $\times 400$ for large images (scale bars, 100 μm); magnification for inset, $\times 800$ (scale bars, 50 μm). Data are means $\pm$ SEM of greater than or equal to seven different xenografts. (C) Cell cycle analysis by fluorescence-activated cell sorting (FACS) in TC32 cells treated with YK-4-279 (3 μM), VCR (30 nM), both, or DMSO. (D) Western blot analysis for p-H3Thr¹¹ in lysates from A4573 cells after treatment with YK-4-279 (3 μM) as indicated. Blots are representative of greater than or equal to three independent experiments. (E and F) Cell cycle analysis by immunohistochemical staining for p-H3Ser¹⁰ in A4573 xenograft tissue from mice. Treatment and quantification were as described for Fig. 1 (F and G). Magnification, $\times 800$

(scale bars, 50 mm). Data are means $\pm$ SEM of greater than or equal to three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, and **** $P < 0.0001$ by unpaired, two-tailed t test.

Fig. 3. Cyclin B1 expression increases after YK-4-279 and VCR treatments, in part, through decreased EWS-FLI1-driven UBE2C.

(A) Cyclin B1 abundance assessed by immunofluorescence staining in TC32 cells after 20 hours of treatment with YK-4-279 (3 μM) or DMSO. Magnification for large images, ×400 (scale bars, 100 μm); magnification for inset, ×800 (scale bars, 50 μm). (B) Western blot analysis for cyclin B1, UBE2C, CDK1, and p-CDK1 (Tyr¹⁵) on lysates from A4573 cells treated with YK-4-279 (3 μM), VCR (10 nM), both, or DMSO. (C and D) Immunohistochemical staining for cyclin B1 (white arrows) of A4573 xenograft tissue from mice; treatment, magnification, and analysis were as described for Fig. 2 (E and F). ** $P < 0.01$ and *** $P < 0.001$ by unpaired, two-tailed t test. (E) RNA-seq analysis for the expression of *UBE2C* in TC32 cells after transfection with either *EWS-FLI1* shRNA or empty vector (EV), or after treatment with YK-4-279 (3 μM) for 12 hours; the depth of all exon reads is represented in fragments per kilobase and permillion RNA-seq fragments of the sample (FPKM). (F and H) Western blot analysis for UBE2C, cyclin B1, and p-H3Ser¹⁰ on A4573 cell lysates after overexpression of DDK-tagged *UBE2C* (OE) (F) or shRNA depletion of cyclin B1 (*CCNB1*) (H), or after treatment with YK-4-279 (3 μM) for 24 hours. Blots are representative. (G) Cell viability assays in A4573 cells after transfection with either DDK-tagged *UBE2C* vector (OE) or control (EV), and after treatment with different

concentrations of YK-4-279 for 72 hours; analysis was as described for Fig. 1A. (I) Cell cycle analysis by FACS in A4573 cells after transfection and treatment as in (H); percentages of cells in G₂-M are indicated. Data are means $\pm$ SEM of greater than or equal to three independent experiments.

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Fig. 4. YK-4-279 potentiates spindle perturbation of VCR and induces chromosomal alignment defects.

(A) Morphological analysis of spindle, chromatin, and centrosome formation assessed by immunofluorescence staining for β -tubulin, DNA, and γ -tubulin in TC32 cells after 8 hours of treatment with YK-4-279 (1 μ M), VCR (30 nM), both, or DMSO. Magnification, $\times 800$ (scale bars, 50 μ m). (B) Western blot analysis for β -tubulin on lysates from A4573 cells treated with YK-4-279 (3 μ M), VCR (10 nM), both, or DMSO. (C) Representative cells with tridentate chromosome formation assessed by confocal microscopy after immunofluorescence staining from (A). White arrows mark residual chromosomes at spindle poles (top) and prominent astral microtubules (middle). Magnification, $\times 1200$ (scale bars, 25 μ m). (D) RNA-seq analysis for the expression of *CENPE*, *KIF22*, and *KIF2C* in TC32 cells with wild-type (WT) *EWS-FLI1* expression, shRNA reduction of *EWS-FLI1* (Δ EF), or treated 12 hours with YK-4-279 (3 μ M). The depth of all exon reads is represented in FPKM. (E) Apoptosis assessed by caspase-3 activity in A4573 cells after single and combinatorial treatments with YK-4-279 (3 μ M) and VCR (10 nM) nonsequentially (No) or sequentially (Yes; 4 hours of pretreatment with either drug). * $P < 0.05$ by unpaired, two-tailed t test.

Fig. 5. YK-4-279 induces alternative proapoptotic splicing of *MCL1* that is confined to ES cell lines and leads to altered protein ratios while reducing Mcl-1L.

(A) RNA-seq analysis and de novo isoform reconstruction from TC32 cells with wild-type (WT) *EWS-FLI1* expression, after shRNA reduction of *EWS-FLI1* (EF), or after treatment with YK-4-279 (3 μ M for 12 hours). Reconstructed transcripts were coded as TCONS and displayed with the adjacent presumed protein isoform. Annotation of TCONS to RefSeq mRNA reference ID is shown in table S4. On the basis of the RefSeq database, the *MCL1* protein-coding region of each transcript is indicated by dashed lines. The aligned reads map to the gene transcript of each condition (WT, EF, or YK). (B) Absolute isoform expression based on RNA-seq reads from each sample (WT, EF, or YK). (C) Validation of *MCL1* splicing by qRT-PCR using specific primer pairs [black arrows in (A)], from exons 1 and 3 in TC32 and HEK293 cells after 18 hours of treatment as indicated (YK, 3 μ M; YK1, 1 μ M; YK3, 3 μ M; VCR, 30 nM). Blots are representative. (D) Annotated is fold change of *MCL1S* expression with respect to DMSO after densitometric quantification of short, two-exon comprising PCR product bands and normalization to 18S ribosomal RNA in TC32 and HEK293 cells after treatment as described for (C). Data are means $\pm$ SEM of greater than or equal to three independent experiments. * $P < 0.05$ by unpaired, two-tailed t test. (E) Western blot analysis for Mcl-1 isoforms on A4573 cell lysates after treatment with YK-4-279 (3

μM), VCR (10 nM), both, or DMSO. Blots are representative. (F) Change in ratio of Mcl-1S/Mcl-1L assessed by densitometric protein quantification after normalization to actin based on Western blot analysis as described for (E). Data are means $\pm$ SEM of greater than or equal to three independent experiments after treatment as described for (E).

Fig. 6. YK-4-279 reverses EWS-FLI1-induced antiapoptotic alternative splicing of *BCL2*, leading to expression of proapoptotic Bcl-2 beta protein isoform after single and combinatorial treatments.

(A and B) RNA-seq analysis for *BCL2* acquired and presented as described for data in Fig. 5 (A and B). (C) Validation of *BCL2* splicing by qRT-PCR using specific primer pairs [black arrows in (A)] from exons 1 and 2 in TC32 and HEK293 cells after treatment as described for Fig. 5C. Blots are representative. (D) Change in the ratio of *BCL2 beta* to *BCL2 alpha* assessed by densitometric RNA quantification after normalization to 18S. Data are means ± SEM of greater than or equal to three independent experiments after treatment as described for Fig. 5C. (E) Western blot analysis for Bcl-2 alpha on TC32 cell lysates after treatment with YK-4-279 (3 μM), VCR (10 nM), both, or DMSO. Blots are representative. (F) Apoptosis assessed by caspase-3 activity in TC32 and NB1643 cells treated with ABT-737. * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$ by unpaired, two-tailed t test.

Fig. 7. Proposed model of synergistic cytotoxicity between YK-4-279 and VCR in ES.

VCR and YK-4-279 induce a cell cycle arrest at the G₂-M transition, a presumed VCR-sensitive stage for microtubule depolymerization. YK-4-279 advances a potent G₂-M arrest that is sustained by persistent amounts of cyclin B1 after reduced expression of UBE2C. VCR and YK-4-279 induce spindle and centrosome perturbation in ES cells including decreased expression of microtubule associated proteins (MAPs). YK-4-279 then flips the final switch to apoptosis by altering the ratios of *MCL1* and *BCL2* transcripts and corresponding protein isoforms.