Dual Targeting of EWS-FLI1 Activity and the Associated DNA Damage Response with Trabectedin and SN38 Synergistically Inhibits Ewing Sarcoma Cell Growth

Patrick J. Grohar1, Laure E. Segars2, Choh Yeung2, Yves Pommier3, Maurizio D’Incalci4, Arnulfo Mendoza2, and Lee J. Helman2

Abstract

Purpose: The goal of this study is to optimize the activity of trabectedin for Ewing sarcoma by developing a molecularly targeted combination therapy.

Experimental Design: We have recently shown that trabectedin interferes with the activity of EWS-FLI1 in Ewing sarcoma cells. In this report, we build on this work to develop a trabectedin-based combination therapy with improved EWS-FLI1 suppression that also targets the drug-associated DNA damage to Ewing sarcoma cells.

Results: We demonstrate by siRNA experiments that EWS-FLI1 drives the expression of the Werner syndrome protein (WRN) in Ewing sarcoma cells. Because WRN-deficient cells are known to be hypersensitive to camptothecins, we utilize trabectedin to block EWS-FLI1 activity, suppress WRN expression, and selectively sensitize Ewing sarcoma cells to the DNA-damaging effects of SN38. We show that trabectedin and SN38 are synergistic, demonstrate an increase in DNA double-strand breaks, an accumulation of cells in S-phase and a low picomolar IC50. In addition, SN38 cooperates with trabectedin to augment the suppression of EWS-FLI1 downstream targets, leading to an improved therapeutic index in vivo. These effects translate into the marked regression of two Ewing sarcoma xenografts at a fraction of the dose of camptothecin used in other xenograft studies.

Conclusions: These results provide the basis and rationale for translating this drug combination to the clinic. In addition, the study highlights an approach that utilizes a targeted agent to interfere with an oncogenic transcription factor and then exploits the resulting changes in gene expression to develop a molecularly targeted combination therapy.
We believe that the disappointing clinical results with this drug can be explained by the corresponding pharmacokinetic data that only transiently achieves serum levels of drug high enough to inhibit EWS-FLI1 (29). This narrow therapeutic index is likely limited by the drug-induced DNA damage that limits the dosing of the drug. Therefore, we theorized that to improve the activity of the drug in the clinic for Ewing sarcoma, it would be necessary to focus on the DNA damage on Ewing sarcoma cells and develop a combination therapy that augments the suppression of EWS-FLI1 activity.

Because EWS-FLI1 is expressed only in Ewing sarcoma cells, we theorized that a common link between trabectedin, EWS-FLI1, and a specific DNA damage pathway could provide a tissue-specific sensitizer to a DNA-damaging agent. It would therefore be possible to utilize trabectedin to block EWS-FLI1 activity and expose a weakness to a second compound essentially creating a molecularly targeted combination therapy.

We utilized our previously published microarray data to interrogate the change in expression of various genes in all major DNA repair pathways and looked for overlap with EWS-FLI1 targets. We found that trabectedin treatment of Ewing sarcoma cells caused substantial suppression of expression of a gene called WRN. The Werner syndrome protein (WRN) is a REC Q helicase, found to be deficient in patients with the premature ageing syndrome known as Werner syndrome (30, 31). Patients with Werner syndrome have notable DNA repair defects responsible for the complex phenotype as well as a predisposition to develop cancer and in particular sarcomas (31). On the molecular level, WRN plays a role in telomere maintenance, transcription, and DNA damage repair based on its ability to modulate the cell cycle to facilitate repair (32). In addition, multiple reports have established that cells deficient in WRN are hypersensitive to camptothecins (33–35).

Our interest in this combination was further piqued when we identified (although did not validate) multiple camptothecins in a previously published high-throughput screen for EWS-FLI1 inhibitors, consistent with a more recent report that shows camptothecin to suppress critical EWS-FLI1 downstream targets (36–38). Therefore, we theorized that in this scenario, trabectedin would block EWS-FLI1 and expose a hypersensitivity to the DNA-damaging effect irinotecan or its active metabolite SN38, which would in turn feed back and augment the suppression of EWS-FLI1 downstream targets.

In this report, we show that trabectedin and SN38 (the active metabolite of irinotecan) are a synergistic molecularly targeted therapy for Ewing sarcoma cells that markedly enhances the cytotoxicity of trabectedin, leading to a low picomolar IC50, cell-cycle effects consistent with the WRN null phenotype and drug synergy in vitro. These effects cause the marked regression of 2 different Ewing sarcoma xenografts with only 4 to 6 doses of drug at a fraction of the dose of camptothecin used in other xenograft studies with no observable toxicity. Together, these results provide the basis and the rationale for the clinical translation of this drug combination for the treatment of Ewing sarcoma. In addition, they provide an example of an approach broadly applicable to a variety of tumors driven by oncogenic transcription factors that integrates the molecular pharmacology of traditional chemotherapeutic agents with the biology of the dominant oncogene to improve the therapeutic index of a drug and molecularly target chemotherapy.

Translational Relevance

The goal of this project is to improve the clinical activity of trabectedin for Ewing sarcoma. The work represents a bedside to bench and back again effort to characterize a novel mechanism of action of trabectedin, the poisoning of EWS-FLI1, and exploit the resulting changes in gene expression to develop a clinically relevant combination therapy.

Materials and Methods

Cell culture

All cell lines are patient derived. TC32, TC71, A673, CHLA9, CHLA10, and TC167 were the gift of Dr. T. Triche (The Saban Research Hospital, Children’s Hospital of Los Angeles, CA). EW8 were the gift of Dr. P. Houghton (Nationwide Children’s Hospital, Columbus, OH). The identity of TC32, TC71, A673, CHLA9, and EW8 Ewing sarcoma cell lines was independently authenticated by reverse-transcription PCR (RT-PCR) with specific primers for the translocation and sequencing of the product. CHLA10 and TC167 were confirmed by Dr. Triche by short tandem repeat genotyping but were not independently authenticated in this study (39). HT1080 cells were obtained from American Type Culture Collection where they are validated by short tandem repeat genotyping and transduced with EWS-FLI1 and GFP as previously described (16). HT1080s were not independently authenticated for this study. The cells were maintained in culture as previously described (39).

Quantitative PCR

TC32 cells were treated with trabectedin for 12 hours, collected using the RNEasy Kit with a Qiashredder (Qiagen) and reverse transcribed using the High Capacity cDNA Synthesis Kit (Life Technologies) in biological and technical replicates of 3 (25°C 10 minutes, 37°C 120 minutes, 85°C 5 minutes). The PCR reaction was performed with 500 ng of template and the TaqMan probes HS99999905 for VIC-GAPDH and FAM-WRN HS01087904 (Applied Biosystems; 50°C 2 minutes, 95°C 10 minutes, 95°C 15 seconds, 60°C 1 minute) using the CFX 96 (Bio-Rad) and quantitated using standard ΔΔCT methods.

Immunoblot analysis

Immunoblotting was performed as previously described (39) using the following antibodies: mouse monoclonal anti-FLI1 (1.5 μg/mL; Becton Dickinson), anti-WRN.
higher than the IC50 of trabectedin or 0.5 nmol/L instead of tency among experiments, we used a concentration slightly puSyn software (ComboSyn; refs. 41 and 42). For consis-
garanteed that did not establish tumors [(TC71: control (irinotecan cohort). Mice were removed from the exper-
Cell viability was measured as previously described (39) using the bioreductive MTS assay (CellTiter 96; Promega) at 490 nm on a VersaMax 96-well plate reader (Molecular Devices). The IC50 was determined using Prism software (GraphPad) from at least 3 independent experiments. The combination index (CI) as a measure of drug synergy was determined over 7 concentrations at a fixed ratio of IC50s according to the method of Chou and Talalay using CompuSyn software (ComboSyn; refs. 41 and 42). For consistency among experiments, we used a concentration slightly higher than the IC50 of trabectedin or 0.5 nmol/L instead of 0.4 nmol/L. Data points were included that fell within the range of 1% to 99% of control. To get the curves to fit the data, points were excluded so that there were equal numbers of points in the individual and combination treatments; however, to minimize bias, the experimental data points are also reported because these points proved to be invariant to the number of points with a narrower dynamic range.

Immunochemistry

TC32 cells were seeded in Lab-Tek II 4 chamber wells (Nunc) at a density of 25,000 cells/well and prepared for ICC (39), probed with primary mouse monoclonal anti-phospho-histone H2A.X (ser 139; γH2AX; 1:1,000 dilution; Millipore) overnight at 4°C, washed with PBS, incubated with Alexa488-labeled anti-mouse immunoglobin G (1:200 dilution; Millipore) for 1 hour in the dark and visualized with a Zeiss 510 confocal microscope in the presence of DAPI in VectaShield mountind medium (Vector Labs) with standard settings that were not changed among treatment groups.

siRNA sensitization experiment

Five microliters of a 400 nmol/L solution of siRNA targeting WRN (Qiagen; #si02663759), EWS-FL1, or a nontargeting siRNA control (siNeg) was complexed with RNaMax (Life Technologies) for 30 minutes, combined with 2000 TC32 or 4000 TC71 cells and incubated for 48 hours. SN38 was subsequently added to achieve a final concentration of 20 nmol/L for the siRNA and 1 or 0.5 nmol/L of SN38 for a total of 110 (TC71) to 120 hours (TC32). Cell viability was monitored using the Incucyte Zoom (Essen Biosciences) imaging microscopy system and percent confluence determined by the system’s imaging algorithm.

Cell-cycle analysis

TC32 Cells were plated, allowed to recover overnight, and then incubated with drug at the specified concentration for 18 hours. Following collection in PBS, the cells were fixed in 70% ethanol for 3 hours at 4°C, washed with PBS, and stained with propidium iodide (0.02 mg/mL) in 0.1% Triton X-100 with RNase A for 30 minutes at room temperature. The cells were filtered and analyzed by with the Fortessa analytical cytometer (BD biosciences). DNA content was used to determine cell-cycle phase.

Luciferase assays

The luciferase and corresponding viability assays were performed in parallel with 8-hour incubations of drug as previously described (16). Bioluminescence was measured after 10 minutes of equilibration on the Victor3 multilabel counter (Perkin Elmer).

Xenograft experiments

Two million TC32 or TC71 cells were inoculated by intramuscular injection in the left gastrocnemius of 4- to 6-week-old female SCID-bg mice (CB17 Cg-Prkdcsid/Lysbg/Crl) (Charles River Laboratories), established to a minimum diameter of 0.5 cm (volume of 65 mm3) and then mice were randomized to treatment groups. Two mice were sacrificed for expression studies on days 4 and 8 (control, trabectedin, combination cohorts) or day 8 (irinotecan cohort). Mice were removed from the experiment that did not establish tumors [(TC71: control cohort (n = 1); TC32: trabectedin (n = 1), irinotecan cohorts (n = 3)) or if the tumor ulcerated (TC32, control, n = 1). Four cohorts of 12 to 15 mice with established TC71 or TC32 xenografts were treated with vehicle controls, 0.15 mg/kg/day of intravenous trabectedin (the MTD) administered via tail vein on days 1 and 8, irinotecan at 5 mg/kg/day administered intraperitoneal on days 4 to 5, and 11 to 12 in TC71 or days 4 to 5 only for TC32 or the combination of trabectedin and irinotecan on the identical dose and schedule. Irinotecan was used at submaximal doses, determined by a dose finding experiment, to demonstrate a combined effect. Irinotecan was used for the in vivo for clinical relevance despite decreased potency in vitro. The volume of the tumors was measured 3 times per week and determined using the

Compounds

Trabectedin was obtained from PharmaMar. Irinotecan and SN38 (Sigma-Aldrich) were aliquoted, stored frozen, and reconstituted immediately before use. SN38 is the active metabolite of irinotecan, which is activated by plasma esterases. To eliminate the confounding effects of FBS serum esterases, SN38 was used for all in vitro experiments. In contrast, irinotecan was used for the in vivo experiments because of the goal of translating this therapy to the clinic.

siRNA silencing

RNAiMax Lipofectamine (4.5 µL per well in a 6-well dish; Life Technologies) was added to siRNA targeting the EWS-FL1 breakpoint site II (40), complexed, combined with cells, and incubated for 36 to 48 hours before collection for Western blot analysis.

Cell viability assays

Cell viability was measured as previously described (39) with standard settings that were not changed among experiments. Immunocytochemistry

TC32 cells were plated, allowed to recover overnight, and then incubated with drug at the specified concentration for 18 hours. Following collection in PBS, the cells were fixed in 70% ethanol for 3 hours at 4°C, washed with PBS, and stained with propidium iodide (0.02 mg/mL) in 0.1% Triton X-100 with RNase A for 30 minutes at room temperature. The cells were filtered and analyzed by with the Fortessa analytical cytometer (BD biosciences). DNA content was used to determine cell-cycle phase.

Luciferase assays

The luciferase and corresponding viability assays were performed in parallel with 8-hour incubations of drug as previously described (16). Bioluminescence was measured after 10 minutes of equilibration on the Victor3 multilabel counter (Perkin Elmer).

Xenograft experiments

Two million TC32 or TC71 cells were inoculated by intramuscular injection in the left gastrocnemius of 4- to 6-week-old female SCID-bg mice (CB17 Cg-Prkdcsid/Lysbg/Crl) (Charles River Laboratories), established to a minimum diameter of 0.5 cm (volume of 65 mm3) and then mice were randomized to treatment groups. Two mice were sacrificed for expression studies on days 4 and 8 (control, trabectedin, combination cohorts) or day 8 (irinotecan cohort). Mice were removed from the experiment that did not establish tumors [(TC71: control cohort (n = 1); TC32: trabectedin (n = 1), irinotecan cohorts (n = 3)) or if the tumor ulcerated (TC32, control, n = 1). Four cohorts of 12 to 15 mice with established TC71 or TC32 xenografts were treated with vehicle controls, 0.15 mg/kg/day of intravenous trabectedin (the MTD) administered via tail vein on days 1 and 8, irinotecan at 5 mg/kg/day administered intraperitoneal on days 4 to 5, and 11 to 12 in TC71 or days 4 to 5 only for TC32 or the combination of trabectedin and irinotecan on the identical dose and schedule. Irinotecan was used at submaximal doses, determined by a dose finding experiment, to demonstrate a combined effect. Irinotecan was used for the in vivo for clinical relevance despite decreased potency in vitro. The volume of the tumors was measured 3 times per week and determined using the
Figure 1. EWS-FLI1 drives expression of WRN, an activity which is blocked by trabectedin treatment. A, mean (±SEM) fold change in WRN expression as a function of GAPDH (2\(^{-\Delta\Delta CT}\)) as measured by qPCR for treatment with solvent control (S) (1.2 ± 0.1), 10 nmol/L (0.03 ± 0.01), 7.5 nmol/L (0.08 ± 0.007), or 5 nmol/L trabectedin (0.23 ± 0.02) for 12 hours, P < 0.0001. Data representative of 3 independent experiments. B, Western blot time course experiment showing expression of WRN as a function of trabectedin treatment over time (hours) relative to NR0B1, ID2, PARP cleavage, and ACTB (loading control). C, Western blot showing expression of WRN as a function of 10 nmol/L trabectedin for 18 hours (ET) or solvent control (SC) in 7 different Ewing sarcoma cell lines. Western blots representative of 3 independent experiments (time course) or 2 to 3 different experiments in 7 cell lines (cell line panel). D, Western blot showing expression of EWS-FLI1, WRN, the EWS-FLI1 target gene ID2, or other DNA damage genes XRCC4, ATM, KU80, and PRKDC for the medium control (C), lipid control (L), or siRNA silencing with a nontargeting siRNA (−) or siRNA targeting EWS-FLI1 (siEF) or SP1 (siSP1) in TC32 Ewing sarcoma cells or 2 other Ewing sarcoma cell lines TC71 or EW8. Data representative of 2 (EW8) or 3 independent experiments (TC32 and TC71).
equation \((D \times d^3)/6 \times 3.12\) (where \(D\) is the maximum diameter and \(d\) is the minimum diameter). Tissue was collected and immediately placed in 10% formalin for immunohistochemistry. The remaining mice were sacrificed when the tumor diameter reached 2 cm in any direction (volume approx. 3,500 mm³). Prism software
Immunohistochemistry was performed in accordance with the guidelines and regulations of and approved by the Animal Care Use Committee at the National Cancer Institute in Bethesda, MD.

**Table 1.** CI as a measurement of synergy for trabectedin and SN38 relative to trabectedin and doxorubicin for experimental data points and as determined by the median effect equation where the value of the CI indicates synergistic (<1), additive (=1), and antagonistic (>1) drug interactions and the smaller the value the greater the synergy

<table>
<thead>
<tr>
<th>Experimental points</th>
<th>2× IC$_{50}$</th>
<th>IC$_{50}$</th>
<th>0.5× IC$_{50}$</th>
<th>Median effect equation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ED$_{50}$</td>
</tr>
<tr>
<td>Trabectedin and SN38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC32</td>
<td>0.84 (±0.02)</td>
<td>0.57 (±0.02)</td>
<td>1.05 (±0.12)</td>
<td>0.51 (±0.06)</td>
</tr>
<tr>
<td>TC71</td>
<td>0.85 (±0.03)</td>
<td>0.72 (±0.04)</td>
<td>0.8 (±0.03)</td>
<td>0.23 (±0.05)</td>
</tr>
<tr>
<td>Trabectedin and doxorubicin</td>
<td>1.18 (±0.06)</td>
<td>0.84 (±0.03)</td>
<td>1.11 (±0.05)</td>
<td>0.87 (±0.05)</td>
</tr>
<tr>
<td>TC32</td>
<td>0.95 (±0.01)</td>
<td>0.85 (±0.03)</td>
<td>1.12 (±0.11)</td>
<td>0.65 (±0.12)</td>
</tr>
<tr>
<td>TC71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data represent the mean value (±SEM) from 3 independent experiments.

(GraphPad) was used to generate survival curves, as determined by time to 2 cm in any direction. All experiments were performed in accordance with the guidelines and regulations of and approved by the Animal Care Use Committee at the National Cancer Institute in Bethesda, MD.

**Results**

**Treatment of Ewing sarcoma cells with trabectedin suppresses expression of WRN mRNA and protein**

To identify genes driven by EWS-FLI1 that were suppressed with trabectedin treatment of Ewing sarcoma cells, we evaluated the expression of all the major DNA repair genes using our previously published microarray data (16). We found that XRCC4 and WRN were highly expressed in Ewing sarcoma cells and changed in expression from a 2-fold change in expression of EWS-FLI1 with trabectedin treatment as previously reported (16).

**siRNA silencing of the EWS-FLI1 transcription factor suppresses WRN expression in Ewing sarcoma cells**

To link WRN expression to EWS-FLI1, we utilized siRNA to silence EWS-FLI1 and found a marked suppression of WRN expression in 3 different Ewing sarcoma cell lines that was not seen with the lipid or nontargeting controls (ref. 40; Fig. 1D). This suppression mirrored that of another EWS-FLI1 target, ID2 (Fig. 1D). Furthermore, other DNA repair genes such as ATM, KU80, or DNA-PK (PRKDC) were not suppressed with EWS-FLI1 silencing. As an additional control, we silenced SP1 with siRNA and found no suppression of WRN.

**Trabectedin-mediated suppression of WRN sensitizes Ewing sarcoma cells to camptothecins to a greater degree than doxorubicin in an EWS-FLI1–restricted manner**

To confirm that we could induce a hypersensitivity to camptothecins in Ewing sarcoma cells by suppressing WRN, we treated TC32 and TC71 cells with the camptothecin SN38 (the active metabolite of irinotecan) over a range of concentrations and found a marked enhancement in cytotoxicity of SN38 at concentrations of trabectedin as low as 0.5 and 0.25 nmol/L or concentrations centered on our previously reported IC$_{50}$ of 0.4 nmol/L (Fig. 2A). To evaluate the shift in IC$_{50}$, we expressed the data normalized to the effect of trabectedin treatment alone and found that 0.5 nmol/L trabectedin shifted the IC$_{50}$ of SN38 from 1.7 nmol/L (95% CI, 1.6–1.8) to 0.02 nmol/L (95% CI, 0.02–0.03) in TC32 cells and from 1.4 nmol/L (95% CI, 1.3–1.4) to 0.06 nmol/L (95% CI, 0.05–0.09) in the TC71 cell line (Fig. 2B). A similar effect was not observed with the topoisomerase II inhibitor doxorubicin, where the addition of 0.5 nmol/L trabectedin does not cause a shift in the dose–response curves leading to nearly identical IC$_{50}$ of 53 nmol/L (95% CI, 44–64) and 41 nmol/L (95% CI, 33–52) for doxorubicin and doxorubicin plus trabectedin, respectively (Fig. 2C).
Figure 3. Trabectedin and SN38 cooperate to increase DNA double-strand breaks and augment the suppression of EWS-FLI1 downstream targets relative to either agent alone. A, single cell imaging showing DNA double-strand breaks as measured by the formation and immunostaining of γH2AX foci (green) with DAPI-stained nuclei for reference (blue) following treatment of TC32 cells with 5 nmol/L trabectedin, SN38, or the combination for 6 hours. B, populations of TC32 cells showing the increased penetrance of γH2AX in the combination therapy relative to each agent alone. White arrows highlight unaffected cells. Quantitation of γH2AX foci in 50 to 65 cells from 8 different images in each of the treatment groups, control (C), trabectedin (T), SN38 (SN), or the combination (T&SN). (Continued on the following page.)
To further establish the link between EWS-FLI1 and cellular sensitivity to the combination of trabectedin and SN38, we transduced HT1080 cells (that normally do not express EWS-FLI1) with EWS-FLI1 and found a marked shift in the sensitivity of the cells that correlated with EWS-FLI1 expression. It is known that forced expression of EWS-FLI1 in other cell types such as rhabdomyosarcoma and neuroblastoma cells changes the morphology and gene expression to resemble an Ewing sarcoma phenotype and genotype (43, 44). More recently, this has been shown to confer drug sensitivity to the recipient cell line (45). We showed that the expression of EWS-FLI1 caused a shift in the IC50 of SN38 from 147 nmol/L in the parent EWS-FLI1 null cells (95% CI, 123–177) to 15 nmol/L in the EWS-FLI1 expressing HT1080 cells (95% CI, 12.6–18.7). The addition of trabectedin to SN38 further augments this sensitization dropping the IC50 to 3.4 nmol/L (95% CI, 3–3.9), almost 7 times lower than the IC50 of 21 nmol/L (95% CI, 17.9–25.2) of the combination in EWS-FLI1 null cells (Fig. 2D).

Finally, we used the method of Chou and Talalay to calculate the CI to determine if the combination of trabectedin and SN38 was synergistic (CI < 1), additive (CI = 1), or antagonistic (CI > 1; refs. 42 and 46). We found that treatment of TC32 and TC71 cell lines with trabectedin and SN38 met criteria for synergy and strong synergy as determined by the median effect equation with values of 0.51 (SEM ± 0.06) and 0.23 (SEM ± 0.05), respectively. Again, we were able to utilize doxorubicin as a reference point and found the predicted degree of synergy. In this case, the combination with doxorubicin showed slight synergy in TC32 with a CI of 0.87 (SEM ± 0.05) and synergy in TC71 with a CI of 0.65 (SEM ± 0.12; Table 1).

**Treatment of Ewing sarcoma cells with trabectedin and SN38 increases DNA double-strand breaks relative to either agent alone**

To evaluate the ability of the drugs to induce DNA damage in Ewing sarcoma cells, we measured the phosphorylation of histone H2AX to generate γH2AX foci by immunocytochemistry and confocal microscopy. We found a significant increase in the number of γH2AX foci in single TC32 Ewing sarcoma cells treated with the combination therapy relative to either agent alone (Fig. 3A). In addition, the DNA damage was more penetrant. Although, trabectedin and SN38 demonstrate considerable γH2AX foci per cell, there is an appreciable variability in this damage as some cells are relatively spared (as indicated by the arrows in Fig. 3B). To quantitate these effects, we randomly selected 50 to 65 cells from 8 different images and manually counted the number of foci per cell. As shown in the dot plot in Fig. 3B, treatment with both SN38 and trabectedin increased the mean number of foci per cell to 9 from 5 and 4, respectively [P = 0.03 (SN38 and Combination); P = 0.0009 (trabectedin and Combination)].

**Trabectedin and SN38 cooperate to suppress EWS-FLI1 activity and downstream target expression**

A recent report indicates that camptothecins directly block the expression of critical EWS-FLI1 downstream targets (36). To evaluate the ability of the combination therapy to interfere with EWS-FLI1 activity, we first evaluated the effect of drug treatment on downstream target promoter activity using our previously published NR0B1 promoter construct (39). We found that the combination had increased suppression of EWS-FLI1–driven luciferase activity relative to either agent alone. At 8 hours of treatment, 5 nmol/L trabectedin suppressed luciferase activity to 69% of control (95% CI, 62–76), 5 nmol/L SN38 to 59% of control (95% CI, 54–63) and the combination suppressed luciferase activity to 41% of control (95% CI, 36–47). The same effect was observed at lower concentrations of SN38, where 1 nmol/L of SN38 led to the suppression of luciferase to 80% of control (95% CI, 76–84), a level that was further suppressed to 53% of control (95% CI, 47–57) when combined with 5 nmol/L trabectedin (Fig. 3C). Furthermore, there was limited suppression of a constitutively active control, where a minimal induction of luciferase activity occurred with 5 nmol/L trabectedin, whereas the combination suppressed to 91% of control (95% CI, 84–98) for 5 nmol/L of both agents and 95% of control (95% CI, 91–99) for 5 nmol/L trabectedin and 1 nmol/L SN38 (Fig. 3C).

This combined suppression of EWS-FLI1 activity translated to the protein level. Both SN38 and trabectedin suppressed the expression of the EWS-FLI1 downstream target ID2 when used alone; however, the combination markedly increased this suppression, particularly at 1 nmol/L [see (‘’ in Fig. 3D)]. In addition, these Western blots confirmed the increase in DNA damage that occurs when these agents are combined as measured by increased phosphorylation of γH2AX (Fig. 3D).

**Silencing of WRN sensitizes Ewing sarcoma cells to SN38 treatment**

To demonstrate that suppression of WRN is a key component of the mechanism of this drug combination, we utilized siRNA to silence WRN and sensitize cells to treatment with the camptothecin, SN38. Silencing of WRN in TC32 Ewing sarcoma cells led to a substantial reduction in viability from 73.3% (SEM ± 2.4) to 36.4% (SEM ± 3.7) in WRN null cells (Fig. 4A). It is notable that siRNA-mediated suppression of EWS-FLI1 expression profoundly suppressed cell growth with a slight,
although statistically significant enhancement with treatment with 1 nmol/L SN38 from 19.9% (SEM ± 0.2) to 12.8% (SEM ± 0.3; P = 0.0001). A similar effect was observed with siRNA targeting of WRN in TC71 Ewing sarcoma cells even at 0.5 nmol/L SN38, resulting in a decrease in percent confluence from 99% (SEM ± 0.2) to 70% (SEM ± 3.8; Fig. 4B).

Trabectedin-mediated suppression of WRN recapitulates the WRN null phenotype

To recapitulate the WRN null phenotype, we measured the effect of treatment with these agents on the cell cycle at a time frame and concentration before substantial cell death (47–49). It has been suggested that the sensitivity of WRN null cells to camptothecins stems from an accumulation of the cells in S-phase because of stalled replication forks that cannot be resolved, leading to increased fork collision, and S-phase-specific apoptosis (33). Consistent with this idea, cells deficient in WRN accumulate in S or G2–M phase with a loss of cells in G1 phase leading to cells with an increased DNA index because of the inability to complete the cell cycle (50, 51).

We found that treatment of Ewing sarcoma TC32 cells with 1 nmol/L trabectedin for 18 hours results in an increase in cells in the S-phase from a mean of 27.3% (±0.3) in the solvent control to 62.4% (±0.5; P < 0.0001) in the trabectedin-treated cells, an effect not observed with the SN38-treated cells that showed no change in the percentage of cells in S-phase (Fig. 4C and D). Indeed, treatment with the combination of trabectedin and SN38 led to a comparable increase of cells in S-phase to 59% (±3.3) as well as an increase in the sub-G0 apoptotic fraction from 4.1% (±0.3) in the solvent control to 14.5% (±0.4; Fig. 4C and D).

The combination of trabectedin and SN38 regress Ewing sarcoma xenografts

To evaluate the effect of combination treatment on the therapeutic index of the drug, we evaluated the combination in xenograft models of Ewing sarcoma. We treated mice at the recommended dose of trabectedin of 0.15 mg/kg/dose q week and 5 mg/kg/dose of irinotecan on days 4 and 5 in the TC32 xenograft and on days 4 and 5, and 11 and 12 in the TC71 xenograft. We used irinotecan for the in vivo experiments because it is a prodrug of SN38, overcomes issues related to administration of SN38 to patients, and has been extensively evaluated in the clinic.

Both individual agents caused some regression of tumor (Supplementary Fig. S2). However, a marked and sustained regression of both tumors was observed in the mice treated with the combination therapy that was more pronounced than treatment with either agent alone, as shown in the dot plot of the TC32 and TC71 xenografts (Fig. 5A and B). On day 15 of treatment, all the mice in the TC71 control cohort had to be sacrificed with a mean tumor volume of 3,768 mm³ (95% CI, 3,298–4,238). Tumor growth was suppressed to 3,066 mm³ (95% CI, 3,284–4,238) in the TC71 treated with 1 nmol/L SN38. A similar result was obtained in the TC32 xenograft at day 24 of treatment, demonstrating mean tumor volumes of 3,432 mm³ (95% CI, 2,884–3,980) in the control treatment, 2,032 mm³ (95% CI, 1,281–2,782) with trabectedin treatment, and 1,347 mm³ (95% CI, 377–2,318) for those mice treated with irinotecan. Again, there was very little if any palpable tumor in the mice treated with the combination therapy that collectively had a mean tumor volume of 77 mm³ (95% CI, 21–132; Fig. 5B). These regressions persisted after treatment ended and no retreatment was performed. It is notable that every mouse in both cohorts treated with the combination therapy had a complete regression to no palpable tumor.

Next, we performed immunohistochemical analysis on the tissue collected from the TC71 xenograft on day 8 to recapitulate the biochemistry of this drug combination in the in vivo setting. Treatment of the mice with a combination of trabectedin and irinotecan led to a substantial increase in the γH2AX staining of tissue (Fig. 5C). Furthermore, treatment with the combination therapy recapitulated the suppression of WRN that we saw in vitro where control cells show intense nuclear and cytoplasmic staining for WRN, and the tissue from the mouse treated with irinotecan and trabectedin show less WRN staining that seems to shift from the nucleus and cytoplasm to the stroma or cytoplasmic membrane (Fig. 5C).

These effects combined to greatly extend the survival of the mice, determined as time to 2 cm, relative to the control mice (Fig. 5D). The TC32 xenograft achieved a roughly doubled survival relative to the single-agent–treated mice with at least 2 mice achieving a complete cure with only one cycle of therapy representing only 4 doses of drug. The TC71 mice also showed an increase in survival that was roughly 6 times that of the control and triple that of the single-agent–treated mice even though dosing of drug occurred only in the first 10 to 11 days of the study. No retreatment was performed because of the local toxicity of trabectedin, a toxicity that would not limit dosing in the clinic in patients with central venous access.

Discussion

The targeting of dominant oncogenes with small molecules has led to impressive clinical responses for some tumor types. For other tumors, particularly those with challenging small molecule drug targets, such as transcription factors, methods are needed to effectively target these oncogenes. In this report, we propose a concept called molecularly targeted chemotherapy. The principle is to utilize an agent to interfere with the activity of the dominant oncogene and then exploit the changes in gene expression to target the toxicity of a second traditional chemotherapeutic agent to the cancer cell.

We have previously shown that trabectedin blocks the activity of the dominant oncogene of Ewing sarcoma cells, the EWS-FLI1 transcription factor. This blockade likely cooperates with other mechanisms of the drug to account
Figure 4. Recapitulation of the WRN null phenotype. A, siRNA mediated silencing of WRN (siWRN) for 48 hours sensitizes TC32 Ewing sarcoma cells to treatment with 1 nM SN38. The controls are a nontargeting siRNA (siNEG) and a siRNA targeting EWS-FLI1 (siEWS-FLI1). B, silencing of WRN for 48 hours sensitizes TC71 Ewing sarcoma cells to treatment with 0.5 nM SN38. The controls are a nontargeting siRNA (siNEG) and a siRNA targeting EWS-FLI1 (siEWS-FLI1). C, cell-cycle effects of ET743 and SN38 that recapitulate the WRN null phenotype. Treatment of TC32 Ewing sarcoma cells with 1 nM Trabectedin and SN38 for 18 hours shifts the cell cycle to S and G2-M as shown in these representative histogram plots. D, column statistics for the treatment groups for the sub-G0, G1, S, and G2-M gates. Treatment groups are untreated (medium), solvent control (solvent), 1 nM Trabectedin (Trabectedin), and 1 nM SN38 (SN38). Data represent the mean (± SD) from at least 2 independent experiments. \( P \) values represent the statistical significance of the changes in S-phase relative to the solvent control and the comparisons are denoted with + or ++.
Figure 5. The combination of trabectedin and SN38 suppresses Ewing sarcoma xenograft growth. A, mean (±SEM) tumor volume for each mouse in the TC71 cohort on day 15 for the 4 treatment groups: solvent control (C; n = 10), 0.15 mg/kg trabectedin (days 1 and 8; Trab., n = 11), 5 mg/kg Irinotecan (days 3, 4, 10, 11; I, n = 13), or the combination of 0.15 mg/kg trabectedin (days 1 and 8) and 5 mg/kg Irinotecan (days 3, 4, 10, 11; Tr and I, n = 13). B, mean (±SEM) tumor volume for each mouse in the TC32 cohort on day 24 for the 4 treatment groups: solvent control (C; n = 10), 0.15 mg/kg trabectedin (days 1 and 8; Trab., n = 10), 5 mg/kg Irinotecan (days 3, 4, I, n = 10), or the combination of 0.15 mg/kg trabectedin (days 1 and 8) and 5 mg/kg Irinotecan (days 3, 4, Tr and I, n = 13). C, immunohistochemical staining of TC71 xenografts with H&E and immunohistochemical staining for WRN and γH2AX for vehicle control and mice treated with trabectedin plus irinotecan. D, survival curves for mice bearing the TC32 and TC71 xenografts demonstrating time to 2 cm in the 4 treatment groups, control (black), trabectedin (red), irinotecan (blue), and combination (green).
the extreme sensitivity of Ewing sarcoma cells to trabectedin treatment in vitro. Unfortunately, the clinical translation of this sensitivity has not been realized. Early clinical experience with trabectedin suggested a particular sensitivity of Ewing sarcoma to this compound with a 25% response rate in one series as well as a documented complete response with single-agent trabectedin therapy in a different phase I study (16–20, 27, 28). Unfortunately, the compound was not active in Ewing sarcoma in a more recent follow-up phase II study (29).

We believe the failure of the drug in the phase II was because of a narrow therapeutic index of the drug that limited the dosing and serum levels of drug to levels insufficient to inhibit EWS-FLI1. In this clinical study, patients achieved serum levels of 3.26 ± 2.95 nmol/L (29). These levels are lower than the 5 to 10 nmol/L concentration of trabectedin that we have previously shown suppresses EWS-FLI1 downstream target expression in vitro. Therefore, the goal of this study was to improve the therapeutic index of the drug by developing a combination therapy with enhanced EWS-FLI1 suppression that focused the collateral drug-induced DNA damage on Ewing sarcoma cells. To develop this combination, we showed that EWS-FLI1 drives the expression of the DNA damage response gene, Werner syndrome helicase (WRN). Because it is known that cells deficient in WRN are hypersensitive to camptothecins, we utilized trabectedin to suppress WRN expression and selectively sensitize Ewing sarcoma cells to treatment with camptothecin (40–42). The camptothecin, in turn, augmented the suppression of EWS-FLI1 targets, making the blockade of EWS-FLI1 achievable in vivo thus causing the regression of 2 established Ewing sarcoma xenograft tumors with only 4 to 6 doses of drug at one tenth the maximum tolerated dose of irinotecan used in other xenograft experiments with virtually no appreciable systemic toxicity (36, 52). We established that the mechanism of this combination was linked to augmented suppression of EWS-FLI1 targets as well as an increase in DNA damage in Ewing sarcoma cells likely mediated by a suppression in WRN expression. Finally, we definitively established the link to EWS-FLI1 by transferring this sensitization to another cellular context, HT1080 cells.

Together, these data and concepts provide the basis for translating this combination therapy to the clinic for the treatment of Ewing sarcoma. It is not clear if other cell types would also have enhanced sensitivity to this combination as a prior report shows activity in rhabdomyosarcoma cells (53). Nevertheless, the results provide the basis for a new strategy of drug targeting for a variety of other tumors driven by dominant oncogenes that integrates the biology of a tumor with the pharmacology of a traditional chemotherapeutic agent to develop a targeted therapy. The end result is a molecularly targeted chemotherapy regimen that would be expected to have activity in the clinic with the hope of improving survival for patients with Ewing sarcoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names or organizations imply endorsement by the U.S. Government. The funders had no role in the design of the study, the collection, analysis, and interpretation of the data; the decision to submit the article; or the writing of the article.

Authors' Contributions
Conception and design: P.J. Grohar, M. D'Incalci, A. Mendoza, L.J. Helman
Development of methodology: P.J. Grohar, L.E. Segars, A. Mendoza, L.J. Helman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.J. Grohar, L.E. Segars, C. Yeung, A. Mendoza
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.J. Grohar, L.E. Segars, Y. Pommier, A. Mendoza
Writing, review, and/or revision of the manuscript: P.J. Grohar, L.E. Segars, Y. Pommier, M. D’Incalci, A. Mendoza, L.J. Helman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.J. Grohar, A. Mendoza
Study supervision: P.J. Grohar, A. Mendoza, L.J. Helman

Acknowledgments
The authors thank C. Warren of the Vanderbilt Medical Center Flow Cytometry for sharing resource core for obtaining the cell-cycle data.

Grant Support
This work has been supported in part by a grant from St. Baldrick’s research foundation (P.J. Grohar and L.E. Segars). P.J. Grohar also receives grant support from the Lily’s Garden Foundation, the Westhaven foundation, Hyundai Hope on Wheels, the Sarcoma Alliance for Research through Collaboration, and the Turner-Hazinski Award at the Monroe Carell Jr. Children’s Hospital at Vanderbilt University. The intramural program at the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400 (L.J. Helman), also funded this project.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 5, 2013; revised October 21, 2013; accepted November 6, 2013; published OnlineFirst November 25, 2013.

References
7. Robin TP, Smith A, McKinsey E, Reaves L, Jedlicka P, Ford HL. EWS/FLI1 regulates EYA3 in Ewing sarcoma via modulation of miRNA-708,

www.aacrjournals.org
Clin Cancer Res; 20(5) March 1, 2014

Downloaded from clincancerres.aacrjournals.org on January 14, 2018. © 2014 American Association for Cancer Research.
13. Rossi ML, Ghosh AK, Bohr VA. Roles of Werner syndrome protein in protection of genome integrity. DNA Repair (Amst) 2010;9:331–44.
or premature mitosis depending on the concentration of 7-hydroxys-
Dual Targeting of EWS-FLI1 Activity and the Associated DNA Damage Response with Trabectedin and SN38 Synergistically Inhibits Ewing Sarcoma Cell Growth


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0901

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/11/25/1078-0432.CCR-13-0901.DC1
http://clincancerres.aacrjournals.org/content/suppl/2014/03/04/1078-0432.CCR-13-0901.DC2

Cited articles
This article cites 51 articles, 23 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/5/1190.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/5/1190.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/20/5/1190.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.