Published in final edited form as:

Clin Cancer Res. 2014 March 01; 20(5): 1190–1203. doi:10.1158/1078-0432.CCR-13-0901.

Dual targeting of EWS-FLI1 activity and the associated DNA damage response with Trabectedin and SN38 synergistically inhibits Ewing sarcoma cell growth

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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 ES cells.

Results—We demonstrate by siRNA experiments that EWS-FLI1 drives the expression of the Werner Syndrome protein (WRN) in ES cells. Since WRN deficient cells are known to be hypersensitive to camptothecins, we utilize trabectedin to block EWS-FLI1 activity, suppress WRN expression and selectively sensitize ES 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.

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Conflicts of Interest: None

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.

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.

Introduction

Ewing Sarcoma (ES) is the second most common malignant bone tumor of childhood with a peak incidence in the second decade of life (1). The molecular feature that drives the malignant phenotype in ES cells is the EWS-FLI1 transcription factor, generated by the t(11;22)(q24;q12) chromosomal translocation (2). This constitutively active transcription factor generates a pro-survival transcriptional program permissive for the evasion of apoptosis and senescence and has been linked to a variety of high-risk features (3–14). In addition, siRNA, antisense DNA and dominant negative methods have established that silencing of EWS-FLI1 leads to a substantial reduction in the ability of the cells to proliferate in vitro or establish tumors in vivo (15). Therefore, small molecule therapies that target EWS-FLI1 would in theory block proliferation and more importantly target the dominant oncogene that drives expression of the genes responsible for high-risk features.

We have recently shown that trabectedin (ET-743, Yondelis™) interferes with the activity of EWS-FLI1, reverses the expression of the EWS-FLI1 induced gene signature and blocks the promoter activity and expression of critical EWS-FLI1 downstream targets (16). In Ewing sarcoma cells, these effects likely cooperate with other established mechanisms of action of trabectedin, that both generate DNA damage and poison the DNA damage response, making ES cells among the most sensitive to the drug in vitro (16–26). Unfortunately, although early studies suggested a particular sensitivity of ES to trabectedin in the clinic, a more recent phase II study did not show activity in this tumor type (27–29).

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 ES, it would be necessary to focus the DNA damage on Ewing sarcoma cells and/or develop a combination therapy that augments the suppression of EWS-FLI1 activity.

Since 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 WRN protein is a REC Q helicase,

found to be deficient in patients with the premature aging syndrome known as Werner's syndrome (30, 31). Werner syndrome patients 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 two different ES xenografts with only 4–6 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.

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 ES 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 (Manassas, VA) 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 Polymerase Chain Reaction (qPCR)

TC32 cells were treated with trabectedin for 12 hours, collected using the RN Easy kit with a QIAshredder (Qiagen, Gaitersburg, MD) and reverse transcribed using the High Capacity cDNA synthesis kit (Life Technologies) in biological and technical replicates of 3 (25°C 10 min, 37°C 120 min, 85°C 5 min). 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 min, 95°C 10 min, 95°C 15 sec, 60°C 1 min) using the CFX 96 (Biorad, Hercules, CA) and quantitated using standard CT methods.

Immunoblot analysis

Immunoblotting was performed as previously described (39) using the following antibodies: mouse monoclonal anti-FLI1 (1.5 µg/mL; Becton Dickinson (BD), Franklin Lakes, NJ), anti-WRN (1:1000, Cell Signaling, Danvers, MA), anti-ATM (1:1000, Cell Signaling), anti-XRCC4 (1:1000, Abcam, Cambridge, UK), rabbit monoclonal anti-ID2 (1:1000 dilution; Cell Signaling), rabbit polyclonal anti-NR0B1 (1:1000 dilution; Abcam), mouse monoclonal anti-PARP (1:1000 dilution, Cell Signaling), rabbit polyclonal anti-ACTB (1:10,000 dilution, Abcam), mouse monoclonal anti-phospho-histone H2A.X (ser 139)(γH2AX) (1:1000 dilution, Millipore, Billerica, MA).

Compounds

Trabectedin was obtained from PharmaMar (Madrid, Spain). Irinotecan and SN38 (Sigma-Aldrich) were aliquoted, stored frozen and reconstituted immediately prior to 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-FLI1 breakpoint site II (40), complexed, combined with cells and incubated for 36 to 48 hours prior to collection for western blot analysis.

Cell viability assays

Cell viability was measured as previously described (39) using the bio-reductive MTS assay (Cell Titer 96®, Promega, Madison, WI) at 490 nm on a Versa Max 96-well plate reader (Molecular Devices, Sunnyvale, CA). The IC₅₀ was determined using Prism® software (GraphPad, La Jolla, CA) from at least 3 independent experiments. The combination index 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, Paramus, NJ)(41, 42). For consistency among experiments we used a concentration slightly higher than the IC₅₀ of trabectedin or 0.5 nM instead of 0.4 nM. Data points were included that fell within the range of 1 to 99% of control. In order 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.

Immunocytochemistry

TC32 cells were seeded in Lab-Tek II™ 4 chamber wells (Nunc, Rochester, NY) 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)(γ H2AX)(1:1000 dilution, Millipore) overnight at 4°C, washed with PBS, incubated with Alexa488-labeled anti-mouse IgG (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, Burlingame, CA) with standard settings that were not changed among treatment groups.

siRNA sensitization experiment

5 μ L of a 400 nM solution of siRNA targeting WRN (Qiagen, #si102663759), EWS-FLI1, or a non-targeting siRNA control (siNeg) was complexed with RNAimax (Life Technologies) for 30 min, combined with 2000 TC32 or 4000 TC71 cells and incubated for 48 hours. SN38 was subsequently added to achieve a final concentration of 20 nM for the siRNA and 1 or 0.5 nM of SN38 for a total of 110 (TC71) to 120 hours (TC32). Cell viability was monitored using the Incucyte Zoom (Essen biosciences, Ann Arbor, MI) 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 (0.02mg/mL) in 0.1% Triton X-100 with RNase A for 30 min 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 (PerkinElmer).

Xenograft experiments

2 million TC32 or TC71 cells were inoculated by intramuscular injection in the left gastrocnemius of 4–6 week old female SCID-bg mice (Charles River Laboratories, Wilmington, MA), established to a minimum diameter of 0.5 cm (volume of 65 mm³) and then mice were randomized to treatment groups. 2 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). 4 cohorts of 12–15 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 4–5, and 11–12 in TC71 or days 4–5 only for TC32 or the combination of trabectedin and irinotecan on the identical dose and schedule. Irinotecan was used at sub-maximal doses, determined by a dose finding experiment, in order 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 equation $(D \times d^2)/6 \times 3.12$ (where D = the maximum diameter and d = 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. 3500 mm³). Prism® software (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 (ACUC) at the National Cancer Institute in Bethesda, MD.

Immunohistochemistry

Tissue sections (10-µm thick) were prepared and stained with hematoxylineosin by American Histo Labs (Gaithersburg, MD) from paraffin blocks. IHC was performed as previously described (39) using rabbit polyclonal anti-WRN (1:200 dilution, Abcam), rabbit polyclonal anti-NR0B1 (1:200 dilution, Abcam) or mouse monoclonal anti-phospho-histone H2A.X (ser 139)(γH2AX)(1:1000 dilution, Millipore) and visualized on a Zeiss 510confocal microscope.

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 ES cells and changed in expression from a Z-score of +1 to –1 or –2 with trabectedin treatment (Supplemental Fig. 1). We confirmed these results by quantitative PCR and found a 350% reduction in mRNA expression of WRN or a 0.29 (95% CI = 0.22 to 0.36) fold change ($P < 0.0001$) (Fig. 1A).

Next, we showed that this suppression of WRN extends to the protein level in ES cells. Treatment of TC32 ES cells with 10 nM trabectedin suppressed WRN starting at about 8 hours of treatment that reached a maximum at 18 hours of treatment (Fig. 1B). The timing of this suppression correlated with the well-characterized EWS-FLI1 downstream target, ID2 and preceded apoptosis as measured by the cleavage of PARP (Fig. 1B). Furthermore, this suppression extended to a panel of ESFT cell lines including TC71, EW8, CHLA9, CHLA10, A673 and TC167 all of which demonstrated a suppression of WRN at 10 nM of treatment for 18 hours (Fig. 1C). There was no 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 ES cells

To link WRN expression to EWS-FLI1, we utilized siRNA to silence EWS-FLI1 and found a marked suppression of WRN expression in three different Ewing sarcoma cell lines that was not seen with the lipid or non-targeting controls (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 ES 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 nM and 0.25 nM or concentrations centered on our previously reported IC₅₀ of 0.4 nM (Fig. 2A). In order to evaluate the shift in IC₅₀, we expressed the data normalized to the effect of trabectedin treatment alone and found that 0.5 nM trabectedin shifted the IC₅₀ of SN38 from 1.7 nM (95% CI = 1.6 to 1.8) to 0.02 nM (95% CI = 0.02 to 0.03) in TC32 cells and from 1.4 nM (95% CI = 1.3 to 1.4) to 0.06 nM (95% CI = 0.05 to 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 nM trabectedin does not cause a shift in the dose response curves leading to nearly identical IC₅₀s of 53 nM (95% CI = 44 to 64) and 41 nM (95% CI = 33 to 52) for doxorubicin and doxorubicin plus trabectedin, respectively (Fig. 2C).

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 nM in the parent EWS-FLI1 null cells (95% CI = 123 to 177) to 15 nM in the EWS-FLI1 expressing HT1080 cells (95% CI = 12.6 to 18.7). The addition of trabectedin to SN38 further augments this sensitization dropping the IC₅₀ to 3.4 nM (95% CI = 3 to 3.9), almost 7 times lower than the IC₅₀ of 21 nM (95% CI = 17.9 to 25.2) of the combination in EWS-FLI1 null cells (Fig. 2D).

Finally, we employed the method of Chou and Talalay to calculate the combination index (CI) to determine if the combination of trabectedin and SN38 was synergistic (CI < 1), additive (CI = 1) or antagonistic (CI > 1) (42, 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 $\pm$ 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 $\pm$ 0.05) and synergy in TC71 with a CI of 0.65 (SEM $\pm$ 0.12)(Table 1).

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

In order to evaluate the ability of the drugs to induce DNA damage in ES cells, we measured the phosphorylation of histone H2A.X to generate γ H2AX foci by immunocytochemistry and confocal microscopy. We found a significant increase in the number of γ H2AX foci in single TC32 ES 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 γ H2AX 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). In order 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). In order 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 nM trabectedin suppressed luciferase activity to 69% of control (95% CI = 62 to 76), 5 nM SN38 to 59% of control (95% CI = 54 to 63) and the combination suppressed luciferase activity to 41% of control (95% CI = 36 to 47). The same effect was observed at lower concentrations of SN38, where 1 nM of SN38 led to the suppression of luciferase to 80% of control (95% CI = 76 to 84) a level that was further suppressed to 53% of control (95% CI = 47 to 57) when combined with 5 nM trabectedin (Fig. 3C). Furthermore there was limited suppression of a constitutively active control where a minimal induction of luciferase activity occurred with 5 nM trabectedin while the combination suppressed to 91% of control (95% CI = 84% to 98%) for 5 nM of both agents and 95% of control (95% CI = 91% to 99%) for 5 nM trabectedin and 1 nM 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 nM. (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 γ H2AX (Fig 3D).

Silencing of WRN sensitizes Ewing sarcoma cells to SN38 Treatment

In order 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 $\pm$ 2.4) confluence in wild type TC32 cells to 36.4% (SEM $\pm$ 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 nM SN38 from 19.9% (SEM $\pm$ 0.9) to 14.8 (SEM $\pm$ 1.6) (P = 0.046). A similar effect was observed with siRNA targeting of WRN in TC71 Ewing sarcoma cellseven at 0.5 nM SN38 resulting in a decrease in percent confluence from 99% (SEM $\pm$ 0.2) to 70% (SEM $\pm$ 3.5) (Fig. 4B).

Trabectedin mediated suppression of WRN recapitulates the WRN null phenotype

In order 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 prior to 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 due to stalled replication forks that can not 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 due to the inability to complete the cell cycle (50, 51).

We found that treatment of Ewing sarcoma TC32 cells with 1 nM trabectedin for 18 hours results in an increase in cells in the S-phase from a mean of 27.3% ($\pm$ 0.3) in the solvent control to 62.4% ($\pm$ 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 (Figs. 4C, D). Indeed, treatment with the combination of trabectedin and SN38 led to a comparable increase of cells in S-phase to 59% ($\pm$ 3.3) as well as an increase in the sub-Go apoptotic fraction from 4.1% ($\pm$ 0.3) in the solvent control to 14.5% ($\pm$ 0.4) (Figs. 4C, D).

The combination of trabectedin and SN38 regress Ewing sarcoma xenografts

In order 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 4–5 in the TC32 xenograft and on days 4–5, and 11–12 in the TC71 xenograft. We used irinotecan for the in vivo experiments because it is a pro-drug 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 (supplemental Fig. 2). 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 (Figs 5A, B). On day 15 of treatment, all the mice in the TC71 control cohort had to be sacrificed with a mean tumor

volume of 3768 mm³ (95% CI = 3298 to 4238). Tumor growth was suppressed to 3066 mm³ (95% CI = 2526 to 3607) with trabectedin treatment and 2233 mm³ (95% CI = 1627 to 2840) with irinotecan. In contrast, there was no detectable tumor in any of the mice treated with the combination yielding a mean tumor volume 78 mm³ (95% CI = 54 to 101) (Fig. 5A). Similar results were obtained with the TC32 xenograft at day 24 of treatment demonstrating mean tumor volumes of 3432 mm³ (95% CI = 2884 to 3980) in the control treatment, 2032 mm³ (95% CI = 1281 to 2782) with trabectedin treatment and 1347 mm³ (95% CI = 377 to 22318) 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 to 132) (Fig. 5B). These regressions persisted after treatment ended and no re-treatment 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 γ H2AX 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 appears 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 doubling in survival relative to the single agent treated mice with at least two 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 the extreme sensitivity of ES 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 due to 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 nM $\pm$ 2.95 nM (29). These levels are lower than the 5 to 10 nM 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. In order to develop this combination, we showed that EWS-FLI1 drives the expression of the DNA damage response gene, Werner Syndrome helicase (WRN). Since it is known that cells deficient in WRN are hypersensitive to camptothecins, we utilized trabectedin to suppress WRN expression and selectively sensitize ES 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 two established ES xenograft tumors with only 4 to 6 doses of drug at 1/10th 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Chris Warren in the Vanderbilt Medical Center Flow Cytometry shared resource core for assistance obtaining the cell cycle data.

Funding

This work has been supported in part by a grant from St. Baldrick's research foundation (PJG& LES). PJG also receives grant support from the Lily's Garden Foundation, the West haven 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 (LJH), also funded this project.

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Statement of Clinical 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.

Figure 1.

EWS-FLI1 drives expression of WRN, an activity which is blocked by trabectedin treatment. A, Mean ($\pm$ 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 nM(0.03 ± 0.01), 7.5 nM (0.08 ± 0.007) or 5 nM 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 nM trabectedin for 18 hours (ET) or solvent control (SC) in 7 different ES cell lines. Western blots representative of 3 independent experiments (time course) or 2–3 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 non-targeting siRNA (–) or siRNA targeting EWS-FLI1 (siEF) or SP1 (siSP1) in TC32 ES cells or two other ES cell lines TC71 or EW8. Data representative of 2 (EW8) or 3 independent experiments (TC32 and TC71).

Figure 2.

Trabectedin sensitizes TC32 and TC71 ES cells to SN38 treatment. A, Dose response curves of cell number at 48 hours of treatment as a function of log concentration SN38 (nM). The curves represent SN38 (circles and solid black) + 0.25 nM trabectedin (Trabec., squares and dotted dark gray) or 0.5 nM trabectedin (Trabec., triangles and solid dark gray). B, Data normalized to solvent control showing the shift in the IC₅₀ of SN38 (black) in TC32 or TC71 cell lines with the addition of 0.5 nM trabectedin (Trabec., gray). C, Identical experiment as (B) showing shift in the IC₅₀ of doxorubicin (black) with 0.5 nM trabectedin (Trabec., Gray). D, Dose response curves of SN38 +/- trabectedin in HT1080 cells transduced with GFP or EWS-FLI1: GFP-SN38 (black solid), GFP-SN38 + trabectedin (Trabec., gray dotted), EWS-FLI1 SN38 (black dotted), EWS-FLI1 SN38 + trabectedin (Trabec., gray solid). The curves are the mean effect determined by non-linear regression for 12 replicates from 3 to 6 independent experiments.

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 γ H2AX foci (green) with DAPI stained nuclei for reference (blue) following treatment of TC32 cells with 5 nM trabectedin, SN38 or the combination for 6 hours. B, Populations of TC32 cells showing the increased penetrance of γ H2AX in the combination therapy relative to each agent alone. White arrows highlight unaffected cells. Quantitation of γ H2AX 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). C, Normalized bioluminescence of stable NR0B1 promoter construct (EWS-FLI1 reporter) treated with solvent control (S) or the designated concentration of trabectedin (Trabec) or

SN38 (* & ^ denote comparisons for $P < 0.0005$). Data represents the mean value normalized to the solvent control for 8 replicates from two independent experiments for each treatment group. D, Western blot showing the effect of SN38 treatment (concentrations above the blot) +/- 5 or 1 nM trabectedin (T) on PARP cleavage, EWS-FLI1 downstream target expression (ID2) and γ H2AX expression. Identical results obtained with three independent experiments. (*) Highlights specific concentrations of trabectedin, SN38 and the combination for comparison.

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 non-targeting 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 non-targeting 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 & G2M as shown in these representative histogram plots. D, Column statistics for the treatment groups for the subG0, G1, S and G2 gates. Treatment groups are untreated (medium), solvent control (solvent), 1 nM Trabectedin (Trabectedin) and 1 nM SN38 (SN38). Data represents the mean (+/- St Dev) from at least two 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 ES xenograft growth. A, Mean ($\pm$ SEM) tumor volume for each mouse in the TC71 cohort on day 15 for the four 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 & I, n = 13) B, Mean ($\pm$ SEM) tumor volume for each mouse in the TC32 cohort on day 24 for the four 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 & I, n = 13) C, Immunohistochemical staining of TC71 xenografts with H&E and immunohistochemical staining for WRN and γ H2AX 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 four

treatment groups, control (black), trabectedin (red), irinotecan (blue) and combination (green).

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Table 1

Combination Index (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. Data represents the mean value ($\pm$ SEM) from 3 independent experiments.

		Experimental Points			Median Effect Equation	
		2X IC50	IC50	0.5X IC50	ED50	ED75
Trabectedin & SN38	TC32	0.84 ($\pm$ 0.02)	0.57 ($\pm$ 0.02)	1.05 ($\pm$ 0.12)	0.51 ($\pm$ 0.06)	0.66 ($\pm$ 0.05)
	TC71	0.85 ($\pm$ 0.03)	0.72 ($\pm$ 0.04)	0.8 ($\pm$ 0.03)	0.23 ($\pm$ 0.05)	0.42 ($\pm$ 0.05)
Trabectedin & Doxorubicin	TC32	1.18 ($\pm$ 0.06)	0.84 ($\pm$ 0.03)	1.11 ($\pm$ 0.05)	0.87 ($\pm$ 0.05)	1.06 ($\pm$ 0.03)
	TC71	0.95 ($\pm$ 0.01)	0.85 ($\pm$ 0.03)	1.12 ($\pm$ 0.11)	0.65 ($\pm$ 0.12)	0.86 ($\pm$ 0.11)