Trabectedin Efficacy in Ewing Sarcoma Is Greatly Increased by Combination with Anti-IGF Signaling Agents

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Abstract

Purpose: Goal of this study was to identify mechanisms that limit efficacy of trabectedin (ET-743, Yondelis) in Ewing sarcoma (EWS). We sought to develop a clinical applicable combination therapy.

Experimental Design: By chromatin immunoprecipitation, we analyzed EWS–FLI1 binding to the promoters of several target genes, such as TGFβR2, CD99, insulin-like growth factor receptor 1 (IGF1R), and IGF1, both in vitro and in xenografts treated with trabectedin or doxorubicin. Combined therapy with trabectedin and anti-IGF1R agents (AVE1642 HAb; OSI-906) was tested in vitro and in xenografts.

Results: We confirm that both trabectedin and doxorubicin were able to strongly reduce EWS–FLI1 (both type I and type II) binding to two representative target genes (TGFβR2 and CD99), both in vitro and in xenografts. However, trabectedin, but not doxorubicin, was also able to increase the occupancy of EWS–FLI1 to IGF1R promoters, leading to IGF1R upregulation. Inhibition of IGF1R either by the specific AVE1642 human antibody or by the dual IGF1R/insulin receptor inhibitor OSI-906 (Linsitinib) greatly potentiate the efficacy of trabectedin in the 13 EWS cell lines here considered as well as in TC-71 and 6647 xenografts. Combined therapy induced synergistic cytotoxic effects. Trabectedin and OSI-906 deliver complementary messages that likely converge on DNA-damage response and repair pathways.

Conclusion: We showed that trabectedin may not only inhibit, but also enhance the binding of EWS–FLI1 to certain target genes, leading to upregulation of IGF1R. We here provide the rationale for combining trabectedin to anti-IGF1R inhibitors.

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Introduction

Ewing sarcoma (EWS) is the second most common primary developmental bone and soft tissue tumor. It has a very aggressive phenotype and preferentially occurs in children and young adults. Despite remarkable progress has been achieved in treatment of localized disease, where overall cure is now approximately 70% (1, 2), there is still an unmet need for therapy amelioration in patients with metastatic disease whose overall survival is lower than 30%. Reduced side effects and improvements in quality of life are also desirable goal. Unfortunately, few new drugs are available for the treatment of patients with EWS and most of the recent results have been achieved thanks to an intensified use of the conventional drugs.

One of the few exceptions in this paucity of new therapeutic alternatives is trabectedin (ET-743, Yondelis), a marine derivate from the Caribbean tunicate Ecteinascidia turbinata (3), which has been shown to combine direct cytotoxic activity toward cancer cells with the peculiar capacity to favorably modify the tumor microenvironment and give potent immunomodulatory effects (for a review see ref. 4). Trabectedin is a tetrahydroisoquinoline molecule that binds to the N2 of guanine in the minor groove, causing DNA damage and affecting transcription regulation in a promoter- and gene-specific manner. Indeed, the specific capabilities of trabectedin to cause a detachment of the FUS-CHOP chimera, the aberrant transcriptional factor that specifically characterizes myxoid liposarcoma (5), from its target promoters is thought to be responsible for the high sensitivity of myxoid/round cell liposarcoma to trabectedin either in vitro and in vivo (6–10). Similarly, trabectedin was found to interfere with the activity of EWS–FLI1, the genetic hallmark and primary oncogenic driver of EWS (11), reversing the expression of the EWS–FLI1–induced gene signature and blocking the promoter activity and expression of critical EWS–FLI1 downstream targets (12). EWS is characterized by the presence of balanced translocations, in which more than 90% of cases present EWS–FLI1 fusion (EWS–FLI1 type I, II, or III chimeras depending on the breakpoint)....
on the exons involved; ref. 13). This hampering effect on EWS–FLI1 transcriptionsal activity is thought to be part of the antitumor efficacy of trabectedin against EWS cells in vitro (14–16). However, phase II clinical trials with trabectedin in EWS reported only a modest activity as single agent (17). In this article, we analyzed the effects of trabectedin with respect to the insulin-like growth factor (IGF) system based on the evidence that cells made resistant to trabectedin overexpress the IGF receptor 1 (IGF1R) and insulin receptor substrate (IRS-1; ref. 18). The IGF system is widely recognized to be very important for EWS cells survival and malignant behavior (19). Accordingly, EWS is the most sensitive tumor to anti-IGF1R agents, with 10% to 14% of clinical responses when anti-IGF1R monoclonal antibodies (HAb) were used as single agents in phase II studies (20–23). EWS–FLI1 is known to interfere with the IGF system either by blocking IGFBP-3 expression (24), a molecule that inhibits IGF signaling by sequestering IGF1 and preventing its interaction with IGF1R (25), or by inducing IGF1 expression (26). In this study, we show that trabectedin, besides causing detachment of the EWS–FLI1 chimera from some well-recognized target promoters, such as TGFb receptor 2 (TGFbR2; ref. 12), is also able to induce attachment of the chimera to the IGF1R promoter, inducing activation of the IGF1R signaling pathway. This finding strongly supports a combination therapy of trabectedin with anti-IGF1R agents.

Materials and Methods

Drugs
Trabectedin was provided as lyophilized formulation and as clinical preparation by PharmaMar S.A., Colmenar Viejo. For in vitro experiments, trabectedin was dissolved in DMSO. For the in vivo studies, the clinical preparation of Yondelis was used. OSI-906 (Linstitinib; Selleck Chemicals) was dissolved in double-distilled water at the final concentration of 10 mmol/L (stock solution). AVE1642, a humanized version of the anti-IGF1R antibody, EM164 (27), was kindly provided by ImmunoGen Inc. and Sanofi. Doxorubicin was purchased from Sigma-Aldrich. Working dilutions of all drugs were prepared immediately before use.

Cell lines
A panel of 13 EWS human cell lines was analyzed. SK-ES-1, SK-N-MC, and RD-ES were provided by the American Type Culture Collection (ATCC). EWS cell lines TC-71 and 6647 were kindly provided by T.J. Triche (Children’s Hospital, Los Angeles, CA). The EWS cell lines LAP-35, IOR/CAR, and IOR/BRZ-2010 as well as the TC-71 variants resistant to trabectedin (TC/ET 12 nmol/L) or to anti-IGF1R agents and referred as TC/AVE (resistant to AVE1642 HAb) were obtained in the Experimental Oncology Lab, Rizzoli Institute (Bologna, Italy) and as previously described (18, 28, 29). The EWS human cell lines WE-68, RM-82, Cado-ES, and STAET2.1 were kindly provided by F. van Valen (Institute of Experimental Musculoskeletal Medicine, University Hospital Münster, Münster, Germany). All cell lines were recently authenticated by short tandem repeat (STR) analysis using genRESVR MPX-2 and genRESVR MPX-3 kits (Serac). The following loci were verified: D16S539, D18S81, D19S433, D21S11, D2S1338, D5S818, D8S1179, FGA, SE33, TH01, and TPOX VWA. The last control was performed in November 2012. Cells were routinely tested for Mycoplasma contamination every 3 months by MycoAlert Mycoplasma detection set (Lonza). Cultures were grown in a humidified incubator at 37°C with 5% CO2 and maintained in standard medium [Isocove Modified Dulbecco’s medium, IMDM (Lonza), or RPMI (Gibco, Life Technologies), plus 10% fetal bovine serum (FBS), or 1% Glutamine (Gibco) and 1% Antibiotics (Gibco)].

In vitro assays
To assess cell growth, the MIT assay (Roche) was used according to the manufacturer's instructions. Cells were seeded into 96-well plates (range, 2,500–10,000 cells/well) in standard medium. After 24 hours, various concentrations of trabectedin (0.3–3 nmol/L) or OSI-906 (0.3–3 μmol/L) were added and cells were exposed up to 72 hours. In combination experiments, cells were treated for 72 hours with drugs alone (control) or combined in fixed ratio 1:1,000.

In vivo antitumor activity
Female athymic nude mice, 6- to 9-week-old obtained from Harlan Italy were used. They were maintained under specific pathogen-free conditions with constant temperature and humidity, according to the institutional [Istituto di Ricerche Farmacologiche Mario Negri (IRFMN), Milan, Italy] guidelines. TC-71 cells (5 × 106) or 6647 cells (10 × 106) were inoculated subcutaneously in the right flank of the mice. The growing tumor masses were measured with the aid of a Vernier caliper, and tumor weights (1 mm3 = 1 mg) were calculated using the formula: length × (width)2/2. When tumor load reached about 100 mg, mice were randomized into experimental groups and treatment was started.

Study groups were listed as follows (at least 8 mice per group): Placebo, 0.9% NaCl; trabectedin, 0.15 mg/kg; doxorubicin, 8 mg/kg; and AVE1642 HAb, 40 mg/kg. Drugs were administered i.v.: trabectedin every 7 days for three times (q7d × 3); doxorubicin every 7 days for two times (q7d × 2); AVE1642 HAb every 3 days for six times (q3d × 6). Drug efficacy was calculated as T/C, where T and C are the mean tumor weights of treated and control groups, respectively. Treatment was considered effective when T/C <42%. Procedures were conducted in conformity with the institutional guidelines that are in compliance with national (Legislative Decree 116 of Jan. 27, 1992 Authorisation 169/94-A issued Dec. 19, 1994 by Ministry of Health) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Standards for the Care and Use of Laboratory
Animals, United States National Research Council, Statement of Compliance A5023-01, November 6, 1998). Animal experiments were reviewed and approved by the IRFMN Animal Care and Use Committee (IACUC) that includes members for ethical issues.

Western blotting
Cells were treated or not (control) with trabectedin (0.5–2.5 nmol/L) or with OSI-906 (400 nmol/L) up to 48 hours or silenced for EWS–FLI1 (75–100 nmol/L siRNA; ref. 30) and lysed as previously described (18). The following primary antibodies (Ab) were used: anti-PARP; cleaved-caspase-3, anti-NF-H 200 kDa (Cell Signaling Technology); anti-IGF1Rβ, anti-FLI, anti-β-actin, and GAP-DH (Santa Cruz Biotechnology); anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare) were used as secondary antibodies.

Chromatin immunoprecipitation
In vitro and in vivo chromatin immunoprecipitation (ChIP) assays were performed as previously described (6, 28) using anti-FLI1 (C-19; Santa Cruz Biotechnology) and/or anti-FLAG (Sigma) antibodies. PCR was performed with primers flanking Ets-containing target promoters fragment (listed in Supplementary Table S1). Amplification products obtained were observed in 1.5% agarose gel with Gel Red staining. For quantitative PCR (qPCR), data are indicated as fold enrichment respect to untreated cells in vitro experiments or to placebo in xenografts and calculated using following formula: % of recruitment = 2ΔΔCt × input chromatin percentage where ΔCt = Ct (input) − CFI1 (FI1 IP) in accordance to Frank and colleagues (31, 32).

Immunofluorescence assays
Cells were seeded in single slide covers placed in 100-mm plates, pretreated with gelatin (Sigma), and grown in standard medium. After exposure to drug/DMSO for 24 hours, cells were fixed in ice-cold-methanol, permeabilized with Triton X-100, and processed for immunofluorescence. Primary antibodies as follows: pH2AX (1:100; Cell Signaling Technology); 53BP1 (1:100; Abcam), anti-tubulin β III (dilution 1:50), or anti-H neurofilament 200 kDa (clone NE14; dilution 1:40; Sigma). Cells were then stained with the secondary antibody Cy5 (Jackson Immunoresearch) and counterstained with DAPI 1 mg/mL (DAKO). Slide covers were mounted in covers with Mowiol fixing agent (Sigma) and cells observed in a Leica Microscope using software LEICA software.

RNA extraction and low-density microarrays by qRT-PCR
RNA extraction was performed using Qiagen RNA extraction kit, following the manufacturer's instructions, as described elsewhere (26, 33). Retrotranscription was performed using 500 ng of total RNA. Low-density microarray, in the form of qRT-PCR 96-well plates Human DNA Repair PCR Array (Qiagen) were performed using the iQ5 thermocycler from Bio-Rad. RT-PCR was run in the iQ5 BIO-RAD thermocycler with the following protocol: 95°C for 10 minutes; 40 cycles of 42°C 10 minutes and 60°C 1 minute. Results were evaluated using the iQ5 software from Bio-Rad and the online platform (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Pathway analysis was performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity system).

Activity of caspase-3 and caspase-7
Caspases-3 and -7 activities were determined in cells exposed to drug/DMSO for 48 hours using the Glo caspase luminescent kit from Promega according to the manufacturer’s instructions. Luminescence was read in a plate reader (Tecan).

Immunohistochemistry
Sections (5 μm) from formalin-fixed, paraffin-embedded TC-71 xenografts were placed on poly-l-lysine–coated slides (Sigma). Avidin-biotin-peroxidase procedure was used for immunostaining, as previously described (34), and slides were stained with anti-tubulin β III (dilution 1:50), anti-H neurofilament 200 kDa (clone NE14; dilution 1:40; Sigma) and IGF1Rβ (Santa Cruz Biotechnology). Detection of Ki-67 was performed on sections as previously described (34). The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed with ApoTag Plus Peroxidase in situ apoptosis kit (Merck Millipore) according to the manufacturer’s instructions.

Statistical analysis
Correlations between two variables were obtained by the Spearman test. IC50 values were calculated from linear transformation of dose–response curves. To define drug–drug interactions (in terms of synergism, additivity, or antagonism), the combination index (CI) was calculated with the isobologram equation (35) by using the CalcuSyn software (Biosoft). Differences among means were analyzed by the Student t test or the ANOVA test.

Results
Trabectedin disrupts EWS–FLI1 binding to some DNA targets but increases recruitment to IGF1R promoter in both in vitro and in vivo models
We used ChIP analysis to monitor the binding of EWS–FLI1 chimera to some well-known target genes, such as TGFβR2 and CD99, reported to be modulated by EWS–FLI1 and proven to have a major role in EWS aggressiveness (28), as well as to IGF1 and IGF1R promoters. ChIP indicated that the amount of EWS–FLI1 chimera bound to the TGFβR2 and CD99 promoters was significantly reduced after 1 hour treatment with trabectedin both in TC-71 cells, displaying EWS–FLI1 type I chimera, and in the 6647 cell line, that displays EWS–FLI1 type II hybrid, at pharmacologic concentrations (IC50 value after 1 hour of treatment; Fig. 1A). The binding of the chimera EWS–FLI1 type I (TC-71) and type II (6647) to the CD99 and TGFβR2 promoters was evaluated also in mouse xenografts after i.v. administration of trabectedin (0.15 mg/kg, every 7 days for three times, q7d × 3) and doxorubicin (8 mg/kg, every 7 days for two times, q7d × 2). As shown in Supplementary Fig. S1, in TC-71 EWS model trabectedin was more active (best T/C 56.2% at days 20) than doxorubicin (reference compound, best T/C 79.5% at days 22). Instead in 6647 xenograft model, doxorubicin was extremely effective...
Figure 1.
Trabectedin caused a dysregulation in EWS–FLI1 chimera binding to specific promoters. ChIP assays were carried out in vitro on TC-71 and 6647 EWS cells, or in xenografts after treatments with trabectedin or doxorubicin. EWS–FLI1 was precipitated by the anti-FLI1 antibody. Decrease in the binding of the chimera EWS–FLI1 type I (TC-71) and type II (6647) to CD99 and TGFβR2 promoters was observed either in in vitro (A) or in xenografts (B) treated as described in Materials and Methods. Results obtained by qPCR are reported as fold enrichment over the controls (untreated in vitro cells; placebo-treated mice) according to the following formula: % of recruitment = $2^{ACt}$, input chromatin percentage where $DCt = Ct (input) - Ct (FLI1 IP)$; refs. 31, 32. P < 0.05; **, P < 0.001, Student t test. C, left, increased recruitment of EWS–FLI1 on IGF1R promoter in TC-71 and 6647 EWS cells treated for 1 hour with trabectedin or doxorubicin. A representative experiment is shown. Data represent recovery of each DNA fragment relative to total input DNA, respect to control. *, P < 0.05; Student t test. Right, upregulation of IGF1R at protein level by Western blotting after exposure to trabectedin (0.5–1 nmol/L) up to 48 hours. GAPDH was used as loading control. Blots are representative of two independent experiments. D, time course of FLI1 association with the IGF1R promoter in TC-71 and 6647 xenografts treated with trabectedin (0.15 mg/kg). *, P < 0.05; Student t test.
Trabectedin was less active (best T/C 48.6% at days 21). In both cases, however, trabectedin and doxorubicin were able to displace EWS–FLI1 chimera from CD99 and TGFβR2 promoters, although with different kinetics (Fig. 1B). Trabectedin was able to cause detachment of the EWS–FLI1 chimera from both CD99 and TGFβR2 promoters already 24 hours after the first dose both in TC-71 and 6647 cells. Reattachment was observed starting 7 days from the third treatment.

Besides inhibitory effects, trabectedin but not doxorubicin also caused a dose- and time-dependent increase in the binding of the chimera to IGF1R promoter in EWS cells (Fig. 1C and Supplementary Fig. S2A). Upregulation of IGF1Rβ was also confirmed at protein level after trabectedin treatment (Fig. 1C), in line with our previous data showing increased transcription and expression of IGF1Rβ in cells made resistant to trabectedin (18). Consistently, silencing of EWS–FLI1 in TC-71 cells induced downregulation of IGF1Rβ protein (Supplementary Fig. S3). Enhancement of EWS–FLI1 occupancy to IGF1R by trabectedin was also observed in vivo in TC-71 and 6647 xenografts (Fig. 1D), further sustaining the relationship between EWS–FLI1 and IGF1R. These findings provided the rationale for testing the combination of trabectedin with anti-IGF1R agents.

**Antitumor activity of the combination between trabectedin and the anti-IGF1R HAb AVE1642**

Antitumor activity of trabectedin alone or in combination with anti-IGF1R AVE1642 HAb was evaluated in TC-71 xenograft model. The combination trabectedin and AVE1642 HAb (best T/C 27.9 at days 20) showed a greater antitumor activity than trabectedin (best T/C 40.3 at days 20) or AVE1642 HAb (best T/C 48.6 at days 15) used as single agents (Fig. 2A). Studies in myxoid liposarcoma indicate that trabectedin besides inhibiting cell proliferation can also act as a differentiating agent by blocking the transactivating ability of the fusion gene product (8). We confirmed the antiproliferative, proapoptotic, and prodifferentiating activity of trabectedin also in EWS (Fig. 2B and Supplementary Fig. S4). Moreover, in keeping with ChIP findings, xenografts treated with trabectedin showed increased expression of IGF1Rβ (Fig. 2B). Combination treatments with AVE1642 HAb further inhibited tumor cell proliferation and IGF1R expression while increased apoptotic rate (Fig. 2B). This supports the combination of trabectedin with anti-IGF1R HAb against EWS.

**Efficacy of the dual inhibitor anti-IGF1R/IR OSI-906 in combination with trabectedin**

Considering that the great majority of EWSs express concomitant high levels of IR, which may overcome the IGF1R blockade...
(29, 36), efficacy of the dual inhibitor anti-IGF1R/IR OSI-906 was evaluated in a panel of 13 EWS cell lines (Table 1), including the TC/ET 12 nmol/L cell line, highly resistant to trabectedin (18) and TC/AVE, resistant to anti-IGF1R AVE1642 HAb (29). Most of cell lines were highly sensitive to the inhibitor with submicromolar IC50 values (Table 1).

The combination of OSI-906 with trabectedin gave synergistic effects in all EWS cell lines, including cells resistant to AVE1642.

Table 1. Efficacy of combined treatments of Trabectedin with OSI-906 in EWS cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>OSI-906 (μmol/L) ± SE</th>
<th>Trabectedin (nmol/L) ± SE</th>
<th>Combination (nmol/L) ± SE</th>
<th>CI ± SE</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-71</td>
<td>0.4 ± 0.15</td>
<td>0.206 ± 0.073</td>
<td>0.157 ± 0.01</td>
<td>0.819 ± 0.01</td>
<td>Synergistic</td>
</tr>
<tr>
<td>WE-68</td>
<td>0.45 ± 0.25</td>
<td>0.140 ± 0.083</td>
<td>0.08 ± 0.04</td>
<td>0.356 ± 0.02</td>
<td>Synergistic</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>0.59 ± 0.18</td>
<td>0.135 ± 0.02</td>
<td>0.07 ± 0.005</td>
<td>0.789 ± 0.08</td>
<td>Synergistic</td>
</tr>
<tr>
<td>LAP-35</td>
<td>0.17 ± 0.07</td>
<td>0.148 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>0.734 ± 0.08</td>
<td>Synergistic</td>
</tr>
<tr>
<td>IOR/CAR</td>
<td>1.402 ± 0.66</td>
<td>0.266 ± 0.009</td>
<td>0.173 ± 0.06</td>
<td>0.789 ± 0.03</td>
<td>Synergistic</td>
</tr>
<tr>
<td>6647</td>
<td>1.25 ± 0.45</td>
<td>0.224 ± 0.04</td>
<td>0.097 ± 0.04</td>
<td>0.687 ± 0.06</td>
<td>Synergistic</td>
</tr>
<tr>
<td>RD-ES</td>
<td>0.63 ± 0.14</td>
<td>0.154 ± 0.05</td>
<td>0.07 ± 0.006</td>
<td>0.646 ± 0.06</td>
<td>Synergistic</td>
</tr>
<tr>
<td>SKE-1</td>
<td>0.76 ± 0.15</td>
<td>0.08 ± 0.004</td>
<td>0.052 ± 0.01</td>
<td>0.784 ± 0.03</td>
<td>Synergistic</td>
</tr>
<tr>
<td>RMB2</td>
<td>0.2 ± 0.14</td>
<td>0.54 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.600 ± 0.09</td>
<td>Synergistic</td>
</tr>
<tr>
<td>Cado-ES</td>
<td>3.77 ± 0.47</td>
<td>0.578 ± 0.058</td>
<td>0.18 ± 0.03</td>
<td>0.770 ± 0.09</td>
<td>Synergistic</td>
</tr>
<tr>
<td>IOR/BRZ-2010</td>
<td>0.605 ± 0.13</td>
<td>0.182 ± 0.003</td>
<td>0.137 ± 0.03</td>
<td>0.834 ± 0.07</td>
<td>Synergistic</td>
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<tr>
<td>TC/AVE 1642</td>
<td>0.402 ± 0.12</td>
<td>1.305 ± 0.263</td>
<td>2.52 ± 0.59</td>
<td>0.411 ± 0.09</td>
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<tr>
<td>TC/ET 12 nmol/L</td>
<td>0.268 ± 0.003</td>
<td>1.982 ± 0.340</td>
<td>0.178 ± 0.004</td>
<td>0.710 ± 0.02</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

IC50 values are referred to trabectedin.
Synergism: CI < 0.9; additive: 0.90 ≤ CI ≤ 1.10; according to ref. 34.

Figure 3. Effects of the dual inhibitor anti-IGF1R/IR OSI-906 in combination with trabectedin. A, caspase cleavage activation (RLU) in TC-71 and WE-68 treated with trabectedin and/or OSI-906 for 48 hours. All treatments are normalized respect to control. Bars, mean of two independent experiments ± SE. **, P < 0.001, Student t test respect to control. B, network and pathway analysis of genes significantly modulated after TC-71 cell exposure to drug combination. Low-density expression array focusing on DNA damage and DNA repair genes was used.
HAb (TC/AVE) or trabectedin (TC/ET 12 nmol/L; Table 1). When OSI-906 was combined to trabectedin, we observed advantageous effects in terms of apoptosis, both in p53wt (WE-68) and p53-mutated cells (TC-71; Fig. 3A). This advantageous proapoptotic cell death may derive from the combination of two different inputs. Although inhibition of the IGF system may block anti-apoptotic effects of IGF1R/IR-A due to disruption of AKT and/or 14.3.3/Raf-1/Nedd4 pathways (37, 38), trabectedin acts as a DNA-damaging agent inducing double-strand breaks (DSB; ref. 3). To further characterize drug effects on DNA repair pathways, we used DNA damage low-density arrays customized to cover homologous recombination (HR), nucleotide excision repair (NER), base excision repair (BER), and non-homologous end joining repair (NHEJ) pathways. Treatment with trabectedin, but not with OSI-906, induced expression of BRCA1, BRCA2, key proteins in HR pathway (39) as well as XRCC1, member of single-strand break repair (SSR) pathway (Supplementary Fig. S5). The drug combination resulted in upregulation of members from the HR pathway (RAD52, BRCA1 and BRCA2), NER proteins (XPA and ERCC1), and SSR pathways (XRCC1; Fig. 3B). As validation, we also studied DNA-damage induction by presence of pH2AX and 53BP1 intranuclear foci after 24 hours treatment (Fig. 4A), showing that trabectedin was a potent DSB inducer, in contrast to OSI-906, and that DNA damage was present when cells were treated with the combination of trabectedin and OSI-906. DNA fragmentation assay showed that besides trabectedin also OSI-906 alone and particularly the combination of the two drugs leads to DNA fragmentation (Fig. 4B). The presence of DNA fragments (<500 bp) after cell exposure to OSI-906 is not surprising because these fragments are likely to be related to DNA degradation by apoptosis. Accordingly, OSI-906 favors expression of apoptotic proteins such as cPARP, fraction 90 kDa (Fig. 4C). PARP cleavage was also observed in the TC/ET 12 nmol/L resistant cell line after treatment with increasing doses of OSI-906, thus confirming complementary proapoptotic effects of the two drugs (Fig. 4D).

**Discussion**

Trabectedin is a newly licensed chemotherapeutic agent, with an acceptable toxicity profile (40). In this article, we show that trabectedin may be advantageously used in combination with inhibitors of the IGF system. Particularly, we provide a rationale for the use of this combination, showing that trabectedin is able to increase IGF1R expression by enhancing EWS–FLI1 occupancy of the IGF1R promoter. EWS–FLI1 drives the malignant phenotype in EWS cells (11), acting either as a transcriptional activator or as a transcriptional repressor in EWS. Importantly, both the activating and repressive functions of EWS–FLI1 are required for its oncogenic functions (41). Trabectedin has been previously found to block the promoter activity and expression of critical EWS–FLI1 downstream targets, and a combination with SN38 (the active metabolite of irinotecan) has been proposed to augment the suppression of EWS–FLI1 activity (16). However, in this article, we show that these inhibitory effects, although probably prevalent, are not exclusive. In fact, while we confirm that both...
Trabectedin and doxorubicin are able to strongly suppress the binding of EWS–FLI1 (both type I and type II) to two target genes (TGFβR2 and CD99) both in vitro and in vivo, a significant enhancement of EWS–FLI1 occupancy on the IGF1R promoter was observed only after exposure to trabectedin. Prior studies have demonstrated that other DNA-binding agents, including mithramycin, and actinomycin D, reduced expression of EWS demonstrated that other DNA-binding agents, including mithramycin, and actinomycin D, reduced expression of EWS and inhibition of cell death messages in all EWS cells, independently from the p53 status. Although treatment with IGF1R antagonists is known to lead to downregulation of the proteins involved in cell survival and inhibition of cell death, thereby recovering cell sensitivity to apoptosis, trabectedin has been previously described as a potent DNA-damaging agent. Trabectedin binds to guanines in the minor groove with some degree of sequence specificity, inducing SSB that rapidly turn into DSB, the most lethal form of DNA damage. Tavecchio and colleagues (50) clearly showed that DSBs are not directly caused by the drug, but are formed during the processing/repair of the drug, requiring a functional HR pathway. In addition, trabectedin also poisons the mechanisms of DNA repair through the formation of ternary NER proteins–DNA–trabectedin complexes (for a review see ref. 51). Our results demonstrated that in EWS cells trabectedin increases expression of BRCA1, BRCA2, key proteins in the HR pathway as well as of XRCC1, which is involved in the SSR pathway, resulting in DNA damage as indicated by phosphorylation of histone H2AX and accumulation of intranuclear foci. The drug combination with OSI-906 maintains and even increases upregulation of members of the XRCC4 and XRCC6 as well as of MSH4 and MSH5, two molecules involved in the maintenance of genomic stability and mitotic DSB repair (52), indicating general alterations of DNA-damage response and repair pathways. This is in line with recent evidence that IGF1R inhibition induces a direct functional defect in DSB repair by both NHEJ and HR, besides indirectly impairing HR through influences on the expression and/or activation of cell-
cycle regulators (53). The importance of trabectedin and IGF system inhibitors in DNA-damage response and repair pathways, which have implications on the therapeutic efficacy and potential toxicity of this combined therapy in the clinic, deserve further research to better elucidate the molecular mechanisms and protein interactions.

Overall, we provide the rationale for combining trabectedin to anti-IGF1R inhibitors. We showed that trabectedin may not only inhibit but also enhance binding of EWS–FLI1 to target genes (Fig. 5). Specifically, IGF1R expression activated after treatment with trabectedin and anti-IGF1R agents improve efficacy of trabectedin in cell lines and xenografts. We thus propose the use of a combination therapy that by exploiting the complementary mechanisms of action of the two drugs may have therapeutic potential.

Disclosure of Potential Conflicts of Interest

M. D’Incalci is a consultant/advisory board member for PharmaMar. No potential conflicts of interest were disclosed by the other authors.

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