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), so as 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 (IGF-1R), and IGF-1 both in vitro and in xenografts treated with trabectedin or doxorubicin. Combined therapy with trabectedin and anti-IGF-1R 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 1 and type 2) 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 IGF-1R promoters, leading to IGF-1R up-regulation. Inhibition of IGF-1R either by the specific AVE1642 human antibody or by the dual IGF-1R/Insulin receptor inhibitor OSI-906 (Linsitinib) greatly potentiate the efficacy of trabectedin in the 13 Ewing sarcoma 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 up-regulation of IGF-1R. We here provide the rationale for combining trabectedin to anti-IGF-1R inhibitors.
Translational Relevance

Trabectedin (ET-743, Yondelis™) is one of the few novel drugs recently proposed for treatment of sarcoma patients. However, in clinical setting the activity observed in Ewing sarcoma was quite modest. This work demonstrates that trabectedin is not only able to inhibit binding to DNA of the transcriptional factor EWS-FLI1, the genetic hallmark of Ewing sarcoma, but also increases its attachment on preferential sequences. Herein, we found enhanced binding to the IGF-1R promoter, which resulted in increased IGF-1R expression. Considering the relevance of the IGF system in Ewing sarcoma, this evidence might very well explain why trabectedin has shown limited efficacy in monotherapeutic regimens in clinical setting and provides the rationale for development of a therapy that combines trabectedin with anti-IGF signaling agents.
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 EWS patients 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 towards cancer cells with the peculiar capacity to favorably modify the tumor microenvironment and give potent immunomodulatory effects [for a review see (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 lyposarcoma 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 over 90% of cases present EWS-FLI1 fusion (EWS-FLI1 type 1, 2 or 3 chimeras depending on the exons involved) (13). This hampering effect on EWS-FLI1 transcriptional 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 paper, 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 (IGF-1R) and insulin receptor substrate (IRS-1) (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-IGF-1R agents, with 10-14% of clinical responses when anti-IGF-1R monoclonal antibodies (HAbs) 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 IGF-1R (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 TGFβ receptor 2 (TGFβR2) (12) is also able to induce attachment of the chimera to the IGF-1R promoter, inducing activation of the IGF-1R signaling pathway. This finding strongly supports a combination therapy of trabectedin with anti-IGF-1R agents.
MATERIALS and METHODS

Drugs. Trabectedin was provided as lyophilized formulation and as clinical preparation by PharmaMar S.A., Colmenar Viejo, Madrid, Spain. For in vitro experiments, trabectedin was dissolved in DMSO. For the in vivo studies the clinical preparation of Yondelis was used. OSI-906 (Linsitinib) (Selleck Chemicals, Houston, TX, USA), was dissolved in double-distilled water at the final concentration of 10mM (stock solution). AVE1642, a humanized version of the anti-IGF-1R antibody, EM164 (27), was kindly provided by ImmunoGen Inc., (Waltham, AM) and Sanofi. Doxorubicin (DXR) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Working dilutions of all drugs were prepared immediately before use.

Cell lines. A panel of 13 EWS human cell lines were analyzed. SK-ES-1, SK-N-MC, and RD-ES were provided by 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, USA). The EWS cell lines LAP-35, IOR/CAR, and IOR/BRZ-2010 as well as the TC-71 variants resistant to trabectedin (TC/ET 12nM) 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 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, Germany). All cell lines were recently authenticated by STR analysis using genRESVR MPX-2 and genRESVR MPX-3 kits (Serac, Bad Homburg, Germany). The following loci were verified: D16S539, D18S51, D19S433, D21S11, D2S1338, D3S1358, D5S818, D8S1179, FGA, SE33, TH01, 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, Basel, Switzerland). Cultures were grown in a humidified incubator at 37°C with 5% CO2 and maintained in standard medium (Iscove Modified Dulbecco’s medium, IMDM.)
(Lonza) or RPMI (Gibco, Life Technologies, Grand Island, NY, USA), plus 10% fetal bovine serum (FBS) or 1% Glutamine (Gibco) and 1% Antibiotics (Gibco).

**In vitro assays.** To assess cell growth, MTT assay (Roche, Indianapolis, IN, USA) was used according to manufacturer’s instructions. Cells were seeded into 96 well-plates (range 2,500-10,000 cells/well) in standard medium. After 24h, various concentrations of trabectedin (0.3-3 nM) or OSI-906 (0.3-3 µM) were added and cells exposed up to 72h. In combination experiments, cells were treated for 72h with drugs alone (control) or combined in fixed ratio 1:1000.

**In vivo antitumor activity.** Female athymic nude mice, 6-9 weeks old obtained from Harlan Italy (Bresso,Italy) were used. They were maintained under Specific Pathogen Free conditions with constant temperature and humidity, according to the institutional (IRFMN) guidelines. 5 x 10^6 TC-171 cells or 10 x 10^6 6647 cells 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 (1mm^3 =1mg) were calculated using the formula: length x (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 eight mice per group): Placebo, 0.9% NaCl; Trabectedin, 0.15 mg/kg; DXR, 8 mg/kg; AVE1642 HAb, 40 mg/kg . Drugs were administered i.v.: trabectedin every seven days for three times (q7dx3); DXR every seven days for two times (q7dx2); AVE1642 HAb every three days for six times (q3dx6). 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 n.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 (0.5-2.5nM) or with OSI-906 (400 nM) up to 48h or silenced for EWS-FLI1 (75-100 nM siRNA) (30) and lysed as previously described (18). The following primary antibodies (Ab) were used: anti-PARP; cleaved-caspase-3, anti- NF-H 200k (Cell Signaling Technology, Beverly, MA, USA); anti-IGF-1Rβ, anti-FLI anti-Beta-actin and GAP-DH (Santa Cruz Biotechnology, San Diego, CA, USA). Anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA) were used as secondary antibodies.

**Chromatin Immunoprecipitation (ChIP).** *In vitro and in vivo* ChIP assay were performed as previously described (6, 28) using anti-FLI1 (C-19, Santa Cruz Biotechnology, Texas, USA) and/or anti-FLAG (SIGMA, St. Louis, MO, USA) antibodies. PCR was performed with primers flanking Ets-containing target promoters fragment, listed in Supplementary Table 1. 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 = $2^{\Delta Ct} \times$ input chromatin percentage where $\Delta Ct = Ct$ (input) - $Ct$ (FLI1 IP) in accordance to Frank SR et al. (31, 32). For TaqMan assay design TFSEARCH - Searching Transcription Factor Binding Sites, version 1.3 free website was used for the prediction of ETS binding sites in the promoter of IGF-1R gene and the sequence spanning from 1041bp to 1051bp was identified as the best. Beacon Designer 4 software was used for the design of the assay spanning from 1005bp to 1114bp. CD99 and TGFβR2 promoter fragments containing ETS consensus sequence were used as EWS-FLI immunoprecipitation controls (28, 30).
Immunofluorescence assays. Cells were seeded in single slide covers placed in 96-well plates, pre-treated with gelatin (Sigma) and grown in standard medium. After exposure to drug/DMSO for 24h, 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); 53BP1 (1:100) (Abcam Cambridge, UK), anti-Tubulin β III (dilution 1:50) or anti-H neurofilament 200kD (clone NE14) (dilution 1:40) (SIGMA). Cells were then stained with the secondary antibody Cy5 (Jackson Immunoresearch, West Grove, Pennsylvania, USA) and counterstained with DAPI 1mg/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 micro arrays by q-RT-PCR. RNA extraction was performed using Qiagen RNA extraction kit, following manufacturer’s instructions, as described elsewhere (26, 33). Retrotranscription was performed using 500 ng of total RNA. Low density micro array, in the form of q-RT-PCR 96 well plates Human DNA Repair PCR Array(Qiagen, Hilden, Germany) were performed using the iQ5 thermocycler from BioRAD. RT-PCR was run in a 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 BIORAD 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 48h using the Glow caspase luminescent kit by Promega (Madison, WI, USA) according to manufacturer’s instructions. Luminescence was read in a plate reader (Tecan Männedorf, Switzerland).
**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 200kD (clone NE14) (dilution 1:40) (SIGMA) and IGF-1Rβ (Santa Cruz). Detection of Ki-67 was performed on sections as previously described (34). TUNEL assay was performed with ApopTag® Plus Peroxidase in situ apoptosis kit (Merck Millipore Billerica MA, USA) according to manufacturer’s instructions.

**Statistical analysis.** Correlations between two variables were obtained by Spearman’s test. IC\(_{50}\) 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 (Biososoft, Ferguson, MO). Differences among means were analyzed by Student’s \(t\) test or ANOVA test.
RESULTS

Trabectedin disrupts EWS-FLI1 binding to some DNA targets but increases recruitment to IGF-1R promoter in both in vitro and in vivo models.

We used Chromatin Immunoprecipitation (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 IGF-1R and IGF-1 promoters. ChIP indicated that the amount of EWS-FLI1 chimera bound to the TGFβR2 and CD99 promoters was significantly reduced after 1h treatment with trabectedin both in TC-71 cells, displaying EWS-FLI1 type1 chimera, and in the 6647 cell line, that displays EWS-FLI1 type2 hybrid, at pharmacological concentrations (IC₅₀ value after 1h of treatment) (Fig. 1A). The binding of the chimera EWS-FLI1 type 1 (TC-71) and type 2 (6647) to the CD99 and TGFβR2 promoters was evaluated also in mouse xenografts after i.v. administration of trabectedin (0.15 mg/kg, every seven days for three times, q7dx3) and DXR (8 mg/kg, every seven days for two times, q7dx2). As shown in Supplementary Fig. S1, in TC-71 EWS model trabectedin was more active (best T/C 56.2% at days 20) than DXR (reference compound, best T/C 79.5% at days 22). Instead in 6647 xenograft model, DXR was extremely effective (best T/C 14.3% at days 21) while trabectedin was less active (best T/C 48.6% at days 21). In both cases, however, trabectedin and DXR 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 24h after the first dose both in TC-71 and 6647 cells. Re-attachment was observed starting seven days from the third treatment.

Besides inhibitory effects, trabectedin but not DXR also caused a dose- and time-dependent increase in the binding of the chimera to IGF-1R promoter in EWS cells (Fig. 1C; Supplementary S2A), while occupancy of the IGF-1 promoter appeared to be only slightly affected (Supplementary Fig. S2B). Up-regulation of IGF-1Rβ was also confirmed at protein level after trabectedin treatment.
(Fig. 1C), in line with our previous data showing increased transcription and expression of IGF-1Rβ in cells made resistant to trabectedin (18). Consistently, silencing of EWS-FLI1 in TC-71 cells induced down-regulation of IGF-1Rβ protein (Supplementary Fig. S3). Enhancement of EWS-FLI1 occupancy to IGF-1R by trabectedin was also observed also in vivo in TC-71 and 6647 xenografts (Fig. 1D), further sustaining the relationship between EWS-FLI1 and IGF-1R. These findings provided the rationale for testing the combination of trabectedin with anti-IGF-1R agents.

Anti-tumor activity of the combination between trabectedin and the anti IGF-1R HAb AVE1642

Anti-tumor activity of trabectedin alone or in combination with anti-IGF-1R 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 anti-tumor 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 anti-proliferative, pro-apoptotic and pro-differentiating activity of trabectedin also in EWS (Fig. 2B, Supplementary Fig S4). Moreover, in keeping with ChIP findings, xenografts treated with trabectedin showed increased expression of IGF-1Rβ (Fig. 2B). Combination treatments with AVE1642 HAb further inhibited tumor cell proliferation and IGF-1R expression while increased apoptotic rate (Fig. 2B). This supports the combination of trabectedin with anti-IGF-1R HAb against EWS.

Efficacy of the dual inhibitor anti-IGF-1R/IR OSI-906 in combination with trabectedin

Considering that the great majority of EWSs express concomitant high levels of IR, which may overcome the IGF-1R blockade (29, 36), efficacy of the dual inhibitor anti-IGF-1R/IR OSI-906 was evaluated in a panel of 13 EWS cell lines (Table 1), including the TC/ET 12nM cell line, highly
resistant to trabectedin (18) and TC/AVE, resistant to anti-IGF-1R 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 HAb (TC/AVE) or trabectedin (TC/ET 12 nM) (Table 1). When OSI-906 was combined to trabectedin we observed advantageous effects in terms of apoptosis, both in p53wt (WE-68) and p53mutated cells (TC-71) (Fig. 3A). This advantageous pro-apoptotic cell death may derive from the combination of two different inputs. While inhibition of the IGF system may block anti-apoptotic effects of IGF-1R/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) (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 (NHEJR) 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 up-regulation of members from 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 24h 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 (<500bp) after cell exposure to OSI-906 is not surprising since 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 12nM resistant cell line after treatment with increasing doses of OSI-906, thus
confirming complementary pro-apoptotic effects of the two drugs (Fig. 4D).
DISCUSSION

Trabectedin is a newly licensed chemotherapeutic agent, with an acceptable toxicity profile (40). In this paper, 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 IGF-1R expression by enhancing EWS-FLI1 occupancy of the IGF-1R promoter. EWS-FLI1 drives the malignant phenotype in EWS cells (11), acting either as a transcriptional activator and 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 paper we show that these inhibitory effects, although probably prevalent, are not exclusive. In fact, while we confirm that both trabectedin and DXR are able to strongly suppress the binding of EWS-FLI1 (both type 1 and type 2) to two target genes (TGFβR2 and CD99) both in vitro and in vivo, a significant enhancement of EWS-FLI1 occupancy on the IGF-1R 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-FLI1 downstream targets and displayed differential specificity, likely due to preferential sequence binding affinities (42, 43). This discovery introduces a certain level of specificity in the action of conventional agents, which is potentially very interesting, but requires further studies since the effects may vary in relation to drugs, transcription factors and cellular context. For example, DXR did not affect the binding of FUS-CHOP to target promoters in myxoid liposarcoma (6), whereas here it was shown to inhibit occupancy of EWS-FLI1 on TGFβR2 and CD99 promoters, indicating differences among transcriptional hybrids and drug action in different cellular contexts. By reporting also an increase and not just a suppression of EWS-FLI1 binding to specific target promoters we introduced another variable that deserves more ample investigation. In the specific context of EWS, the increase in
IGF-1R expression is absolutely reasonable from a biological point of view considering the importance that the IGF system has in maintenance of EWS malignancy (19, 44), and was indeed confirmed also in cells made resistant to trabectedin (18). From a clinical point of view, increased expression of IGF-1R in response to in vitro and in vivo exposure to trabectedin, provides the rationale for a combined use of trabectedin with anti-IGF-1R agents. Here, we demonstrate the advantages of this combination either using HAb AVE1642, a well-tolerated agent which binds human IGF-1R specifically and with high affinity (45, 46), or the dual inhibitor IGF-1R/IR, OSI-906, a small molecule shown to have anti-tumoral activity against several tumors (47, 48), including osteosarcoma (49). The association of OSI-906 with trabectedin gave synergistic effects in all of the 13 EWS cell lines here considered, including cells resistant to trabectedin (18) or to anti-IGF-1R agents (29). This appears to be due mainly to the complementary pro-apoptotic effects of the two drugs which by affecting different pathways give rise to a combination able to deliver cell death messages in all EWS cells, independently from the p53 status. While treatment with IGF-1R antagonists is known to lead to down-regulation of the proteins involved in cell survival and inhibition of cell death (38, 47), 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 single strand breaks (SSB) that rapidly turn into double strand breaks (DSB), the most lethal form of DNA damage. Tavecchio et al.(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 (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 intra-nuclear foci. The drug combination with OSI-906 maintains and even increases up-regulation of members of HR (RAD52, BRCA1 and
BRCA2), NER (XPA, ERCC1), and SSR (XRCC1) pathways but also induces a strong down-regulation of 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 IGF-1R 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-IGF-1R inhibitors. We showed that trabectedin may not only inhibit but also enhance binding of EWS-FLI1 to target genes (Fig. 5). Specifically, IGF-1R 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.

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REFERENCES


FIGURES LEGENDS

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 DXR. EWS-FLI1 was precipitated by the anti-FLI1 antibody. Decrease in the binding of the chimera EWS-FLI1 type 1 (TC-71) and type 2 (6647) to CD99 and TGFβR2 promoters was observed either in in vitro (A) or in xenografts (B) treated as described in Materials and Methods section. 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 = $2^{\Delta Ct} \times \text{input chromatin percentage}$ where $\Delta Ct = Ct \text{ (input)} - Ct \text{ (FLI1 IP)}$ (31, 32) * p<0.05; ** p<0.001, Student’s t test.

C, Left: Increased recruitment of EWS-FLI1 on IGF-1R promoter in TC-71 and 6647 EWS cells treated for 1h with trabectedin or DXR. A representative experiment is shown. Data represent recovery of each DNA fragment relative to total input DNA, respect to control. * p<0.05; Student’s t test.

Right: up-regulation of IGF-1R at protein level by western blotting after exposure to trabectedin (0.5-1 nM) up to 48h. GAP-DH was used as loading control. Blots are representative of two independent experiments. D, Time course of FLI1 association with the IGF-1R promoter in TC-71 and 6647 xenografts treated with trabectedin (0.15mg/kg). * p<0.05; Student’s t test.

Figure 2. Efficacy of the combination of trabectedin with anti-IGF-1R HAb AVE1642 against TC-71 xenografts. A, Drugs were administered i.v. as follows: trabectedin 0.15 mg/kg, every seven days for three times (q7dx3) and AVE1642 40 mg/kg, every three days for six times (q3dx6). ANOVA test: * P < 0.05; ** P < 0.01; ***P < 0.001 compared with controls (Placebo). Points, tumor weight means; bars, SE. B, Representative immunohistochemical evaluation of Ki-67, apoptotic rate by TUNEL assay and IGF-1Rβ (magnification, x200), in untreated or treated tumors. Percentages ± SE of positive cells are indicated. * p<0.05; ** p<0.001, Student’s t test.
Figure 3. Effects of the dual inhibitor anti-IGF-1R/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 48h. All treatments are normalized respect to control. Bars, mean of two independent experiments ±SE. ** p<0.001, Student’s 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.

Figure 4. DNA damage induction after cell exposure to trabectedin and/or OSI-906. A, Induction of H2AX phosphorylation (γ-H2AX) and 53BP1 expression in TC-71 cells after exposure to trabectedin, and/or OSI-906 (200-400nM) for 24h. (magnification x400). B, DNA fragmentation induced in TC-71 cells after 12-24h exposure to drugs. C, D Evaluation of Caspase 3 and PARP cleavage by western blotting after 24h exposure to drugs. Equal loading was monitored with beta-actin blotting.

Figure 5. Schematic representation of trabectedin activity in EWS. Trabectedin may not only inhibit (1) but also enhance the binding of EWS-FLI1 to target genes. Specifically, IGF-1R expression is activated after treatment with trabectedin (3), therefore providing the rationale for combined treatments with anti-IGF-1R agents.
FIGURE 1
**FIGURE 2**

(A) Graph showing the tumor weight (mg) over days from tumor inoculum for different treatments: Placebo, Trabectedin, AVE1642, and Trabectedin+AVE1642.

(B) Images of Ki-67, TUNEL, and IGF-1Rβ staining for Placebo, Trabectedin, AVE1642, and Trabectedin+AVE1642 groups.

- **Ki-67**
  - Placebo: 56.4 ± 4.2 %
  - Trabectedin: 29.1 ± 4.2 %
  - AVE1642: 30.2 ± 4.8 %
  - Trabectedin+AVE1642: 5.8 ± 1 %

- **TUNEL**
  - Placebo: 1.44 ± 0.2 %
  - Trabectedin: 1.8 ± 0.54 %
  - AVE1642: 1.2 ± 0.5 %
  - Trabectedin+AVE1642: 10.8 ± 1.7 %

- **IGF-1Rβ**
  - Placebo
  - Trabectedin
  - AVE1642
  - Trabectedin+AVE1642
FIGURE 3
FIGURE 4
Figure 5
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 (uM) ±SE</th>
<th>Trabectedin (nM) ±SE</th>
<th>Combination (nM) ±SE*</th>
<th>CI±SE**</th>
<th>Effects</th>
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<tr>
<td>TC-71</td>
<td>0.4 ± 0.15</td>
<td>0.206±0.013</td>
<td>0.137±0.01</td>
<td>0.819± 0.01</td>
<td>Synergistic</td>
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<td>WE-68</td>
<td>0.45 ± 0.25</td>
<td>0.140±0.083</td>
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<td>0.356± 0.02</td>
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<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>
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<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>
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<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>
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<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>
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<td>RD-ES</td>
<td>0.63 ± 0.14</td>
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<td>0.07±0.006</td>
<td>0.646± 0.06</td>
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<td>SKES-1</td>
<td>0.76 ± 0.15</td>
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<td>0.032±0.01</td>
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<td>RM82</td>
<td>0.2± 0.14</td>
<td>0.54±0.012</td>
<td>0.05±0.01</td>
<td>0.600± 0.09</td>
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<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>
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<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>
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<td>TC/ET12nM</td>
<td>0.402±0.12</td>
<td>1305±263</td>
<td>2.32±0.59</td>
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<td>TC/AVE1642</td>
<td>0.268±0.003</td>
<td>1982±340</td>
<td>0.178±0.004</td>
<td>0.710± 0.02</td>
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* IC₅₀ values are referred to trabectedin;

** (synergism: CI < 0.9; additive: 0.90 ≤ CI ≤ 1.10 according to ref 34)
Trabectedin efficacy in Ewing sarcoma is greatly increased by combination with anti-IGF signaling agents.

Ana Teresa Amaral, Cecilia Garofalo, Roberta Frapolli, et al.

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