Combined Transcriptional and Translational Targeting of EWS/FLI-1 in Ewing’s Sarcoma

Silvia Mateo-Lozano, Prafulla C. Gokhale, Viatcheslav A. Soldatenkov
Anatoly Dritschilo, Oscar M. Tirado, and Vicente Notario

Abstract

Purpose: To show the efficacy of targeting EWS/FLI-1 expression with a combination of specific antisense oligonucleotides and rapamycin for the control of Ewing’s sarcoma (EWS) cell proliferation in vitro and the treatment of mouse tumor xenografts in vivo.

Experimental Design: EWS cells were simultaneously exposed to EWS/FLI-1–specific antisense oligonucleotides and rapamycin for various time periods. After treatment, the following end points were monitored and evaluated: expression levels of the EWS/FLI-1 protein, cell proliferation, cell cycle distribution, apoptotic cell death, caspase activation, and tumor growth in EWS xenografts implanted in nude mice.

Results: Simultaneous exposure of EWS cells in culture to an EWS/FLI-1–targeted suppression therapy using specific antisense oligonucleotides and rapamycin resulted in the activation of a caspase-dependent apoptotic process that involved the restoration of the transforming growth factor-β–induced proapoptotic pathway. In vivo, individual administration of either antisense oligonucleotides or rapamycin significantly delayed tumor development, and the combined treatment with antisense oligonucleotides and rapamycin caused a considerably stronger inhibition of tumor growth.

Conclusions: Concurrent administration of EWS/FLI-1 antisense oligonucleotides and rapamycin efficiently induced the apoptotic death of EWS cells in culture through a process involving transforming growth factor-β. In vivo experiments conclusively showed that the combined treatment with antisense oligonucleotides and rapamycin caused a significant inhibition of tumor growth in mice. These results provide proof of principle for further exploration of the potential of this combined therapeutic modality as a novel strategy for the treatment of tumors of the Ewing’s sarcoma family.

In recent years, it has become abundantly clear that the EWS/FLI-1 translocation is the most important molecular determinant of the pathobiology of the Ewing’s sarcoma family of tumors (ESFT; ref. 1). Over 90% of ESFT carry EWS/FLI-1 chimeric transcripts and their protein products, consisting of the NH2 terminus of EWS fused to the COOH-terminal portion of FLI-1 proteins (2). Despite aggressive treatment strategies, such as high-dose chemotherapy combined with surgery and/or extended radiotherapy, the prognosis for Ewing’s sarcoma (EWS) patients with large primary tumors or metastatic disease remains poor (2). Consequently, approaches that efficiently target the EWS/FLI-1 proteins may improve therapeutic strategies for ESFT treatment.

Antisense-based gene expression knockdown is a developing, promising area of cancer therapeutics, as confirmed by recent clinical trials (3). Antisense oligonucleotides are designed to bind to RNA through Watson-Crick hybridization (4). The best characterized antisense mechanism of action involves cleavage of the target gene transcript by endogenous cellular nucleases, such as RNase H (5). Several chemical modifications have been developed to enhance the properties and effectiveness of antisense oligonucleotides. One of the oligonucleotide chemistries most frequently used involves their phosphorothioate modification (5), the extent of which renders the antisense oligonucleotide molecules increasingly resistant to nuclease degradation (6). Although inhibition of gene expression is the predominant mechanism by which antisense oligonucleotides exert their biological activity, non-antisense effects have also been described to contribute, sometimes in a dose-dependent fashion (7), to the overall action of certain antisense oligonucleotides (5).

The mammalian target of rapamycin (mTOR) protein kinase controls translation initiation through two pathways, activation of ribosomal S6K1 to enhance translation of mRNAs that bear a
5’ terminal oligopyrimidine tract and suppression of 4E-BPs to allow cap-dependent mRNA translation (8). The fact that the mTOR pathway is up-regulated in many human cancers identifies it as a novel therapeutic target (8). Rapamycin and its derivatives (CCl-779, RAD001, and AP23576) are immunosuppressor macrolides that block mTOR functions and have shown antiproliferative activity against a variety of neoplastic malignancies (9). We previously reported that mTOR signaling plays a central role in EWS cell pathology, and that rapamycin targets the EWS/FLI-1 proteins to mediate its antitumor effect on EWS cells (10), thus showing that rapamycin provides a viable alternative for ESFT treatment.

It seemed reasonable that combining a mRNA-targeting strategy, such as using antisense oligonucleotides, with another approach targeting the protein product of the same gene would not only enhance the gene expression suppressive effect but also circumvent the need of high dosage for either individual treatment, thus minimizing possible toxicities to normal cells. Moreover, this notion could be especially valid for EWS because the EWS/FLI-1 target is present in tumor cells and absent in normal cells. We now show that concurrent administration of an EWS/FLI-1–specific antisense oligonucleotide (AS-12) with rapamycin triggers EWS cell death in culture through a process involving the transforming growth factor-β (TGF-β)–induced apoptotic pathway and a substantial inhibition of tumor growth in mouse xenographs. Overall, our results provide proof of principle for the inclusion of this combined therapeutic modality as a novel strategy for ESFT treatment.

Materials and Methods

Culture conditions, antibodies, and general reagents. EWS cells were cultured and maintained as described (10). Polyclonal antibodies against Survivin, Bcl-xl, phospho-p70s6k, cleaved caspase-3, cleaved caspase-7, IRS-1, and monoclonal anti-phospho-(Ser473)AKT were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-X-linked inhibitor of apoptosis (anti-XIAP) was from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-FLI-1 and anti-TGF-β receptor II (TGF-βRII) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TGF-β neutralizing antibody was from Abcam Ltd. (Cambridge, United Kingdom). β-Actin (Abcam) was used as a loading reference. All antisense oligonucleotides and sequence-scrambled (SC) control oligonucleotides were synthesized by Bio-Synthesis, Inc. (Lewisville, TX) with two phosphorothioate-modified bonds from each end. Rapamycin was from LC Laboratories (Woburn, MA). Recombinant human TGF-β1 was from Chemicon International, Inc. (Temecula, CA). Trypan blue was from Life Technologies (Grand Island, NY). All other general reagents were from Sigma-Aldrich (St. Louis, MO).

Apoptosis and cell cycle assays. Apoptosis was evaluated by viable cell counting and/or terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assays. Cell viability was determined as described (10). TUNEL assays were done for in situ detection of apoptotic cells using the red-based TMR In situ Death Detection kit from Roche Diagnostics (Indianapolis, IN). Cells were cultured in chamber slides (Nunc, Naperville, IL) to a density of 5 × 10^4. Sixteen hours after exposure to EWS/FLI-1 antisense oligonucleotide (10 μmol/L), rapamycin (10 ng/mL), or their combination, cells were washed with PBS, fixed in freshly prepared paraformaldehyde (4% in PBS) for 30 minutes at room temperature, rinsed thrice in PBS, permeabilized with 0.2% Triton X-100 in PBS for 30 minutes, and incubated with the TUNEL reaction mixture for 1 hour at 37°C in a humidified atmosphere in the dark. TUNEL-positive cells were visualized with a Nikon E600 fluorescence microscope. Cell cycle distribution of cells harvested 24 hours after exposure to similar treatments was evaluated by flow cytometric analysis as described (10), on a FACScan instrument (Becton Dickinson, San Jose, CA), using the ModFit LT Analysis System (Verity Software House, Topsham, ME) at the Flow Cytometry/Cell Sorting Shared Resource of the V.T. Lombardi Comprehensive Cancer Center. Parallel experiments were done using SC oligonucleotide (10 μmol/L) as control. The same TUNEL kit described above was used on deparaffinated 5-μm tumor sections. All treatments were carried out in triplicate. Oligonucleotides were added directly to the EWS cell cultures, without any auxiliary delivery system.

Caspase assays. A4573 cells (2 × 10^4 per well) were seeded into 96-well plates. After overnight incubation, cells were treated for 24 hours with either AS-12 (10 μmol/L), rapamycin (10 ng/mL), their combination, or DMSO vehicle (as negative control), each in the presence or absence of the Ac-DEVD-CHO caspase-3/7 inhibitor (20 μmol/L). All treatments were done in triplicate. Parallel control experiments were done using SC-12 (10 μmol/L). After treatment, caspase-3 alone and caspase-3/7 activity determinations were done using, respectively, CaspACE and Apo-ONE Homogeneous Caspase-3/7 assays (Promega, Madison, WI) following manufacturer’s protocols.

Reverse transcription-PCR. Conditions for RNA preparation, reverse transcription, amplification, and PCR product analysis and quantification were as described (11). Primers ACAAGCAGCAACAAACGAGG (upper) and ATCGGGATGCCAAAGAGG (lower) were used to amplify EWS/FLI-1; primers used to amplify TGF-β1 were TGTGGCCGC-TGCTGCTAC (upper) and GCACCTCCCCGTGCTC (lower); and the primers CTTGGTTAGAAGTGTC (upper) and AGGCGAGGT-TAGGTTG (lower) were used to amplify human TGF-βRII. Primers for actin amplification were described previously (11). For each set of primers, the number of cycles was adjusted so that the reaction end-points fell within the exponential phase of product amplification, thus providing a semiquantitative estimate of relative mRNA abundance (12, 13).

Immunologic techniques. Procedures for Western immunoblotting, immunofluorescence, and immunohistochemistry were previously described (10, 11, 14). Antibodies against FLI-1, php70s6k, cleaved caspase-3, cleaved caspase-7 (all at 1:50 dilution), TGF-βRI (at 1:100 dilution), or TGF-β1 (at 1:250 dilution) were used for immunohistochemistry assays. ELISA was used to determine the amount of active TGF-β1 secreted into the culture media by cells treated for 48 hours with SC-12, rapamycin, AS-12, or their combinations; these were analyzed for active TGF-β1 levels using the Emax ImmunoAssay System (Promega) according to the manufacturer’s directions. Three independent experimental replicas were used for these analyses.

In vivo studies. Male BALB/c athymic (nu/nu) nude mice (5–6 weeks old) were obtained from the U.S. National Cancer Institute. Mice were housed in the animal facilities of the Division of Comparative Medicine, Georgetown University. All animal work was done under protocols approved by the Georgetown University Animal Care and Use Committee. Procedures for tumor induction and tumor volume evaluation were as described (14). Once tumors reached a mean volume of about 200 mm^3, mice were randomized into six groups (12 animals per group), and treatment was initiated. Treatment groups included AS-12 alone, SC-12 alone, rapamycin alone, AS-12 plus rapamycin, SC-12 plus rapamycin, and vehicle control. Oligonucleotides (given i.t. at a dose of 5 mg/kg) and rapamycin (given i.p. at a dose of 1.5 mg/kg) were all administered daily. Animals in the vehicle control group received i.p. injections of the rapamycin carrier solution alone. All animals were treated for two 5-day series with a 2-day break in between. At specified times during the treatment, or whenever tumors reached the maximum volume allowed by institutional tumor burden guidelines, representative animals from each treatment group were sacrificed by asphyxiation with CO_2. Animals treated with either rapamycin alone or with the oligonucleotide/rapamycin combinations were euthanized 4 weeks after initiation of the experiment. Tumors were immediately excised from sacrificed animals, measured, and used for protein, TUNEL, and immunohistochemical assays.
Statistical analysis. Unless otherwise indicated, reverse transcription-PCR and Western blot analyses were repeated at least thrice. Data from densitometric quantification analyses were expressed as mean ± SD. For these and other assays involving statistical analysis, ANOVA or Student’s t tests were used to assess the significance of differences between groups or individual variables, respectively. P ≤ 0.01 was regarded as significant.

Results

A 12-mer antisense oligonucleotide reduces EWS/FLI-1 protein expression levels and inhibits the proliferation of A4573 and TC-71 cells but not of SK-ES-1 cells. A panel of five antisense oligonucleotides of different lengths (12-30 nucleotides) targeting either the translation junction or the translation initiation codon (AS-AUG) of the EWS/FLI-1 gene were used to treat A4573 cells. SC control oligonucleotides matched in length to each antisense sequence (Fig. 1A) were used as controls. The most effective cell proliferation inhibitor was the 12-mer antisense oligonucleotide (AS-12; Fig. 1B), which also caused the greatest reduction in EWS/FLI-1 protein levels (>83%), relative to its matched scrambled (SC-12) control (Fig. 1C). Consequently, AS-12 was selected for subsequent experiments. To test its target specificity, AS-12 was used to treat TC-71 and SK-ES-1 EWS cells, which carry EWS/FLI-1 fusion types 1 and 2, respectively, whereas A4573 cells carry a type 3 fusion. Interestingly, AS-12 efficiently inhibited the proliferation of TC-71 cells (data not shown) and also reduced their EWS/FLI-1 protein levels to about 50% but had no apparent effect on SK-ES-1 cells (Fig. 1D). Unless stated otherwise, essentially identical results were obtained from treatments with either vehicle (PBS) or the SC-12 control oligonucleotide in these and other experiments. Because EWS/FLI-1 is known to transcriptionally repress the expression of the type II receptor for TGF-β (TGF-RII; ref. 15), we tested whether AS-12 treatment had any effect on TGFβ-RII mRNA levels. Results (Fig. 1E) showed that, relative to SC-12-treated controls, the down-regulation of EWS/FLI-1 protein caused by AS-12 was paralleled by an up-regulation of TGFβ-RII mRNA in both TC-71 and A4573 cells, with no effect on SK-ES-1 cells (Fig. 1E), thus showing the efficacy of AS-12 treatment to revert the transcriptional activity of EWS/FLI-1.

Combining AS-12 with rapamycin results in additive antiproliferative effects on EWS cells. To maximize the suppressive effects on EWS/FLI-1, A4573 cells were treated with a combination of AS-12 and rapamycin, a drug previously shown to down-regulate the EWS/FLI-1 protein expression in EWS cells (10). Cells were treated with AS-12 and rapamycin alone or in combination, and their effect on cell proliferation was determined 48 hours later. Results (Fig. 2A) showed that, relative to controls treated with SC-12 alone, treatment with rapamycin or AS-12 alone reduced cell proliferation by about 40% and 60%, respectively. The effects of the SC-12/rapamycin combination in this and other experiments were essentially identical to those of rapamycin alone, indicating that SC-12 did not modify the cellular response to rapamycin. Contrary, the AS-12/rapamycin combination diminished cell proliferation by around 80%. This inhibitory outcome correlated with the down-regulating effect of each treatment on EWS/FLI-1 protein expression, which was nearly abolished by the AS-12/rapamycin combination (Fig. 2B). We reported previously that rapamycin inhibited EWS cell proliferation by inducing G1-phase cell cycle arrest (10), and others have published that certain antisense oligonucleotides against EWS/FLI-1 reduced cell proliferation by inducing cell cycle arrest also (16). Consequently, we tested whether AS-12 reduced EWS cell proliferation by inducing cell cycle arrest, and whether the AS-12/rapamycin combination had a more pronounced effect. As shown in Fig. 2C, AS-12 alone induced a minor 5% G1 phase cell cycle arrest, and rapamycin alone had a more marked effect (30% increased G1), whereas an additive effect (35% increased G1) was observed for cells treated with the AS-12/rapamycin combination.

Recent reports described that rapamycin and its derivatives, by inhibiting mTOR, activate insulin-like growth factor I (IGF-I) signaling by increasing the levels of IRS-1 and phospho-AKT, paradoxically attenuating in this way their own anti-tumor effect (17, 18). Because this may pose a particularly serious problem for ESFT, given that IGF-1/IGF-IR signaling is an important determinant of the malignant properties of these tumors (19, 20), we examined whether the antiproliferative activity of the AS-12/rapamycin combination might be limited by a similar mechanism. As shown in Fig. 2D, rapamycin alone did increase the levels of both IRS-1 and phospho-AKT (by about 3.5-fold). Interestingly, however, although the AS-12/rapamycin combination also increased the levels of IRS-1, it had a much smaller effect on the levels of phospho-AKT (Fig. 2D). The simultaneous treatment with AS-12 prevented the induction by rapamycin of a greater increase in phospho-AKT most likely because the down-regulation of EWS/FLI-1 brought about by AS-12 resulted simultaneously decreased the levels of both IGF-I and IGF-IR (Fig. 2E), thereby favoring the antiproliferative activity of rapamycin.

AS-12 treatment induces apoptosis in A4573 cells. The marked reduction in A4573 cell proliferation produced by AS-12 treatment (Fig. 2A) could not be explained by the minor (~5%) G1-arresting effect of AS-12 on the cell cycle (Fig. 2C). This, and the fact that antisense oligonucleotide treatment directed against the EWS/FLI-1 gene has been shown to induce apoptosis in EWS cells in some instances (16), prompted us to test whether AS-12 treatment induced apoptosis in A4573 cells. Indeed, relative to untreated and vehicle- or sequence-scrambled treated controls, AS-12 alone induced a marked level (25-30%) of cell death, which was significantly enhanced (to about 50%) when AS-12 was combined with rapamycin (Fig. 3A, left). Induction of cell death was partially prevented by a general caspase inhibitor (INH; Fig. 3A), although, interestingly, no variations in the levels of caspase-3 activity were detected after any of the treatments (Fig. 3A, right). However, the use of a dual caspase-3/7−specific assay allowed the detection of marked increases in caspase activity induced by both the AS-12 alone and the AS-12/rapamycin combination (Fig. 3B), which were inhibited by the Ac-DEVD-CHO caspase-3/7−specific inhibitor (Fig. 3B), suggesting that the apoptotic process triggered by AS-12 was most likely caspase-7 dependent. Furthermore, detection of cleaved caspase-7 by TUNEL and immunofluorescence showed (Fig. 3C) that both AS-12 alone and AS-12/rapamycin induced a caspase-7-dependent apoptotic process, and that the combined treatment was a more effective apoptosis inducer than either drug alone (Fig. 3D). To better understand the mechanism by which AS-12 triggered apoptosis and the reason for the increased effectiveness of the
combined treatment, Western blot analyses were used to test for possible changes caused by the various treatments in the expression of key apoptotic effectors. Although rapamycin treatment alone induced the down-regulation of the Survivin, XIAP, and Bcl-xL proteins, it did not result in the accumulation of detectable levels of cleaved caspase-7 (Fig. 3E). However, treatment with AS-12 alone resulted in a remarkable extent of caspase-7 cleavage, along with a significant reduction of Bcl-xL (Fig. 3E). When AS-12 and rapamycin were combined, the expression of the survival proteins, particularly Bcl-xL, was down-regulated, and caspase-7 cleavage was also observed (Fig. 3E). In agreement with its ability to down-regulate the expression of type 1, but not type 2, EWS/FLI-1 proteins (Fig. 1D), treatment with AS-12 alone also induced caspase-7 cleavage in TC-71 cells but not in SK-ES-1 cells (Fig. 3F).

**Combined AS-12/rapamycin therapy inhibits the growth of ESFT-derived tumors in vivo by triggering a caspase-3/7-dependent apoptotic response.** A nude mouse tumor xenograft model system established as described in Materials and Methods was used to evaluate the effects of AS-12, rapamycin, and their combination *in vivo*. When tumors reached a volume of ~200 mm³, animals were randomized into six groups, treated as described in Materials and Methods, and tumor growth was followed over a period of up to several weeks. No appreciable differences in tumor growth were observed among animals in the PBS and SC-12 control groups. However, as illustrated in Fig. 4A, at the time when animals from control groups had to be sacrificed following Institutional Animal Care and Use guidelines on maximum allowable tumor burden (days 14-15), AS-12 alone, rapamycin alone, SC-12/rapamycin, and AS-12/rapamycin had already caused significant delays in tumor development (*P* < 0.0013, for the last three experimental groups). After the second 5-day treatment series, treatment with rapamycin alone was more effective at delaying tumor growth than was the treatment with AS-12 alone. No differences in tumor growth were observed between animals treated with rapamycin alone and those treated with the SC-12/rapamycin combination, whereas the AS-12/rapamycin combination resulted in a more robust inhibition than that caused by rapamycin alone for the duration of the experiment (Fig. 4A).

![Fig. 1.](image_url) A 12-mer antisense oligonucleotide (AS-12) targeted against the type 3 junction of the EWS/FLI-1 gene reduces EWS/FLI-1 protein levels and the proliferation of EWS cells. A, A4573 cells were treated for the indicated times with a set of five different antisense oligonucleotides (10 μmol/L); four directed against the translocation breakpoint sequence and one against the region containing the translation initiation codon (AS-AUG). Columns, mean percentage of cell proliferation relative to the proliferation of cells treated with the corresponding sequence-scrambled, control oligonucleotides; bars, SD. B, Western blot analysis of EWS/FLI-1 levels comparing each antisense oligonucleotide with its matched sequence-scrambled oligonucleotide control. Actin was used as the loading control. Densitometric determinations (mean ± SD) of signal intensity from three independent experiments. C, Western blot analysis of EWS/FLI-1 expression levels in TC-71 and SK-ES-1 cells treated with AS-12 or control SC-12 oligonucleotides. D, mRNA levels of TGFβ-RII analyzed by reverse transcription-PCR in the EWS cells shown after treatment with AS-12 or control SC-12 oligonucleotides (10 μmol/L, in both cases). Actin was used as internal control for normalization purposes.
To correlate the antitumor effects of these treatments with the levels of EWS/FLI-1 expression, we examined EWS/FLI-1 protein expression in tumors from control and treated animals. On day 12 (Fig. 4A), tumors were harvested, paraffin embedded, sectioned, and immunostained for EWS/FLI-1. No appreciable differences in EWS/FLI-1 expression levels were observed among animals in the two control groups and, as shown in Fig. 4B, EWS/FLI-1 expression was strong and localized in the nucleus of cells in tumors from control mice. In tumors from mice treated with AS-12 or rapamycin, the immunoreactive EWS/FLI-1 stain decreased, being nearly undetectable in tumors from mice treated with the AS-12/rapamycin combination (Fig. 4B). Western immunoblot analyses of tumor samples corroborated immunohistochemical assays (Fig. 4C). Following the approach used in some clinical trials to evaluate the efficacy of mTOR-specific inhibitors (21), we tested the efficacy of rapamycin in our system by analyzing the phosphorylation status of p70s6k, the downstream substrate of mTOR, in tumor samples from control and treated mice. Results (Fig. 4D) showed a remarkable cooperativity between AS-12 and rapamycin to completely suppress p70s6k phosphorylation, an effect much more pronounced than the inhibition levels caused by each individual treatment.

To verify whether the different treatments also induced apoptosis in vivo, we analyzed tumor cell apoptosis by TUNEL assay in serial sections of the tumors described above. As shown in Fig. 5A, in samples representative of tumors from both groups of control animals the number of apoptotic, TUNEL-positive cells was minimal. In contrast, increasing levels of apoptosis were observed in tumors derived from animals treated with rapamycin, AS-12, and the AS-12/rapamycin combination. Furthermore, these increasing levels of apoptosis observed in treated animals correlated with the expression of cleaved forms of caspase-3 and caspase-7, as detected by immunohistochemical analysis in the same samples (Fig. 5B).

**Treatment with sublethal concentrations of TGF-β1 together with rapamycin results in EWS cell apoptosis.** Because elevated expression levels of the TGF-β1 receptor were restored as a consequence of the down-regulation by AS-12 of EWS/FLI-1, its transcriptional repressor, it seemed possible that the re-establishment of a TGF-β1/TGF-β1 proapoptotic loop (22) in AS-12-treated cells may contribute to its apoptotic effect. Therefore, we analyzed possible changes in the levels of TGF-β1 mRNA and active secreted protein in A4573 cells brought about by treatments with AS-12 alone, rapamycin alone, or the AS-12/rapamycin combination. Relative to SC-12 controls, treatment of A4573 cells with rapamycin alone resulted in an almost complete suppression of TGF-β1 mRNA expression (Fig. 6A) and a significant reduction of the levels of secreted active TGF-β1 (Fig. 6B), whereas the levels of TGF-β1 mRNA and secreted protein observed in cells subjected to treatments involving AS-12 were not significantly different from those in SC-12 controls (Fig. 6A and B). These data suggested that AS-12, by an unknown mechanism, prevented the suppressive effect of rapamycin on TGF-β1 expression and secretion and contributed to the establishment of a TGF-β1 proapoptotic autocrine loop in EWS cells. To provide further support for the involvement of TGF-β in the process of AS-12-induced apoptosis, we first treated A4573 cells with increasing concentrations of TGF-β1, to determine their dose response, with cell death as the end point (data not shown). Next,
we treated A4573 cells with AS-12 alone or with AS-12 plus a TGF-β1 neutralizing antibody and followed the effect on apoptosis by measuring caspase-3/7 activity levels 48 hours later. Results (Fig. 6C) showed that blocking TGF-β1 significantly decreased the level of caspase-3/7 activity induced by AS-12. Conversely, simultaneous treatment of the cells with a sublethal dose of TGF-β1 (0.1 ng/mL) plus rapamycin induced levels of apoptosis, as measured by caspase-3/7 activity, similar to those observed after treatment with AS-12 alone (Fig. 6C), whereas the levels of apoptosis induced by TGF-β1 alone were as low as those elicited by treatment with rapamycin alone. These results provided strong evidence in support of the existence of a TGF-β1/TGF-βRII loop contributing to the proapoptotic activity of AS-12 on EWS cells. This notion was further strengthened by the observation with immunohistochemical methods of a similar situation, with regard to levels of expression of TGF-β1 and TGF-βRII, in tumors induced in nude mice by injection of cells treated with AS-12 alone, rapamycin alone, or their combination (Fig. 6D), thus recapitulating in vivo the conditions detected in cultured cells: although both rapamycin and AS-12 increased the expression of TGF-βRII, contrary to the cases involving AS-12 treated cells, TGF-β1 production in tumors induced by cells treated with rapamycin alone seemed restricted to non-tumor cells present in the specimens (Fig. 6D).

**Discussion**

In recent years, aggressive multidisciplinary approaches have significantly improved the prognosis for ESFT patients. Nevertheless, despite these aggressive treatment modalities, the prognosis for patients with metastases or early relapse remains...
very poor (23). Consequently, more effective therapeutic options are needed. The EWS/FLI-1 protein plays a key role in the pathogenesis of ESFT (24). As an aberrant transcription factor, it regulates the expression of a variety of genes (15, 25–31) that modulate important functions in tumor cells, such as cell proliferation, apoptosis, and differentiation. Therefore, the EWS/FLI-1 protein represents a unique therapeutic target for the treatment of ESFT (24). In the present study, we show the effectiveness of a treatment that simultaneously targeted the EWS/FLI-1 protein at the transcriptional level, with an antisense oligonucleotide directed against the translocation junction of the EWS/FLI-1 gene, as well as post-transcriptionally, by inhibiting translation of the EWS/FLI-1 protein with rapamycin. The AS-12/rapamycin combination efficiently induced apoptosis of EWS cells in vitro and led to a marked inhibition of EWS tumor growth in vivo.

Inhibition of the expression of the EWS/FLI-1 gene by using antisense oligonucleotide or small interfering RNA has been investigated by other groups (1, 16). Most of the sequences targeting the translocation junction used in previous studies ranged from 15 to 25 nucleotides in length (16). However, in agreement with previous reports showing that shorter sequences, even as short as heptanucleotides, behaved as more potent and specific gene expression suppressive agents (32–34), a 12-mer antisense oligonucleotide (AS-12) directed against the breakpoint junction of a type 3 EWS/FLI-1 translocation was clearly the most effective of the antisense oligonucleotides used in our study. Although AS-12 treatment had little effect on EWS cell cycle, it was a potent inducer of apoptosis both in vitro and in vivo, significantly reducing the growth of A4573-derived EWS tumors in mice. Interestingly, this same antisense oligonucleotide (AS-12) was also effective targeting the type 1 EWS/FLI-1 translocation present in TC-71 cells. This is noteworthy because type 1 and type 3 EWS/FLI-1 translocations involve the same sequence from exon 6 of the FLI-1 gene (35), and molecular modeling of EWS/FLI-1 mRNA secondary structure around the breakpoint region predicted that the nucleotides available for pairing with antisense oligonucleotides targeted against the junction sequence would be predominantly located within the FLI-1 side of the hybrid transcript (36). Therefore, it seems that, although the AS-12 spans both sides of the junction point, its interference with the FLI-1 portion is sufficient to cause the observed effects on EWS cells. The absence of such specific sequence in type 2 translocations would explain its lack of activity on SK-ES-1 cells. These data agree with results from other studies in which

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antisense oligonucleotides or small interfering RNA directed against the translocation point were used to knockdown EWS/FLI-1 expression, which generally indicated that the most effective oligonucleotides were those targeting the FLI-1 portion of the translocation (1, 37, 38). Furthermore, our results indicate that AS-12 could be effective against the majority of EWS tumors because most patients present with type 1 translocations (2) and, including those with type 3, the range of action of AS-12 may approach the 98% to 99% of the cases.

We described previously that rapamycin down-regulated the expression of the EWS/FLI-1 protein and inhibited the proliferation of EWS cells in vitro (10). We hypothesized that combining rapamycin with antisense oligonucleotides specifically designed against EWS/FLI-1 could overcome some of the possible limitations of the use of antisense oligonucleotides, such as their susceptibility to degradation and generally poor uptake (39), thus enhancing their effectiveness. Indeed, results showed that combined treatments were markedly more efficient than individual treatments at inhibiting EWS/FLI-1 expression, reducing cell proliferation, and, most importantly, at inducing co-operative apoptotic effects both in vitro and in vivo. In light of reports (17, 18) that rapamycin, by increasing IRS-1 and phospho-AKT levels, could attenuate its own antitumor activity, combining AS-12 and rapamycin became mutually beneficial: rapamycin contributing a potent cell cycle arresting activity, and AS-12 counteracting the potential self-attenuating, antiapoptotic effect of rapamycin not only by reducing the expression of both IGF-I and IGF-IR but also by maintaining the normal secreted levels of active TGF-β1 in the presence of rapamycin, which would have down-regulated them otherwise.

TGF-β has been shown to inhibit cell proliferation and induce apoptosis in a variety of cancer cells (40, 41). Given the antiproliferative and proapoptotic functions of TGF-β, it is not surprising that this pathway is disrupted in some cancers either by somatic mutations within the TGF-βRII gene (22) or, as in the case of ESFT, by EWS/FLI-1–dependent TGF-βRII transcriptional suppression (15). The differential effect between AS-12 and rapamycin on TGF-β expression may explain why whereas AS-12 induced apoptosis, rapamycin predominantly inhibited cell proliferation (10). Although both AS-12 and rapamycin (10) restored high levels of expression of the TGF-βRII receptor in vitro and in vivo, in contrast with the effects observed in cases involving AS-12-treated cells, the strong down-regulation of TGF-β1 expression caused by rapamycin in vitro and on EWS tumor cells in vivo would disrupt the activity of the TGF-β1-induced apoptotic pathway. Although understanding the mechanism by which, when in combination, AS-12 prevents the down-regulating effect of rapamycin on TGF-β1 expression requires further experimentation, it seems a crucial event for eliciting the enhanced apoptotic activity of the AS-12/rapamycin combination on EWS cells and their inhibitory activity on tumor growth in vivo.

Our results agree with the recent report that EWS/FLI-1 silencing induced apoptosis in EWS cells (31). Although some caspase-3 cleavage was observed in AS-12-treated cells
(data not shown), caspase activity assays and dual caspase-7 immunofluorescence/TUNEL analyses showed that the apoptotic process induced by AS-12 was mainly caspase-7 dependent in vitro and in vivo. Interestingly, the apoptotic levels elicited by the AS-12/rapamycin combination were greater than those induced by AS-12 treatment alone, although AS-12 by itself caused a greater extent of caspase-7 cleavage. This can be explained by the differential effect of AS-12 alone or in combination with rapamycin on the levels of Survivin and XIAP, members of the IAP family of proteins, which have been identified as potent caspase inhibitors (42). Whereas XIAP is known to inhibit activated caspase-3 and caspase-7 through direct interactions, the mechanism by which Survivin inhibits caspases is less clear (38). In our study, although both AS-12 alone and in combination with rapamycin down-regulated Survivin and XIAP, the extent of down-regulation of both Survivin and XIAP induced by treatment with the AS-12/rapamycin combination was substantially greater than the down-regulation induced by AS-12 alone. The increased abundance of Survivin and XIAP in AS-12-treated cells, relative to AS-12/rapamycin–treated cells, may result in a more efficient inhibition of the active forms of caspase-7 in spite of their increased levels after the former treatment.

Sequence-specific down-regulation of EWS/FLI-1 expression in EWS xenografts has been shown to correlate with tumor growth inhibition (43, 44). However, the delivery system for antisense and small interfering RNA oligonucleotides still remains an important unsolved issue. In this regard, it is important to point out that in our study individual treatments with naked AS-12 oligonucleotide or low-dose rapamycin (1.5 mg/kg/d) significantly delayed tumor growth, and their combination markedly inhibited tumor growth. Moreover, the extent of tumor growth inhibition correlated with the effectiveness of the various treatments to target the expression of the EWS/FLI-1 fusion protein. Remarkably, contrary to what was observed in vitro, all treatments induced different grades of apoptosis that was associated not only with caspase-7 but also with caspase-3 activation. Although the reason for this difference between the in vivo and in vitro apoptotic processes remains to be elucidated, rapamycin-induced apoptosis in vivo has been reported to occur even when only cytostatic effects are induced in vitro (45, 46). In our study, it is possible that in vivo TGF-β1 may be provided by autocrine and paracrine mechanisms that would allow higher levels of the activity of the TGF-β1 apoptosis-induced pathway, thus resulting in a greater involvement of caspase-3 in the process than in vitro. However, this mechanistic possibility requires further characterization.

In summary, simultaneous administration of EWS/FLI-1–targeted antisense oligonucleotides and rapamycin induces the...
apoptotic cell death of EWS cells through a process involving the restoration of the TGF-β1/TGF-β1RII proapoptotic pathway. In vivo experiments with the antisense/rapamycin combination conclusively show a robust inhibition of the growth of tumor xenografts in mice, thus providing proof of principle supporting further exploration of the potential of this combined therapeutic modality as a novel strategy for the treatment of tumors of the ESFT.

References

Combined Transcriptional and Translational Targeting of EWS/FLI-1 in Ewing's Sarcoma

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