**New Strategies in Ewing Sarcoma: Lost in Translation?**

Fernanda I. Arnaldez and Lee J. Helman

**Abstract**
Ewing sarcoma is the second most common pediatric malignant bone tumor. Aggressive multimodality therapy has led to an improvement in outcomes, particularly in patients with localized disease. However, therapy-related toxicities are not trivial, and the prognosis for patients with relapsed and/or metastatic disease continues to be poor. In this article, we outline some of the promising therapies that have the potential to change the Ewing sarcoma therapeutic paradigm in the not-too-distant future: insulin-like growth factor receptor inhibitors, targeting of the fusion protein, epigenetic manipulation, PARP inhibitors, and immunotherapy. *Clin Cancer Res*; 20(12); 3050–6. ©2014 AACR.

**Background**
Ewing sarcoma is the second most common pediatric malignant bone tumor, and it can also present as a soft-tissue malignancy. Approximately 225 new cases are diagnosed each year in the United States in patients between the ages of 1 and 20 years (1). The presence of macrometastatic disease continues to be the single most significant predictor of poor clinical outcome (2). Patients with localized disease treated with multimodality therapy can achieve a 5-year event-free survival rate of 70% (3, 4), whereas the 5-year overall survival rate of patients who present with overt bone or bone marrow metastatic disease at diagnosis is less than 20% (1, 5, 6). Traditional therapeutic approaches include local control of the primary lesion that involves surgery and/or radiation therapy, and treatment of disseminated disease with multiagent cytotoxic chemotherapy.

These approaches have led to significant improvements in outcomes over the past decades, particularly in patients with localized disease (7). However, novel therapeutic approaches are clearly needed, not only to increase survival in patients with relapsed or metastatic disease (2), but also to continue to improve survival of patients with localized disease as well as to decrease the acute and chronic toxicities associated with current cytotoxic drugs.

The molecular hallmark of Ewing sarcoma is the translocation between EWS, an FET family protein, and an ETS transcription factor (8). The FET family includes nuclear proteins such as FUS, EWS, and TAF15, which are involved in abnormal rearrangements with transcription factors (9). In 85% of cases, the t(11;22)(q24;q12) translocation between the *EWSR1* and *FLI1* is detected. However, many other fusions have been described (10, 11). This fusion gives origin to a chimeric transcription factor that is responsible for the Ewing sarcoma oncogenic program. This aberrant factor modifies the transcription machinery, activating or quite often repressing transcription of target genes (12, 13). Although the cell of origin has been much debated, growing evidence suggests that Ewing sarcoma could possibly originate in mesenchymal stem cells (14).

Despite a growing body of knowledge about Ewing sarcoma biology, the successful application of basic discoveries to the clinic has remained elusive. Preclinical results have not always been predictive of clinical trial outcomes, highlighting the need for better models with the capability to identify targetable drivers of disease. Improved preclinical models and application of innovative clinical trial designs are needed, including the incorporation of combinational therapy in early-phase therapeutic development. However, several recent contributions hold promise for the future and are discussed below (Table 1).

**On the Horizon**

**Targeting the EWS–FLI1 fusion protein**

Efforts to suppress the oncogenicity of the fusion protein can be described in two fronts: inhibition of the transcription factor activity and inhibition of selected downstream target genes (Fig. 1). The discovery of the EWSR1–FLI1 (EF) fusion protein was reported in 1992 (8). This fusion protein generated by the Ewing-specific t(11;22) translocation functions as an aberrant transcription factor that leads to an altered transcriptional profile of both upregulated and downregulated transcripts (12, 13). Preclinical models support the potential of the EF fusion protein as a therapeutic target in Ewing sarcoma. Synthetic RNAi targeting of the fusion led to inhibition of tumor growth in vivo and in vitro. In these models, RNAi knockdown was associated with decreased expression of the EF fusion protein and downregulation of EF transcriptional targets such as c-Myc (15). Inhibition or knockdown of EWS–FLI1 in vitro leads to decreased cell viability in vitro (16). Concurrent administration of rapamycin and antisense oligonucleotides...
showed delayed tumor growth in murine xenografts (17). Although these results are encouraging, in vivo delivery of RNAi has proved to be challenging. Using a synthetic polymeric nanoparticle as a carrier, an RNAi directed toward the EWS–Fli1 fusion is being studied in murine xenografts. The strategy used to target tumor cells is to couple the siRNA-carrying nanoparticles with a monoclonal antibody against CD99, an antigen widely expressed in the surface of Ewing sarcoma cells. Efficacy measures of this creative approach will be of interest in the near future (18).

Thus, it has become clear that the EF fusion transcription factor is necessary for tumor growth, and targeting the mutant transcription factor itself or critical downstream targets of this protein are attractive therapeutic strategies. However, direct inhibition of transcription factors has proved to be difficult. The fusion protein has been shown to interact with RNA helicase A (RHA) using phage display and chromatin immunoprecipitation techniques (19). Follow-up studies showed that RHA stimulated the activity of promoters regulated by EWS–Fli1. Using Plasmon resonance screening, YK-4-279, an inhibitor of the EWS–Fli1–RHA interaction has been identified. This compound was associated with decreased tumor growth in orthotopic xenografts, but has not progressed to clinical development yet (20, 21).

The transcriptional signature of the EF fusion has been described (12), and as noted above, another way to target the EF-mutant transcription factor is to develop approaches to alter the downstream targets. Creative approaches have been used to screen compounds that are able to negatively regulate the expression of EWS–FLi1 target genes. A functional screen of a library of 1,040 compounds was performed in search for an EWS–FLi1 “off” signature, and identified cytarabine as a negative modulator of transcriptional activity (22). Unfortunately, cytarabine did not show benefit in a phase II clinical trial and was associated with significant hematologic toxicity (23). In a similar fashion, although preclinical work showed that trabectedin could interfere with the transcriptional activity of EWS–Fli1 (24), only 1 patient with Ewing sarcoma achieved stable disease (SD) in a recent phase II trial (25).

More recently, 1,280 compounds were functionally screened for suppression of EWS–Fli1 activity. Midostaurin, a multikinase inhibitor, was shown to modulate the expression of EWS–Fli1 target genes, and it was associated with decreased tumor growth in xenografts (26). Although midostaurin is undergoing clinical evaluation for the treatment of hematologic malignancies, no clinical data in solid tumors are available at this time. Finally, a library of 50,000 compounds was functionally screened using a reporter of EWS transcriptional activity (27), and mithramycin, an antineoplastic antibiotic, was identified as the leading hit. Interestingly, reports of Ewing sarcoma responses to this drug had been reported decades ago (28). Mithramycin treatment led to decreased tumor growth in xenograft models, as well as inhibition of the EWS–Fli1 signature. A phase I/II clinical trial evaluating the activity of mithramycin in patients with Ewing sarcoma is ongoing.

### Table 1. Novel therapeutic approaches in Ewing sarcoma

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Agent</th>
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<th>Outcome for phase II studies</th>
<th>Refs.</th>
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<tr>
<td>Inhibition of EF RNAi</td>
<td>YK-4-279</td>
<td>Preclinical</td>
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<td></td>
</tr>
<tr>
<td>Inhibition of EF transcriptional signature</td>
<td>Cytarabine</td>
<td>Clinical (phase II)</td>
<td>Negative</td>
<td>(22)</td>
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<tr>
<td></td>
<td>Trabectedine</td>
<td>Clinical (phase II)</td>
<td>Negative</td>
<td>(23, 24)</td>
</tr>
<tr>
<td></td>
<td>Mithramycin</td>
<td>Clinical (phase II)</td>
<td>Ongoing</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>Midostaurin</td>
<td>Preclinical</td>
<td></td>
<td>(25)</td>
</tr>
<tr>
<td>Inhibition of tumor growth signaling pathways</td>
<td>Insulin growth factor receptor I blockade</td>
<td>Clinical (phase II)</td>
<td>Positive</td>
<td>(34)</td>
</tr>
<tr>
<td>Disruption of DNA repair</td>
<td>PARP inhibitors</td>
<td>Clinical (phase II)</td>
<td>Negative</td>
<td>(45)</td>
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<tr>
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<td>HDAC inhibitors (vorinostat)</td>
<td>Clinical (phase I)</td>
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<td>Immune-based therapies</td>
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<td></td>
<td>NK cell-based therapies</td>
<td>Clinical (phase I)</td>
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<tr>
<td></td>
<td>CAR</td>
<td>Preclinical</td>
<td></td>
<td>(67, 68)</td>
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Abbreviations: CAR, chimeric antigen receptor; HDAC, histone deacetylase.

### Insulin-like growth factor receptor blockade

The relevance of insulin-like growth factor receptor (IGF1R) signaling in Ewing sarcoma has been established. The fusion protein positively regulates the expression of IGF1R (29, 30), which has been shown to be necessary for EWS–FLi1-mediated transformation of fibroblasts (31). Ewing sarcoma cells are sensitive to IGF1R inhibition in vitro and in vivo (32–35). A phase II trial of R1507, a fully human IGF1R-blocking antibody, showed an overall
complete response (CR)/partial response (PR) rate of 10% (36). Similarly, the single-agent activities of cixutumumab (IMC-A12) and figitumumab, different human antibodies targeting the same receptor, were reported as 10% and 14%, respectively, in patients with refractory Ewing sarcoma (37, 38). A major limitation of these studies was the inability to identify strategies to select patients most likely to respond to this therapeutic intervention.

Even in responding patients, the majority of responses are short lived; thus, as in other targeted therapies, acquired resistance to IGF1R blockade has also emerged as a major problem in targeting the IGF1R in Ewing sarcoma, and it is very likely that combination strategies will be needed in the future to optimize the likelihood of sustained responses. There is some emerging evidence of mTOR and ERK activation in patients who develop resistance to these therapies (39). A recent trial combining cixutumumab with temsirolimus reported that 35% patients had SD longer than 5 months or CR/PR (40). In addition, activation of MAPK and insulin receptor (41) could be involved in resistance to IGF1R inhibition.

Given that the activity of these IGF1R-blocking agents has been established, it is imperative that we identify predictors of response as well as mechanisms of acquired resistance so that the most rational combinatorial therapies are used on the properly selected patients.

**PARP inhibitors**

PARP inhibitors are an area of growing interest for the Ewing sarcoma community. In 2012, a marked sensitivity of Ewing sarcoma to olaparib, a PARP inhibitor (AZD2281), was observed in a high-throughput screen of 639 cancer cell lines aimed to detect drug sensitivity patterns as a function of genomic features (42). In a different study, sensitivity of Ewing sarcoma cells to olaparib was documented both in vitro and in tumor xenografts (43). Ewing sarcoma cells were more sensitive to PARP inhibition than prostate cancer cells harboring the TMPRSS2-ERG translocation. Remarkably, the combination of temozolamide and olaparib was synergistic in abrogating progression of Ewing sarcoma xenografts (43). An effect of the EWS–FLI1 fusion transcript in the DNA damage response was suggested more than a decade ago (44). Interestingly, a high expression of PARP in Ewing sarcoma cells has been reported (45). However, the exact role of PARP in Ewing sarcoma biology continues to be an area of active research.

The enthusiasm about these results resulted in a phase II clinical trial of olaparib in recurrent/metastatic Ewing...
sarcma following failure of prior chemotherapy. Unfortunately, no CR/PR was seen with 4 of 12 patients achieving SD at a maximum of 18.4 weeks with a median time to progression of 5.7 weeks. Further accrual to this trial was discontinued (46). Unfortunately, molecular diagnosis was not a requisite for enrollment, and it is hard to speculate about the biologic reasons for these results, which could be related not only to lack of the FET–ETS translocation but also to general lack of predictiveness of current preclinical models as well as pharmacologic factors. However, it is quite possible that other PARP inhibitors, or, combination therapies, such as with temozolomide, might have a more auspicious outcome.

**Epigenetic therapies**

**Polycomb repressor genes.** One of the known downstream targets of EWS–FLI1 is EZH2, which is the catalytic subunit of the polycomb repressor gene 2 associated with "stemness" features in tumor cells (47). Expression of EWS–FLI1 leads to EZH2 upregulation in mesenchymal stem cells (48) and expression of both EZH2 and BMI1 in human neural crest cells, although BMI1 is not a direct transcriptional target of EWS–FLI1 (49). These findings suggest a rationale for the exploration of the use of the new EZH2 inhibitors in this tumor (50, 51).

**Histone deacetylases.** It has been shown that EWS–FLI1 has a transcriptional repressive function (13). One of the downstream targets of the fusion protein that is required for oncogenic transformation is NKX2.2 (52). This gene encodes for a transcription factor with both activating and repressing domains. NKX2.2 is thought to exert its transcriptional repression via TLE (transducin-like enhancers of split–associated recruitment of histone deacetylases (HDAC)). TLE proteins are the homologues of Groucho in humans. They are a family of proteins that act as transcriptional modulators. Expression of individual TLE genes correlates with immature epithelial cells that are progressing toward their terminally differentiated state, suggesting a role during epithelial differentiation (53). TLE proteins are expressed in Ewing sarcoma and are believed to exert their repressive function via recruitment of HDACs. This is a possible mechanism that could explain preclinical activity of HDAC inhibitors in these tumors (54). In vitro HDAC inhibition using vorinostat in the Ewing sarcoma A673 cells led to growth inhibition by abrogation of the transcriptional-repressive function (54), which is consistent with findings from prior studies in which Ewing sarcoma xenografts showed sensitivity to HDAC inhibition (55). Moreover, combination of 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation, and an HDAC inhibitor in vitro showed reactivation of tumor suppressor genes and decreased clonogenicity in vitro in Ewing sarcoma cell lines (56). Although initial clinical trials of this approach have not shown responses (57), this avenue has not been fully explored yet.

**Immunotherapy**

Immunotherapy should be considered as a valid approach to Ewing sarcoma therapy. The recent developments in cancer immunotherapy, particularly the positive results seen after PD-1 blockade in solid tumors (58, 59), have renewed enthusiasm about therapeutic manipulation of the immune system with the aim of tumor eradication.

A trial of consolidative immunotherapy for high-risk pediatric sarcomas, including Ewing sarcoma, using autologous T cells and dendritic cells pulsed with peptides derived from tumor-specific translocation, was performed at the NCI. This approach proved to be feasible and led to 31% 5-year overall survival (60).

TRAIL is a member of the TNF superfamily with antitumor activity secreted primarily by natural killer (NK) cells. Ewing sarcoma cells express the TRAIL death receptors, and have been shown to be sensitive to TRAIL-induced caspase-8–mediated apoptosis in vitro. Tumor progression using xenografts and transgene TRAIL expression showed association of ligand expression with delayed tumor progression (61). In a recent phase I trial evaluating lextatumumab, a fully human agonistic antibody against TRAIL receptor 2 in which 4 patients with Ewing sarcoma were enrolled, the agent was well tolerated but no CRs or PRs were observed (62). Interestingly, there is potential for synergistic combination of immune-based therapies and HDAC inhibitors. Ewing sarcoma cells treated with vorinostat had increased sensitivity to TRAIL-induced apoptosis via increased activation of caspase 8 (63).

Preclinical studies have demonstrated sensitivity of Ewing sarcoma cells to expanded NK cells in vitro and in vivo (64). This is congruent with the previous findings that NK cells are able to recognize and destroy Ewing sarcoma cells by signaling through NKG2D and DNAM-1 receptors (65). Clinical trials exploring the feasibility of NK-based therapy with and without stem cell transplantation in patients with high-risk sarcomas, including Ewing sarcoma, are ongoing (clinicaltrials.gov: NCT01287104 and NCT01875601).

Once again, HDAC inhibition has been linked with increased expression of NGK2D ligands in Ewing sarcoma cells that increased sensitivity to NK cell–mediated cytolysis (66). Ligand upregulation has also been linked to DNA damage—for instance using radiation—(67), all suggesting that optimal combination or sequential therapies may enhance this therapeutic approach.

Finally, chimeric antigen receptor (CAR)–based therapy is currently being developed for Ewing sarcoma. Modified T cells have shown promising results in hematologic malignancies (68). Surface receptors expressed in Ewing sarcoma, such as the ganglioside antigen GD2, are being actively explored as potential targets for Ewing sarcoma CAR therapy (69, 70).

In summary, several recent discoveries are opening a new era of clinical investigation in Ewing sarcoma. Inhibition of the aberrant fusion protein, PARP inhibition, epigenetic manipulation, IGF1R blockade, and immune therapies all hold the promise of achieving improved outcomes in patients with Ewing sarcoma.

However, despite a sizable number of scientific discoveries in Ewing sarcoma biology, these findings have not led
to a significant improvement in outcomes, particularly in patients presenting with metastatic disease. A recent Children’s Oncology Group trial demonstrated benefit of interval-compressed chemotherapy in patients with localized disease (7), but as was the case for previous improvements in the treatment of Ewing sarcoma, this approach had no impact on the outcome of patients with advanced metastatic disease. Although the reasons for this remain elusive, some assessment of possible reasons for a disconnect between these advances and clinical advances seems warranted.

The lack of reproducibility of preclinical data, particularly in oncology, has emerged as a growing concern. Reasons for this could be multifactorial: technical difficulties, lack of adequate models or even well-meaning experimental bias, and error. However, it has been argued that the increasing pressure in the current “publish or perish” culture, difficulties in publishing negative results, and scarcity of funding might have an impact in variable experimental results.

One result of this well-documented phenomenon could be lack of preclinical rigor required before observations are tested in the clinic. For example, many published articles using xenograft models demonstrate that a novel intervention leads to a slowing of tumor growth, but very few publications demonstrate actual tumor shrinkage. Although tumor shrinkage per se may not predict clinical efficacy, clearly better preclinical predictors are necessary. In a previous report comparing activity of novel cytotoxic agents in xenografts with activity in phase II studies, the best predictor of clinical activity was when a compound had activity in at least 33% of multiple histologies in multiple xenograft models (71). As noted above, these data were generated using cytotoxic agents, and currently we simply do not know what best predicts for clinical activity with newer “targeted” agents other than a specific activating mutation in a kinase predicting for response to a specific kinase inhibitor. Unfortunately, no such activating kinase mutations have been found in Ewing sarcomas, reinforcing the point that better predictors for clinical activity are necessary for this tumor.

Another issue that may be impeding progress in this rare, highly aggressive sarcoma is the paucity of preclinical and clinical models testing rational combinations of signaling pathway–targeted agents. With the limited number of patients available for phase II testing, we may be better served by early testing of combination-targeted agents based upon scientific rationale, rather than insisting upon single-agent activity in the phase II setting.

Finally, inadequate correlative studies early on in the development of agents for Ewing sarcoma have markedly hindered progress. Although tissue acquisition in pediatric oncology continues to be an area of heated debate, one could argue the dubious ethics of enrolling patients in a trial in which key biologic questions will remain unanswered.

In summary, although much biologic insight has been gained in understanding Ewing sarcoma, we must work harder to ensure these gains are translated to the clinic. Combination of different approaches in a rational and creative manner continues to be a challenge for the future. To overcome this hurdle, it will be necessary to foster collaboration between very distinct investigative approaches and to appeal to innovative clinical trial designs. If the recent and exciting biologic discoveries can be translated into effective therapies able to optimize outcomes while minimizing toxicities, we will be able to convey renewed optimism to patients affected by this deadly tumor who are in desperate need of new therapeutic approaches.

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

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Conception and design: F.I. Arnaldez, L.J. Helman
Development of methodology: F.I. Arnaldez
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.J. Helman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.J. Helman
Writing, review, and/or revision of the manuscript: F.I. Arnaldez, L.J. Helman

Received August 8, 2013; revised April 4, 2014; accepted April 13, 2014; published OnlineFirst April 22, 2014.


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Clinical Cancer Research

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