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# The Current and Future Therapies for Human Osteosarcoma

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**Abstract:** Osteosarcoma (OS) is the most common non-hematologic malignant tumor of bone in adults and children. As sarcomas are more common in adolescents and young adults than most other forms of cancer, there are a significant number of years of life lost secondary to these malignancies. OS is associated with a poor prognosis secondary to a high grade at presentation, resistance to chemotherapy and a propensity to metastasize to the lungs. Current OS management involves both chemotherapy and surgery. The incorporation of cytotoxic chemotherapy into therapeutic regimens escalated cure rates from <20% to current levels of 65-75%. Furthermore, limb-salvage surgery is now offered to the majority of OS patients. Despite advances in chemotherapy and surgical techniques over the past three decades, there has been stagnation in patient survival outcome improvement, especially in patients with metastatic OS. Thus, there is a critical need to identify novel and directed therapy for OS. Several Phase I trials for sarcoma therapies currently ongoing or recently completed have shown objective responses in OS. Novel drug delivery mechanisms are currently under phase II and III clinical trials. Furthermore, there is an abundance of preclinical research which holds great promise in the development of future OS-directed therapeutics. Our continuously improving knowledge of the molecular and cell-signaling pathways involved in OS will translate into more effective therapies for OS and ultimately improved patient survival. The present review will provide an overview of current therapies, ongoing clinical trials and therapeutic targets under investigation for OS.

**Key Words:** Bisphosphonates, BMP, IGF, IGFBP5, limb-salvage, osteosarcoma, OS99.

## INTRODUCTION

Osteosarcoma (OS) generally occurs in pediatric patients and young adults, most commonly during the adolescent years when bone growth is rapid. In fact, there exists a direct relationship between rapid bone growth and OS [1,2]. OS is a highly malignant bone tumor, the most common primary malignancy of bone in children and adolescents and the fifth most common malignancy overall in children and young adults [3]. The incidence of OS is slightly higher in males than females, which is attributable to a longer duration of skeletal growth in males [4]. OS most frequently occurs in the metaphyseal area of long bones adjacent to or involving the growth plate, with approximately 75% of all cases occurring in the distal femur and proximal tibia [1,5,6]. Approximately 20% of OS patients present with clinically detectable metastatic disease at diagnosis, and the most common site of metastases is the lung [7]. In a study by Kager *et al*., the presence of greater than five metastatic lesions was associated with a 5-year survival rate of only 19% [8]. Critical determinants of long-term survival in patients presenting with metastatic disease are location of disease and surgical resectability, number of metastases at diagnosis, location of metastases, surgical remission and tumor necrosis following neoadjuvant chemotherapy [9-12]. While there are a number of histologic subtypes, there are three major subtypes of OS classified by tumor matrix predominance, osteoblastic, chondroblastic and fibroblastic; although treatment and patient outcomes do not differ among subtypes [13,14]. Subtypes also differ depending on location; making up less than 5% of OS cases, parosteal, central low-grade and periosteal OS have distinct morphologies but portend an improved

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prognosis [14-19]. Pathological diagnosis of OS depends on interpretation of osteoid matrix type produced by cancer cells since no diagnostic immunohistochemical or genetic studies yet exist [13].

The current standard of care treatment for OS patients is comprised of surgery with wide excision of the primary tumor and cytotoxic chemotherapy, a treatment which often proves difficult for patients due to the systemic toxicities of these agents [20]. Currently, chemotherapy plays a major role in the treatment of OS. The benefit of early neoadjuvant chemotherapy is greater than surgery alone, and this is due to the frequent presence of subclinical micrometastatic disease [21-23]. Chemotherapy alone can eradicate these micrometastases if initiated when the micrometastatic disease burden is low [24], statistically improving survival even in patients diagnosed with localized high-grade OS. Prior to neoadjuvant chemotherapy regimens, amputation was the sole therapy for OS and resulted in a 5-year overall survival rate of only 10-20% [25-27]. Over the past half century the development of effective chemotherapeutic agents, improvement in local tumor control and the incorporation of these modalities into therapeutic regimens has increased OS cure rates from <20% prior to the 1970's, even with full local tumor control, to 65-75% today [22,24,28,38]. While chemotherapy has revolutionized OS therapy, surgical and imaging techniques have also improved considerably over the past three decades, allowing for limb-salvage procedures in greater than 90% of patients compared to 1980s limb-salvage rates of 53%. Limb-salvage procedures benefit patients from a psychological and cosmetic standpoint while having local recurrence and survival rates similar to amputation [21,25, 30,39,]. In fact, improvements in surgical techniques permitting limb-salvage procedures have paralleled improvements in patient survival outcomes [21].

While modifications in chemotherapeutic regimens, surgical procedures and imaging have allowed limb-salvage procedures to become commonplace in treating OS and have improved overall patient survival, there have been no significant improvements in survival outcomes for patients with metastatic OS over the past three decades. Survival rates for patients with pulmonary metastases, even following aggressive multimodal treatment, is currently only 25-30% and has not improved over the past 25 years [11,28,47-55]. Extrapulmonary metastases developing in uncommon sites such as the kidneys, brain, heart, mediastinum and epidural space present especially difficult challenges [28,56]. Thus, there is a critical need to identify novel and directed therapy for OS. Several clinical trials are currently ongoing for various sarcoma therapies that have demonstrated objective responses in OS. Novel drug delivery mechanisms are also at the clinical trial phases. Furthermore, there is an abundance of preclincial research which holds great promise in the development of future OS-directed therapeutics. This review aims to discuss today's standard therapies for OS, recently completed and current clinical trials that may soon be implemented into OS treatment regimens and preclinical studies that will hopefully improve OS patient outcomes in the coming years.

## History of Chemotherapeutics in OS

The history of chemotherapeutic treatment for OS began in the early 1960s when Sutow *et al.* demonstrated antitumor activity against OS with L-phenylalanine mustard, resulting in a 14% disease-free survival rate [28,57]. In 1969, shortly after the combination of vincristine, dactinomycin and cyclophosphamide (VAC regimen) was shown to be effective in treating rhabdomyosarcoma, Sutow *et al.* achieved a 33% disease-free survival rate in patients treated with a pulse VAC regimen consisting of intermittent intensive pulses of cyclophosphamide with the traditional VAC regimen [28,58,59]. After doxorubicin demonstrated antitumor activity in OS, Sutow *et al.* substituted doxorubicin for dactinomycin in the pulse VAC regimen and also added L-phenylalanine mustard in a regimen named "Co*n*padri " or Co*n*padri I [28,57,60], resulting in a 55% disease-free survival rate. Addition of methotrexate to the regimen, called "Co*m*padri" or "Co*m*padri II" (each successive number indicating an evolution in the regimen), delayed the development of pulmonary metastases [28,61,62]. Subsequently, the Compadri IV and V regimens increased the dosages of methotrexate and doxorubicin, using aggressive "front loading." Together, the Compadri regimens comprise the first successful attempt at utilizing combination chemotherapy, with different mechanisms of action and minimal overlapping toxicities, as adjuvant OS therapy. Prior to the 1970s, survival rates for patients with metastatic OS were below 2% but improved to approximately 30% after 1970, demonstrating the profound impact of the incorporation of chemotherapy to OS treatment regimens [28,63]. The Compadri studies laid the foundation for clinical trials performed throughout the 1980s and 1990s that utilized multi-agent regimens consisting of doxorubicin, cisplatin, bleomycin, cyclophosphamide, ifosfamide, dactinomycin and high-dose methotrexate. In 1976, Rosen *et al.* introduced the concept of neoadjuvant chemotherapy for OS in an effort to provide ample time for the manufacturing of custom-made prostheses following limb-salvage procedures while reducing preoperative tumor volume [64].

## Current Chemotherapeutics in OS

Today, standard chemotherapeutics include high-dose methotrexate with leucovorin rescue, doxorubicin (Adriamycin), cisplatin, cyclophosphamide and ifosfamide.

## High-dose Methotrexate with Leucovorin Rescue

Methotrexate works by binding stoichiometrically and irreversibly to dihydrofolate reductase, inhibiting the formation of tetrahydrofolate and preventing the synthesis of thymidylate causing cell death [28]. Leucovorin is the antidote to methotrexate and serves to replenish tetrahydrofolate stores depleted by methotrexate. This regimen (MTX-L), a mainstay in OS chemotherapy, was established by Jaffe *et al.* and is comprised of IV doses of 10-12.5 g/m$^2$ over 4-6h with leucovorin rescue starting 24h after the onset of methotrexate infusion [28,64-67]. When used in combination therapy, the interval between doses of MTX-L is generally 21-28 days. Doxorubicin is typically administered 8-10 days after the initial dose of MTX-L. When administered as a single adjuvant agent following surgical resection of the primary tumor,

MTX-L resulted in a 40% disease-free survival. However, when combined with other agents as pre- and post-operative therapy, disease-free survival rates of 65-75% were achieved [28,64,66-72]. Methotrexate has been shown to potentiate the effects of radiation therapy [29].

Methotrexate blood levels of 700-1000 μmol/L at 4-6h following infusion are required for optimal results, and Jaffe *et al.* of the University of Texas MD Anderson Cancer Center find that concentrations of 1500 μmol/L or higher at 4-6h are desirable and more likely to be achieved by limiting pre- and intra-therapeutic intravenous (alkaline) hydration to 3L/m$^2$/24h [28]. With optimal dosages administered over 4-6h along with appropriate hydration, peak MTX levels can be expected at the following time points after the initiation of infusion. Excess of the following values may result in toxicities [28,73-75]: 24h: 30-300 μmol/L; 48h: 3-30 μmol/L; 72h: <0.3 μmol/L.

Leucovorin is administered according to algorithms available in most institutions. Most protocols include an IV dose of 10 mg after completion of the methotrexate infusion followed by subsequent doses every 6 h until the MTX level falls below 0.1 μmol/L; this typically requires 72h and 12 doses.

Candidates for MTX-L therapy must have normal renal and hepatic function, a normal complete blood count (CBC) and the absence of infection. These prerequisites can be determined by a corrected creatinine clearance, basic metabolic panel, urinalysis, liver function tests and a complete blood count prior to each course of therapy. Although uncommon, toxicity may clinically manifest with gastrointestinal mucosal ulceration, myelosuppression and hepatorenal failure. Mucositis and nephrotoxicity are commonly associated with delayed MTX clearance and precipitation of methotrexate within the renal tubules, a process that can be adversely affected by administration of other nephrotoxic drugs such as cisplatin [76,77]. Treatment of toxicity includes increasing fluid intake to 4L/m$^2$/24h, increasing leucovorin dose to 50-100 mg every 6h, administering carboxypeptidase G-2 in the event of oliguria/anuria and renal dialysis.

**Doxorubicin**

Doxorubicin is used in most OS combination chemotherapy regimens and has been called the single most effective agent [28,78]. Doxorubicin is an anthracyline chemotherapeutic which functions by intercalating into DNA, inducing single- and double-strand breaks. Initial studies using doxorubicin in patients with pulmonary metastases resulted in response rates of 35-40%, including the complete disappearance of lung lesions in some patients and an overall 40% reduction in tumor volume [28,79-81]. Similar to methotrexate, doxorubicin can potentiate the therapeutic effects of radiation therapy, albeit with occasional complications of erythema and ulceration of skin when used intra-arterially; these adverse effects can preclude limb-salvage procedures [28,82,83].

The major toxicity of doxorubicin is delayed congestive heart failure but can generally be prevented by limiting the cumulative dose to 300 mg/m$^2$ in children below the age of six, 450-500 mg/m$^2$ in adolescents and approximately 600 mg/m$^2$ in adults [28,84]. Dexrazoxane, a derivative of the metal-chelating agent EDTA, has been administered in combination with doxorubicin to prevent cardiac failure [28,85]. This agent functions to chelate iron and reduce the number of metal ions that complex with doxorubicin, thus decreasing the production of cytotoxic superoxide radicals [86]. Newer liposomal formulations of doxorubicin are less cardiotoxic but not yet approved for the treatment of OS despite significant activity in some soft tissue sarcoma subtypes [87-90]. Candidates for doxorubicin treatment require a cardiac assessment consisting of an electrocardiogram and echocardiogram; these should be obtained prior to each course of treatment, with further assessments obtained at regular intervals for long-term survivors.

**Cisplatin**

Cisplatin (*cis*-diamminedichloroplatinum II) is a platinum-containing chemotherapeutic that, although lacking an alkyl group, is also classified as an alkylating agent. It is thought to function by binding and crosslinking DNA, interfering with transcription and DNA replication [91]. Cisplatin can be administered intravenously or intra-arterially. Initial studies in patients with unresectable or metastatic OS demonstrated responses of 30-50% when administered intravenously as a single agent or in combination with doxorubicin [28,92-94]. Surprisingly, response rates were as high as 60-90% when administered intra-arterially as a single agent for primary tumor treatment [28,95,96]. Intra-arterial administration increases local cytotoxic concentrations, thereby improving tumor penetration [28,96-100]. Studies at the University of Texas MD Anderson Cancer Center utilized a regimen consisting of 120 mg/m$^2$ of cisplatin intra-arterially over 4h with 95 mg/m$^2$ doxorubicin concurrently administered over 24h in a series of four preoperative courses administered at 4-week intervals. Although tumoricidal effects are achieved more rapidly with intra-arterial therapy, similar cytotoxic effects can be achieved with intravenous therapy administered in several courses [28]. Toxicities of cisplatin include auditory and renal dysfunction [28,101,102]. Hearing impairment may occur in approximately 40% of patients [87]. Patients can also develop peripheral neuropathies. Another platinum-containing chemotherapeutic agent, carboplatin, has been used in combination with other chemotherapeutics and will be discussed later in this review [9,20,28,103,104].

**Oxazaphosphorines**

Alkylating agents of the oxazaphosphorine family used in treating OS include cyclophosphamide and ifosfamide. Alkylating agents function by adding alkyl groups to guanine nucleotides, inducing DNA damage and resulting in cytotoxicity. Furthermore, the addition of etoposide, a topoisomerase inhibitor which interferes with DNA structural changes during the normal cell cycle, to cyclophosphamide may synergize in treating both primary and metastatic OS. The administration of regimens containing ifosfamide and etoposide (IE) to poor responders to chemotherapy demonstrated outcome improvements similar to those of good responders [13,54]. Intensification of IE chemotherapy in patients with limited tumor necrosis following first-line therapy is currently being evaluated in a clinical trial [13,105]. Alky-

lating agents are not cross-resistant, and patients who experience relapse after treatment with one alkylating agent may achieve responses when using another [28,106,107].

The major toxicity of the oxazaphosphorines is hemorrhagic cystitis, an effect caused directly by the metabolite acroline. Hemorrhagic cystitis can be prevented by administration of Mesna, an agent which absorbs acroline, along with generous administration of fluids [28]. Other less common toxicities include moderate myelosuppresion and renal dysfunction; renal dysfunction is less likely with the use of cyclophosphamide. In one study, investigators at the University of Texas MD Anderson Cancer Center used high-dose ifosfamide (17.5 $g/m^2$) and recommend limiting this dose to four courses due to the development of moderate renal failure following the fifth course [28]. When there is evidence of renal dysfunction, it is recommended that cyclophosphamide is substituted for ifosfamide. Azospermia occurs in nearly all patients receiving a total ifosfamide dose great than 75 $g/m^2$ [87].

## Chemotherapy Regimens

Regimens consisting of neoadjuvant and adjuvant chemotherapy in addition to definitive surgical resection of the primary tumor have become the current standard of care for OS. Nearly all OS chemotherapy regimens consist of multiple agents with different mechanisms and minimal overlapping toxicities. The most commonly employed regimens use combinations of the chemotherapeutics cisplatin, doxorubicin, ifosfamide and high-dose methotrexate with leucovorin rescue [5,36,60,93,108-112]. Regimens containing methotrexate, doxorubicin (Adriamycin) and cisplatin are termed *MAP* [13]. Current multi-agent chemotherapy regimens combined with surgical resection result in 5-year survival rates of approximately 70% [87,113], and the quality of response to neoadjuvant chemotherapy is an important predictor of future survival. In fact, patients with greater than 90% necrosis in the resected tumor following neoadjuvant chemotherapy have an increased 5-year survival rate of 90% [87,114].

Different recommendations regarding adjuvant and neoadjuvant chemotherapy exist depending on the grade and stage of the tumor. Neoadjuvant chemotherapy is preferred for all patients with high-grade tumors prior to wide excision. Adjuvant chemotherapy is recommended for patients with low-grade or periosteal lesions with surgical pathological findings of high-grade disease. All patients with high-grade OS should receive both neoadjuvant and adjuvant chemotherapy following surgical resection. Despite National Comprehensive Cancer Network (NCCN) guidelines which offer the option of switching chemotherapeutic regimens in patients with poor histologic responses to neoadjuvant chemotherapy, there is no data yet to support this recommendation [87].

As one of the major challenges of OS therapy, the optimal treatment for patients with relapsed or metastatic OS remains to be defined. In the event of relapse, surgical resection if accessible and/or second-line chemotherapy are recommended [87,115]. Recommended regimens for relapsed or refractory metastatic OS include docetaxel and gemcitabine; cyclophosphamide and etoposide; cyclophosphamide and topotecan; gemcitabine, ifosfamide and etoposide; ifosfamide, carboplatin and etoposide; and high-dose methotrexate, etoposide and ifosfamide. There have been no reports to date identifying any markers detecting silent pulmonary metastases. Although a promising therapeutic avenue, molecular profiles of gene expression patterns and phenotypes of OS that may lead to personalized, less toxic regimens have not yet been well described. Man *et al.* set out to predict responses of OS patients to neoadjuvant chemotherapy based on genetic profiles by developing a multigene classifier. A total of 45 genes were identified, and overexpression of these genes was seen in poor responders [108,116,117]. Poor responders with overexpression of these specific genes were found to more frequently experience relapse and pulmonary metastases despite aggressive chemotherapy [108,118].

## The Surgical Treatment of OS

Amputation was the primary modality of therapy of OS before the advent of chemotherapy and limb salvage. Prior to neoadjuvant chemotherapy regimens, amputation was the only surgical treatment and alone had a 5-year overall survival rate of 10-20% [25-27]. Today, surgery remains an essential component of OS treatment regimens. The primary objective of surgical therapy is complete excision of the primary tumor with conservation of the limb as a secondary goal and amputation a last resort [5,108]. Nonetheless, amputation is indicated if resection to disease-free margins results in a non-functional limb, tumor extent is beyond resectability or the patient desires the functionality of a prosthesis to the cosmesis of limb-salvage. In fact, advances in prosthetic joints which now utilize microprocessor technology have improved functional outcomes in patients undergoing amputation [113,119]. Amputation may also result in improved local control if extensive contamination of tumorous tissue is found at biopsy [21]. Young patients who are not candidates for limb-salvage procedures may sometimes undergo rotationplasty, an intercalary resection of a segment of bone followed by reconstruction of the limb by rotating 180 degrees cranio-caudally with the rotated ankle serving as a knee joint [13,120]. This alternative has outstanding oncologic, functional and psychosocial results for young patients [13,120-122].

Limb-salvage, a procedure involving allograft bone and/or prosthetic joint replacement, is often performed following complete, margin-free resection of the primary tumor and must result in reconstruction of a viable and functional limb [5,108,118]. In the 1980s and early 1990s, a 5-cm margin above or below the tumor or articular cartilage was considered adequate in preventing tumor spread [21,123,124]. Today, a 2-cm margin above or below the tumor is considered a safe margin for resection [21,40,125]. Current evidence supports the notion that closer resection margins are acceptable in patients with good responses to neoadjuvant chemotherapy and do not increase local recurrence rates [21,126,127]. Furthermore, smaller bone resection margins are more likely to allow for preservation of the growth plate as well as a larger area for endoprosthetic fixation [76].

Advances in imaging technique, neoadjuvant chemotherapy regimens and improvements in surgical methods have

allowed limb-salvage surgery to become the predominant alternative to amputation [25,30,39,41-46]. Specifically, improvements in MRI have helped surgeons to better delineate the extent of the primary tumor and its relation to the neurovascular margin, precisely reducing margins and making limb-salvage more feasible [21,22,29]. By combining current chemotherapeutic regimens with limb-sparing operations, greater than two-thirds of patients with non-metastatic OS of the extremities are long-term survivors with an overall limb preservation rate of greater than 80% [21,38]. Limb-salvage surgery results in improved limb functionality, appearance and psychological outcomes while having local recurrence and survival rates similar to those undergoing amputation [25,21,39,40]. However, because of the removal of the primary tumor with adequate margins, limb-salvage procedures often result in large bone and soft tissues losses and associated long recovery times, poor joint stability and partial loss of function [25,39,44,45]. Nonetheless, since limb-salvage more successfully preserves limb function and provides improved psychological and physiological benefits, it is preferred to amputation in the vast majority of OS cases [21,25,40,44,128-131].

A retrospective study by Ayerza *et al.* studied 251 patients with high-grade OS and compared survival rates, limb-salvage surgery rates and rates of amputation following limb-sparing operations over three periods of time between 1980 and 2004 [21]. The 5-year survival rate during the first period in the 1980s was 36%, while it was 60% and 67% in the 1990s and early 2000s, respectively. Similarly, the limb-salvage surgery rate in the 1980s was 53%, whereas it was 91% and 97% in the 1990's and early 2000's, respectively. The overall limb-salvage rate, accounting for both primary and secondary amputation, was 36% in the 1980s, whereas it was 91% and 93% in the 1990s and 2000's, respectively. This important study demonstrated that OS patients treated during the past 25 years had higher rates of limb-salvage operations and a parallel improved overall survival rate with lower rates of secondary amputation. Survival rates in this study were similar to other studies [21,30-32,126,132,133].

In order to evaluate the efficacy of limb-sparing surgery, rates of amputation following limb-sparing operations, or secondary amputation, must be considered [25,130]. In a retrospective study by Tan PX *et al.* 120 patients with OS of the knee underwent joint reconstruction using hinged knee prosthesis and had a secondary amputation rate of 10% (n=12). Secondary amputation resulted from local tumor recurrence in eight patients and deep infection in four patients, comparable to previous reports [21,25-27,30,40,128-131]. Aside from local recurrence and deep infection, long-term complications of prosthetic replacement include aseptic loosening with poor bone/implant interface contact, mechanical wear and peri-prosthetic fractures [21,25,42,109]. The Memorial Sloane-Kettering Cancer Center reports that 15.8% of prosthetic knee reconstructions require revision for aseptic loosening, with overall failure-free rates of 82%, 71% and 50% at 3, 5 and 10 years, respectively [109,134]. Tan *et al.* demonstrated a median long-term prosthesis survival of 6.2 years [25]. With this, young survivors of OS must anticipate multiple prosthetic revisions during their lifetimes.

In very young children with osteosarcoma involving the long bones, surgical resection poses challenges and often causes limb length discrepancies [13]. Measures taken by surgeons to minimize limb length discrepancies include lengthening following resection or extendible prostheses mediated by an externally applied magnetic field which lengthens the prosthesis as the patient grows [13,135]. If the joint surface is spared, intercalary allografts with or without vascularized fibular transfer have demonstrated excellent outcomes and may be considered in this group of patients [13,136,137]. Farfalli *et al.* recently investigated the relative performance of biologically fixed prostheses, methods which may improve endoprosthetic reconstruction fixation [13,138]. Bone distraction has been successfully used in patients with tumors not invading the physis to create newly formed bone distally and provide a margin for tumor excision followed by intercalary joint-sparing reconstruction; this method should not be used in poor responders to neoadjuvant chemotherapy [13,138-140].

While most forms of osteosarcoma require chemotherapy in addition to surgical resection, low-grade central, parosteal and periosteal OS regimens consist of surgery only and have significantly decreased risks of distant metastases [13,15-19,141,142]. To prevent the unnecessary administration of chemotherapy and its associated adverse effects, it is of the utmost importance to appropriately diagnose these subtypes.

## The Role of Radiation Therapy in OS

Although not a cornerstone in OS therapy, a malignancy once considered radioresistant, radiation therapy does have a role in the treatment of certain patients. For unresectable, incompletely resectable and metastatic tumors especially within the axial skeleton, radiotherapy is recommended as part of a multimodal treatment program [13,108]. More specifically, radiation therapy can be used for the treatment of chemoresistant pulmonary metastases, inoperable primary tumors and the alleviation of cord compression and bone pain [28,111,143-145]. High-dose photon irradiation (50-70 Gy) in combination with chemotherapy can be used when tumors are located in inaccessible sites including the pelvis, vertebrae and the base of the skull as well as in patients who are not surgical candidates or refuse surgery [108,146,147]. To reduce recurrence risk and to increase the success rate in cases of limb amputation, radiotherapy may be considered as adjunct therapy [108,118].

A recent study by Ciemik *et al.* used proton radiotherapy with a median dose of 68.4 Gy in patients with unresectable or incompletely resected OS and demonstrated local control at 3 and 5 years of 82% and 72%, respectively [13,148]. Heavy ions may be advantageous in treating radioresistant tumors and have been investigated in OS: Utilization of high-dose carbon ions (70.4 Gy) demonstrated local control at 3 years of 92% in patients with unresectable head and neck OS [13, 149, 150]. As a palliative measure for patients with painful, inoperable bone metastases, radiotherapy with external beam radiation or radioisotopes including samarium or bisphosphonates can be considered [151,152]. Anacak *et al.* described an approach of intraoperative extracorporeal irradiation in which 15 patients underwent primary tumor resection with limb-salvage and single-dose extracorporeal

**Table 1. Recently Completed Clinical Trials for OS Therapies**

| Clinical Trial | Phase Level | Agent(s) Tested | Mechanism of Agent(s) | Study Results | Ref. |
|---|---|---|---|---|---|
| Carboplatin/Ifosfamide Window Therapy for Osteosarcoma: OS91 Trial | II | Carboplatin, Ifosfamide | Carboplatin: Platinum-containing chemotherapeutic, Ifosfamide: Alkylating chemotherapeutic | Carboplatin/ifosfamide-containing regimen demonstrated similar response rates to cisplatin-containing therapies with lower toxicity | Meyer *et al.* 2001 |
| Frontline treatment of localized osteosarcoma without methotrexate: OS99 Trial | II | Carboplatin, Ifosfamide, Methotrexate | Methotrexate: Dihydrofolate reductase inhibitor | Carboplatin/ifosfamide-containing regimen demonstrated similar response rates to both cisplatin-containing or methotrexate-containing regimens | Daw *et al.* 2011 |
| Addition of pamidronate to chemotherapy for the treatment of osteosarcoma | I | Pamidronate | Bisphosphonate | Pamidronate was safely incorporated into OS chemotherapy regimens without altering efficacy and may improve durability of reconstruction | Meyers *et al*. 2011 |
| The Addition of Muramyl Tripeptide to Chemotherapy Improves Overall Survival: Intergroup Study 0133 | III | Liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE) | Non-specific immune modulator activates pulmonary inflammatory cells | Addition of ifosfamide to cisplatin/doxorubicin and MTX did not improve EFS or overall survival; addition of L-MTP-PE significantly improved overall survival with a trend toward improved EFS | Meyers *et al*. 2008 |
| Addition of muramyl tripeptide to chemotherapy for patients with newly diagnosed metastatic OS | III | Liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE) | Non-specific immune modulator activates pulmonary inflammatory cells | Addition of L-MTP-PE did not demonstrate improvement in outcomes among patients with metastatic OS | Chou *et al*. 2009 |
| Inhaled GM-CSF for First Pulmonary Recurrence of Osteosarcoma: Effects on Disease-Free Survival and Immunomodulation | I | Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) | Immunostimulatory protein secreted by WBCs augments proliferation and function of WBCs | Inhalation of GM-CSF was feasible with low toxicity, but no detectable immunostimulatory effects or improved outcomes were demonstrated | Arndt *et al*. 2010 |
| Phase II Trial of Trastuzumab in Combination With Cytotoxic Chemotherapy for Treatment of Metastatic Osteosarcoma With HER2R Overexpression | II | Trastuzumab | Humanized monoclonal antibody to human epidermal growth factor receptor 2 (HER2) | Addition of trastuzumab to cytotoxic chemotherapy in patients with HER2-positive OS did not improve patient outcomes. Trastuzumab was safely administered without short-term cardiotoxicity | Ebb *et al*. 2012 |

irradiation at 50 Gy with re-implantation of involved bone segments. This method resulted in no local recurrence or graft failure in irradiated bone during a mean follow-up period of 22 months, however this novel method merits further investigation before its widespread incorporation into limb-salvage-containing regimens [108,153].

## Recently Completed and Ongoing Clinical Trials for OS Therapy

While evolving regimens containing conventional cytotoxic chemotherapeutics have revolutionized OS treatment, there has been stagnation in the improvement of patient outcomes over the past three decades in patients with metastatic OS. Furthermore, many patients with metastatic and/or recurrent OS receive nearly all standard chemotherapeutic agents during the initial phase of treatment, limiting therapeutic options secondary to cumulative organ toxicity and/or resistance to previously used agents [154]. Thus, there is a critical need to develop novel and effective therapies for the treatment OS in order to improve patient survival. The following is a review of recently completed clinical trials as well as targets in current clinical trials for osteosarcoma (Table **1**).

### St. Jude Children's Research Hospital OS99 and OS91Trials-HDMTX and Carboplatin

As discussed earlier, methotrexate has been a mainstay in OS chemotherapy for decades. However, the necessity of high-dose methotrexate (HDMTX) within a multi-agent regimen has come into question as many theorize that its toxicity interferes with the dose-intensive delivery of other agents [76,77,155-157]. Furthermore, HDMTX requires sophisticated pharmacokinetic monitoring and leucovorin rescue dose-adjusted to MTX levels, a process which can be

difficult for institutions that cannot adequately provide this [76,77]. The recently completed St. Jude Children's Research Hospital OS99 Trial investigated the efficacy of a regimen comprised of carboplatin, ifosfamide and doxorubicin without HDMTX in patients with newly diagnosed, localized, resectable OS [76]. The previously performed OS91 trial utilized a regimen comprised of carboplatin, ifosfamide, doxorubicin and HDMTX, yielding similar outcomes with comparably less toxicity to cisplatin-based regimens [76,103].

In the OS99 trial, treatment consisted of 12 cycles of chemotherapy administered over 35 weeks: 3 weeks of carboplatin (8 mg/mL x min on day 1) and ifosfamide (2.65 $g/m^2$ x 3d) and one cycle of doxorubicin (25 $mg/m^2$ x 3d) prior to surgical resection. Following resection, 2 cycles of carboplatin combined with ifosfamide and 3 cycles each of doxorubicin (25 $mg/m^2$ daily x 2d) combined with ifosfamide or carboplatin were administered [76]. Out of 72 eligible patients with a median age of 13.4 years enrolled from 1999 to 2006, 40 of 66 (60.6%) evaluable patients demonstrated good histologic responses (>90% tumor necrosis) following neoadjuvant chemotherapy. Estimated 5-year event-free survival rate was 66.7%±7.0% for the OS99 trial compared to 66.0%±6.8% for the OS91 trial (p=.98), which contained HDMTX treatment. Estimated 5-year survival rate was 78.9±6.3% for the OS99 trial and 74.5%±6.3% for the OS91 trial (P=0.40).

The OS99 trial also investigated whether resection of the primary tumor with a 3-cm rather than 5-cm (as used in the OS91 trial) bone margin could be safely performed without increasing the rate of local recurrence [76]. The use of a 3-cm bone resection margin did not adversely affect the rate of local control as compared to the OS91 trial, findings that may also be partially attributable to improved surgical and imaging techniques over the past decade [76]. In fact, a local recurrence rate of 3% with a 3-cm margin in the OS99 trial is on the low end of the range cited by other studies [12,36,54,76,158,159].

The results of the OS99 trial demonstrate that the regimen tested produces outcomes comparable to those of both cisplatin-containing and HDMTX-containing regimens. HDMTX-free regimens may provide safe alternatives to patients who demonstrate intolerance to HDMTX, especially those with renal dysfunction, as well as institutions that cannot provide adequate pharmacokinetic monitoring for HDMTX [76]. Furthermore, use of the OS99 regimen may reduce the cost and treatment complexity associated with administration of HDMTX.

The OS91 trial demonstrated that carboplatin-containing regimens resulted in similar outcomes with less toxicity than cisplatin-containing regimens while the OS99 trial demonstrated that HDMTX-free regimens resulted in similar outcomes as HDMTX-containing compounds in patients with localized OS. As most OS chemotherapy regimens today utilize methotrexate and/or cisplatin, the findings of the OS91 and OS99 trials may provide less toxic and costly alternatives with equivalent efficacy to the current standard of care.

**Bisphosphonates**

Bisphosphonates including zoledronate, minodronate, risedronate and alendronate have recently demonstrated antitumor activity in OS [108,117,160-163]. Bisphosphonates are analogues of endogenous pyrophosphates, molecules delivered to sites of increased bone formation and resorption which strongly bind to hydroxyapatite on bone surfaces [109]. Bisphosphonates are frequently used for the treatment of hypercalcemia of malignancy, and it was originally thought that these drugs exclusively stabilized bone. However, bisphosphonates have shown direct effects on tumor cells, with *in vitro* studies demonstrating that bisphosphonate treatment of myeloma cells causes inhibition of proliferation and induction of apoptosis; these effects were also seen in breast cancer cell lines [109,164-166]. Bisphosphonates likely inhibit adhesion of tumor cells to bony matrix and may also inhibit matrix metalloproteinases, enzymes utilized by tumor cells to invade tissue and metastasize [109,167,168]. In addition to interfering with mechanisms used by tumor cells to establish metastases, several of the genes upregulated in OS cells may be inhibited by bisphosphonates [109]. Numerous *in vitro* and *in vivo* studies of both human and animal OS cell lines have demonstrated activity of the bisphosphonates alendronate, clodronate, minodronate, pamidronate and zoledronate against OS, both alone and in combination with standard chemotherapy [109,117,160-163,169-184].

In a study by Mintz *et al*., expression profiling demonstrated significantly different expression of many genes involved in osteoclastogenesis and bone resorption including osteoprotegerin, annexin 2, SMAD and TGFβ-1 between groups of patients with favorable and non-favorable responses to chemotherapy, suggesting that the interaction of tumor and microenvironment is a critical determinant of chemotherapeutic response and may be altered by the therapeutic use of bisphosphonates [109,185].

A study by Horie *et al.* combined zoledronate with paclitaxel or gemcitabine and demonstrated antitumor effects in mouse OS cells. While zoledronate alone was found to have anti-OS activity, the agent acted synergistically when combined with paclitaxel or gemcitabine. Furthermore, pretreatment of OS cells prior to doxorubicin administration augmented OS cell sensitivity [108,176].

A Phase I clinical trial performed by Meyers *et al.* investigated the safety and feasibility of adding pamidronate, the most widely used bisphosphonate in children and young adults, to standard OS chemotherapy regimens [109,186-193]. Forty OS patients were enrolled in the study, 29 without clinically detectable metastatic disease and 11 with clinically detectable metastatic disease at presentation. They were treated with cisplatin, doxorubicin and methotrexate with the addition of pamidronate 2mg/kg/dose (max 90 mg) monthly for 12 doses. Endpoints included overall survival, event-free survival (EFS) and durability of prosthetic reconstruction. Patients with localized disease demonstrated 5-year EFS of 72% and overall survival of 93%. Patients with metastatic disease demonstrated 5-year EFS of 45% and overall survival of 64%. These survival rates are similar to previous studies [21,30-32,126,132,133]. Toxicity for patients treated with pamidronate was similar to those treated with chemotherapy alone. Allograft reconstruction resulted in 2 graft

failures, 4 delayed unions and 6 successful grafts with 5 of 33 overall reconstruction failures and no stress fractures or growth disturbances.

The results of this clinical trial demonstrate that pamidronate can be safely incorporated into standard chemotherapeutic regimens for the treatment of OS while not impairing the efficacy of chemotherapy and possibly improving the durability of limb reconstruction in limb-salvage surgery. These promising results provide justification for a prospective randomized trial with the addition of bisphosphonates to chemotherapy for the treatment of OS. While pamidronate was selected for this clinical trial, newer and more potent bisphosphonates, such as zoledronate, may have more potent anti-tumor effects. In fact, more preclinical data exists supporting the anti-OS effects of zoledronate than any other bisphosphonate [109,117,160,170-172,175-178,182-184,194]. However, employing zoledronate in future trials may increase risk of osteonecrosis of the jaw or atypical fractures, necessitating more vigilant observation for toxicity during therapy [109,195]. Two OS clinical trials are currently under way using zoledronate [196,197].

With a multitude of promising preclinical studies, a successful phase I clinical trial and ongoing phase II/III clinical trials, bisphosphonates may soon be incorporated into OS treatment regimens. These drugs may not only improve patient survival but also the durability of reconstruction following limb-salvage surgery.

### Liposomal Muramyl Tripeptide Phosphatidylethanolamine (L-MTP-PE)

Liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE) or MEPACT (Milennium: The Takeda Oncology Company, Cambridge, Massachusetts, USA) functions by causing infiltration of inflammatory macrophages into pulmonary metastases [28,113,198,199]. MTP-PE is a synthetic analog of a component of bacterial cell walls which acts as a non-specific immune modulator [200]. MTP-PE has been incorporated into liposomes, allowing targeted delivery of MTP-PE to monocytes and macrophages in locations such as the lungs, the most common site of OS metastasis [200,201]. MTP-PE is a ligand for the nucleotide-binding oligomerization domain 2 (NOD2) receptor, a receptor expressed by monocytes, macrophages, dendritic cells and granulocytes [202,203,204]. MTP-PE binding with NOD2 causes activation of monocytes, stimulating increased expression of adhesion molecules and pro-inflammatory mediators including TNF-α, IL-1, -6, and −8 and monocyte chemotactic protein-1 (MCP-1) [205,206]. Several preclinical studies have demonstrated and confirmed the anti-OS effects of L-MTP-PE in rodent and canine models [200,201,207-217]. Importantly, concurrent administration of standard cytotoxic chemotherapy does not interfere with the antitumor effects of L-MTP-PE [200,213].

In the 1990s, the Children's Cancer Group and Pediatric Oncology Group performed a prospective, randomized phase III clinical trial of newly diagnosed OS patients named Intergroup Study 0133, the largest ever randomized trial in OS. The study was a 2x2 factorial design with one group of patients receiving either a 3-drug regimen containing doxorubicin, cisplatin and HDMTX or a 4-drug regimen containing the same three drugs as well as ifosfamide. The second group of patients was randomly assigned either the 3- or 4-drug regimen with L-MTP-PE. Patients received one of four randomized treatments and underwent complete resection of the primary tumor. There was a significant improvement in overall survival (p=0.03, 70% vs. 78%, hazard radio 0.71, 95% CI 0.52-0.96) and a trend toward improved event-free survival (p=0.08) in patients receiving L-MTP-PE, however no benefit for patients receiving the 4-drug regimen versus the 3-drug regimen [113,200]. Both chemotherapy regimens resulted in similar EFS and overall survival: EFS for all patients treated with the three-agent chemotherapy regimen was 65% and 63% at 4 and 6 years, respectively and 66% and 64% for all patients receiving the four-agent regimen (p=0.91).

In a prospective randomized phase III clinical trial for patients presenting with metastatic OS, Chou *et al.* of the Children's Oncology Group (COG) investigated the efficacy of L-MTP-PE when added to a standard 3-agent chemotherapy regimen of cisplatin, doxorubicin and HDMTX in 91 patients. 46 patients received three-agent chemotherapy and L-MTP-PE, while 45 patients received only three-agent chemotherapy. There was a trend but non-significant improvement in 5-year EFS for patients receiving L-MTP-PE (42% vs. 26%, RR 0.72, p=0.23, 95% CI 0.42-1.2). There was also a trend but non-significant improvement in 5-year overall survival for patients receiving L-MTP-PE (53% vs. 40%, RR 0.72, p=0.27, CI 0.4-1.3). The results of this study do not strongly suggest that the addition of L-MTP-PE to standard three-agent chemotherapy can improve outcomes in patients with metastatic OS, however the outcome pattern is similar to that seen in the statistically significant non-metastatic cohort from the Intergroup Study 0133, the largest prospective randomized OS trial performed to date [200]; the comparatively small number of patients in the COG study likely precludes precise evaluation of treatment efficacy. L-MTP-PE was recently approved by the European Medicine Agency and currently is used in Europe for treatment of resectable, non-metastatic high-grade OS. It is also available through a Compassionate Investigational New Drug (CIND) application in some trials in the United States [198,199,200].

The L-MTP-PE clinical trials demonstrate that this novel drug may improve survival as part of a treatment regimen in patients presenting with non-metastatic OS. Furthermore, future larger clinical trials may demonstrate improved patient outcomes in patients presenting with metastatic OS. With recent approval for the treatment of non-metastatic high-grade OS in Europe, we may soon see benefit from the use of this drug that will facilitate its approval for use in the United States.

### Inhaled Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Granulocyte-macrophage colony stimulating factor is a protein secreted by several types of WBCs that stimulates the proliferation and differentiation of hematopoietic cells and augments the function of WBCs including neutrophils, monocytes, macrophages and dendritic cells [218]. GM-CSF also has immunomodulatory and immunostimulatory effects that promote recruitment and increased cytotoxicity of acti-

vated macrophages, increased numbers of CD4 T-cells, and enhanced function of natural killer and dendritic cells [154,219-221]. As the lung is the most common location for OS metastasis, an immunostimulatory approach directly targeting the lungs may provide a therapeutic avenue for patients with pulmonary recurrence of OS. Preclinical data has demonstrated antitumor effects of GM-CSF [154,222,223]. Additionally, initial phase I data on inhalational GM-CSF in humans exists [154,218,224,225]. OS cells metastatic to the lung have shown decreased cell surface expression of Fas and Fas ligand (FasL) [154,226,227], and induction of Fas on lung metastases using aerosol therapy has been demonstrated to induce tumor regression in a mouse model [154,228].

Arndt *et al.* of the Children's Oncology Group (COG) recently performed a phase I clinical trial using inhaled GM-CSF in patients with first isolated pulmonary recurrence of OS. Forty three eligible patients received inhaled GM-CSF at doses ranging from 250 to 1750 μg twice daily on alternating weeks. After two cycles, thoracotomy was performed to resect tumors and analyze pulmonary nodules for expression of Fas/Fas L and dendritic cells via immunostaining for CD1a, clusterin and S100. Patients then received 12 adjuvant cycles of therapy on alternating weeks or until disease progression.

Dose escalation to 1750 μg twice daily was found to be feasible without toxicity. 3-year EFS and 3-year overall survival were 7.8% and 35.4%, respectively, similar to multiple prior studies [35,38,51,53,154,229-232]. However, only 11 of 30 nodules following inhalation stained positive for CD1a, 4 of 30 for S100 and 6 of 30 for clusterin. While the results of this study demonstrate that the administered doses of inhaled GM-CSF do not result in toxicity and aerosolized delivery of a biological agent to patients is feasible, no detectable immunostimulatory effects in pulmonary metastases or improved patient outcomes occur following administration of this drug.

While exciting preclinical data has demonstrated the anti-OS effects of GM-CSF, this phase I clinical trial established safety at administered doses without any significant improvement in patient outcomes or immunostaining, a surrogate measure of the immunostimulatory effects of inhaled GM-CSF. With safety of this therapy established, a future larger prospective randomized trial may demonstrate improved outcomes in OS patients.

### Human Epidermal Growth Factor Receptor 2

As a correlation has been established between overexpression of the human epidermal growth factor receptor 2 (HER2) and poor OS patient outcomes, this receptor has become a therapeutic target [233-237]. Trastuzumab, a humanized monoclonal antibody which specifically binds HER2, has demonstrated therapeutic responses when combined with cytotoxic chemotherapy in breast cancer patients with elevated expression levels of HER2 [238-244]. While a promising target, cardiotoxicity is an adverse effect associated with trastuzumab therapy and is particularly concerning when combined in regimens with doxorubicin or other anthracyclines [233].

Ebb *et al.* investigated the feasibility and safety of treating children newly diagnosed with metastatic OS with trastuzumab combined with cytotoxic chemotherapy [233]. Prior to enrollment, all patients underwent testing for HER2 expression using immunohistochemical staining and grading. Only patients with overexpression of HER2 were treated with trastuzumab. The chemotherapeutic agents used in the study were doxorubicin, methotrexate, cisplatin, ifosfamide and etoposide. To minimize the risk of cardiotoxicity, dexrazoxane was included in the treatment regimen. 96 evaluable patients were enrolled; 41 were HER2 positive and 55 were HER2 negative.

For HER2-positive patients, 30-month EFS was 32% with a 30-month overall survival of 59%. Among HER2-negative patients, 30-month EFS was 32% with a 30-month overall survival of 50%, with differences between HER2-positive and negative groups not significant ($p=0.54$ for EFS, $p=0.58$ for overall survival). Regarding cardiotoxicity, no patient developed clinical evidence of congestive heart failure, significant differences in blood pressures or left ventricular fractional shortening (LVFS). While these results demonstrate that trastuzumab can be safely delivered in combination with anthracycline-containing regimens and dexrazoxane, the therapeutic benefit in HER2-positive patients remains uncertain. However, while HER2-negaitve patients and HER2-positive patients demonstrated similar EFS and overall survival outcomes, it is possible that the addition of trastuzumab to the regimen of HER2-positive patients may have diminished the adverse prognostic impact of HER2 overexpression in this cohort of patients.

While the safety of trastuzumab was demonstrated in this phase II clinical trial, administration of this monoclonal antibody against HER2 was not randomized and thus its survival benefits cannot be concluded from this study alone. A prospective randomized study will be essential in determining this agent's true efficacy. A recently completed study by the Children's Oncology Group describing the impact of HER2 status and response/survival in non-metastatic OS patients will likely be the basis of future prospective randomized trials with trastuzumab.

### Insulin-like Growth Factor Pathway

The insulin-like growth factor pathway has wide implications in a variety of different cancer types, and overexpression of IGF-1, IGF-2 and IGF-1 receptor (IGF-1R) has been demonstrated in OS [13,245-247]. Therapies targeting the IGF pathway are currently being explored in clinical trials for Ewing's Sarcoma and OS [13,248-250], including inhibitors of the IGF-1 receptor (IGF-1R) [251,252]. Ganitumab, a fully human monoclonal antibody to IGF-1R, has been shown to decrease proliferation in OS cell lines and inhibit OS tumor growth *in vivo* [253,254]. Recently, a phase II clinical trial investigated the effects of ganitumab in patients with metastatic Ewing family tumors (EFT) or desmoplastic small round cell tumors (DSRCT) [252]. Patients received ganitumab monotherapy every 2 weeks which demonstrated significant anti-tumor activity in these inevitably fatal and aggressive tumors. Although tolerated, adverse effects included fatigue, thrombocytopenia, hyperglycemia and infusion reactions, findings also reported in the first in-human

study with ganitumab [255]; the deaths in the study were all due to disease progression.

While ganitumab monotherapy was found to demonstrate modest clinical activity in patients with EFT/DSRT, there have been no published clinical trials to date demonstrating anti-tumor activity in OS. The results of these clinical trials in EFT/DRST are the foundation on which current clinical trials targeting the IGF pathway in EFT and OS are based. The promising results from these initial trials will hopefully demonstrate anti-tumor activity in metastatic OS as well.

### Angiogenesis

Targets of angiogenesis as potential OS therapies include inhibitors of vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) with agents such as bevacizumab and ridaforolimus, respectively. These agents are being studied at both the preclinical and clinical level [13,256-259]. Angiogenesis is critical for tumor development, invasion and metastasis and is regulated by pro-angiogenic factors including vascular endothelial growth factor (VEGF), a factor expressed in nearly all human tumors and highly associated with increased tumor vascularity [260]. Antibodies of VEGF have demonstrated antitumor activity in both preclinical and early phase I/II trials in adults [261]. However, there is a concern for toxicity when monoclonal VEGF-neutralizing antibodies are used in the pediatric population which is commonly affected by OS. A recent study demonstrated a severe adverse events (SAEs) rate of 17% in pediatric patients with recurrent or poor-prognosis malignancies including Ewing Sarcoma and rhabdomyosarcoma, rates higher than previous reports [262,263]. Further investigation with larger cohorts will be needed to assess the safety profile and benefits of bevacizumab in pediatric cancer patients.

A recent clinical phase II trial with ridaforolimus assessed its antitumor activity in patients with advanced sarcomas including OS [256]. Of 212 patients treated, four patients demonstrated partial responses including two patients with OS. All patients reported adverse effects, although generally mild, including fatigue, stomatitis and hypertriglyceridemia. While demonstrating promising progression-free survival rates among patients with advanced sarcoma, the true clinical benefit would be best addressed by a randomized clinical trial. This is currently being evaluated in patients with advanced bone and soft tissue sarcoma in the ongoing Sarcoma mUlti-Center Clinical Evaluation of the Efficacy of riDaforolmus (SUCCEED) trial.

While these recently completed clinical trials do not specifically demonstrate anti-OS activity, ongoing clinical trials will hopefully better describe the efficacy and safety of these drugs and their potential role in OS therapy.

### Other Clinical Trials

Recombinant human apoptosis ligand 2/tumor necrosis factor-related apoptosis-inducing ligand (rhu Apo2L/TRAIL) (ligand for the death receptor), also called dulamnermin, is a proapoptotic receptor agonist which binds death receptor 4 and death receptor 5 (also called TRAIL 1 and 2, respectively) and triggers cell death independently of the p53 pathway via activation of the intrinsic pathway [198,264]. A recent phase I study of Apo2L/TRAIL demonstrated significant partial responses (PRs) in chondrosarcoma patients and is now being used in phase I trials for OS patients. The underlying mechanisms of response and resistance of this drug in sarcoma patients remains to be determined [198].

A bone-seeking radiopharmaceutical Samarium-153 ethylene diamine tetramethylene phosphonate (153Sm-EDTMP) was found in one study to be effective in treating metastatic OS, but follow-up studies have not confirmed these findings [87,265]. As earlier, symptomatic bone metastases have also been treated with high-dose 153Sm-EDTMP, resulting in significant relief but also resulting in severe myelosuppression [28,266].

Cediranib (Recentin, AZD2171, AstraZeneca Pharmaceuticals, Wilmington, Delaware, USA) is an orally available small molecule which inhibits the tyrosine kinase activity of vascular endothelial growth factor receptor 1 (VEGFR-1), VEGFR-2 and VEGFR-3, receptors which mediate both angiogenesis and lymphangiogenesis. In a pediatric phase I study in OS patients with pulmonary metastases, objective responses were demonstrated in patients with Ewing sarcoma, synovial sarcoma and osteosarcoma [198,267,268]. Phase I studies using a combination of cediranib with bevacizumab (Avastin, Genentech/Roche, and CA) are in progress (ClinicalTrials.gov number NCT00458731) [198]. A phase II study is currently in development [267].

Rexin-G (Epeius Biotechnologies, San Marino, California, USA) is designed as a tumor-targeted retrovector nanoparticle consisting of a collagen matrix binding motif on its surface and a dominant negative cyclin G1 construct which functions by inhibiting the G1 phase of the cell cycle [198,269]. A recently completed independent phase I/II and phase II study in chemotherapy-resistant OS patients receiving escalating doses of IV Rexin-G demonstrated median progression-free survival of 3.7 months and median overall survival of 7.8 months at high doses, showing a dose-response relationship in the Phase I/II trial. Results of the phase II study were similar. The results of this phase study demonstrate that Rexin-G therapy is safe with no dose-limiting toxicity at the doses administered and may improve survival in chemotherapy-resistant sarcoma and osteosarcoma [211].

Other specific targets of phase I/II clinical trials for which OS patients may be enrolled include p53/mouse double minute (MDM2) inhibitor, MET, histone deacetylase (HDAC) and notch/gamma secretase inhibition [198].

## Molecular Basis of Osteosarcoma

OS tumors arise during times of high bone turnover, most commonly during the adolescent growth spurt [146,270]. Mutations involving oncogenes and tumor suppressors arising from chromosomal amplifications, deletions, rearrangements or translocations may explain some of the molecular underpinnings of OS [146,270,271]. Aberrations in various signaling pathways have also been associated with OS tumor growth and metastasis. Furthermore, recent studies have suggested that OS may arise from differentiation defects in which affected cells are arrested as undifferentiated precur-

sors [272]. Therefore, current research in the development of novel OS therapies seeks to address molecular targets and differentiation defects in order to reduce disease severity, improving patient prognoses.

### Tumor Suppressors

Mutations in Rb and p53 tumor suppressor pathways are associated with OS pathogenesis. An important regulator of the G1/S transition, hypophosphorylated Rb binds to and inactivates the transcription factor E2F, leading to cell cycle arrest. Rb itself is inactivated through phosphorylation by the Cyclin D1/CDK4 complex, liberating E2F to promote cell entry into S phase. Nearly 70% of sporadic OS cases have been associated with an abnormality in the Rb1 locus [146,271-276]. Furthermore, in addition to being linked to a poor prognosis following OS diagnosis, individuals heterozygous for an inactivation of Rb1 have a 1,000-times greater risk of developing OS

Another tumor suppressor in the Rb pathway, $p16^{INK4A}$, is associated with development of OS [277]. Normally, $p16^{INK4A}$ inactivates CDK4 and therefore prevents the G1 to S-phase transition. Mutations in $p16^{INK4A}$ can mimic Rb inactivation, resulting in loss of tumor suppressor ability and thus uninhibited cell cycle progression. Also similar to Rb, $p16^{INK4A}$ alterations are associated with a poor OS prognosis, especially in pediatric patients [271,278].

Also implicated in OS, the p53 gene is involved in regulating cell cycle progression within the context of apoptosis and DNA repair mechanisms [279-282]. Point mutations, gene rearrangements and loss of the p53 allele at the 17p13 region are frequently demonstrated in OS patients [271,283]. In such cases, inactivation of this important tumor suppressor allows cells to bypass repair checkpoints and proliferate without adequate regulation.

### Oncogenes

Several oncogenes have been associated with the development of OS. The c-Myc gene, encoding a transcription factor involved in cell proliferation and tumor growth, appears to be amplified in nearly 12% of OS cases; it has been suggested that patients with overexpression of c-Myc have a higher rate of tumor recurrence [284-289]. MDM2 is involved in inactivating and marking the tumor suppressor p53 for degradation and is amplified at the 12q13 locus in about 10% of OS patients [280-283,290-292]. CDK4, which forms a complex with Cyclin D1 and promotes cell cycle progression, also appears to have higher levels of expression in OS, resulting in increased cell proliferation and tumor growth [293]. Additional oncogenes for which activating mutations have been associated with OS include CCND1, FOS and ERBB2 [270].

### Signaling Pathway Dysregulation

Aberrant signaling in a variety of pathways has been implicated in osteosarcoma development. As one of the most extensively studied pathways in OS, variations in the TGF-β signaling pathway, consisting of three proteins (TGF-β1-3) involved in cellular differentiation, growth and apoptosis, have been demonstrated to cause tumor progression [294-297]. Increased expression levels of TGF-β1 and TGF-β3 have been demonstrated in OS patients, with tumor progression strongly correlated to increased expression [298].

Defects in the insulin-like growth factor (IGF) signaling axis also have been implicated in tumor growth and the metastatic propensity of OS. IGF-1 and IGF-2 are growth hormones critical in the development of long bone, particularly during the adolescent growth spurt [299]. By binding to the IGF-1 receptor (IGF-1R), either ligand can promote cell proliferation while also weakening cell-to-cell and cell-to-extracellular matrix interactions [300]. When overexpressed, IGF hormones can lead to tumor growth. Similarly, overexpression of IGF-1R can lead to a malignant OS phenotype [301]. Furthermore, insulin-like growth factor binding protein 5 (IGFBP5), one of six proteins that modulates the activity of IGF hormones, is significantly underexpressed in OS and may explain both the highly proliferative and metastatic potential of these tumors [299].

Other signaling pathways also implicated in OS include Shh, FGFR2 and MET/HGF, though their molecular underpinnings are not well understood [302-304]. Finally, pathways associated with Wnt proteins and Runx2, which will be discussed in detail later in this review, are associated with defects in osteogenic differentiation and seem to be involved in OS tumor development.

### OS-Associated Syndromes

There are several medical conditions that have been implicated in patients who also develop OS. Rothmund-Thomson syndrome leads to photosensitivity, cataracts and skeletal dysplasias due to a mutation in a RECQ helicase [305]. Patients afflicted with this autosomal recessive condition have a higher tendency to develop OS, with rates suggested as high as 32%, and at a younger age than typical OS patients. Approximately 1% of patients with Paget's disease develop OS. Resulting from an imbalance in osteoblast and osteoclast activity, Paget's contributes to large deformations in bone which may play a critical role in the development of OS, especially among older patients developing this malignancy [306]. Neurofibromatosis 2 (NF2) is thought to contribute to development of OS through destabilization of p53. Patients with NF2 have decreased expression of merlin, an ERM-related tumor suppressor that inhibits MDM2-mediated degradation of p53 [307,308]. Mice heterozygous for NF2 have been shown to have a higher propensity for poorly differentiated and highly metastatic OS tumors.

## Osteosarcoma Arises From Defects in Mesenchymal Stem Cell Differentiation

Stem cells are undifferentiated precursor cells defined by their ability to self-renew and proliferate which can give rise to many tissue types [309]. Specifically, mesenchymal stem cells (MSCs) are bone marrow stromal cells with the capacity to differentiate into bone, muscle, tendon and adipose tissue [310-313]. Various endogenous and environmental factors play a critical role in tightly regulating osteogenic differentiation, leading to proper bone formation [272]. There are also several markers of osteoblastic differentiation indicating specific time points along the cascade, including connective tissue growth factor (CTGF), alkaline phospha-

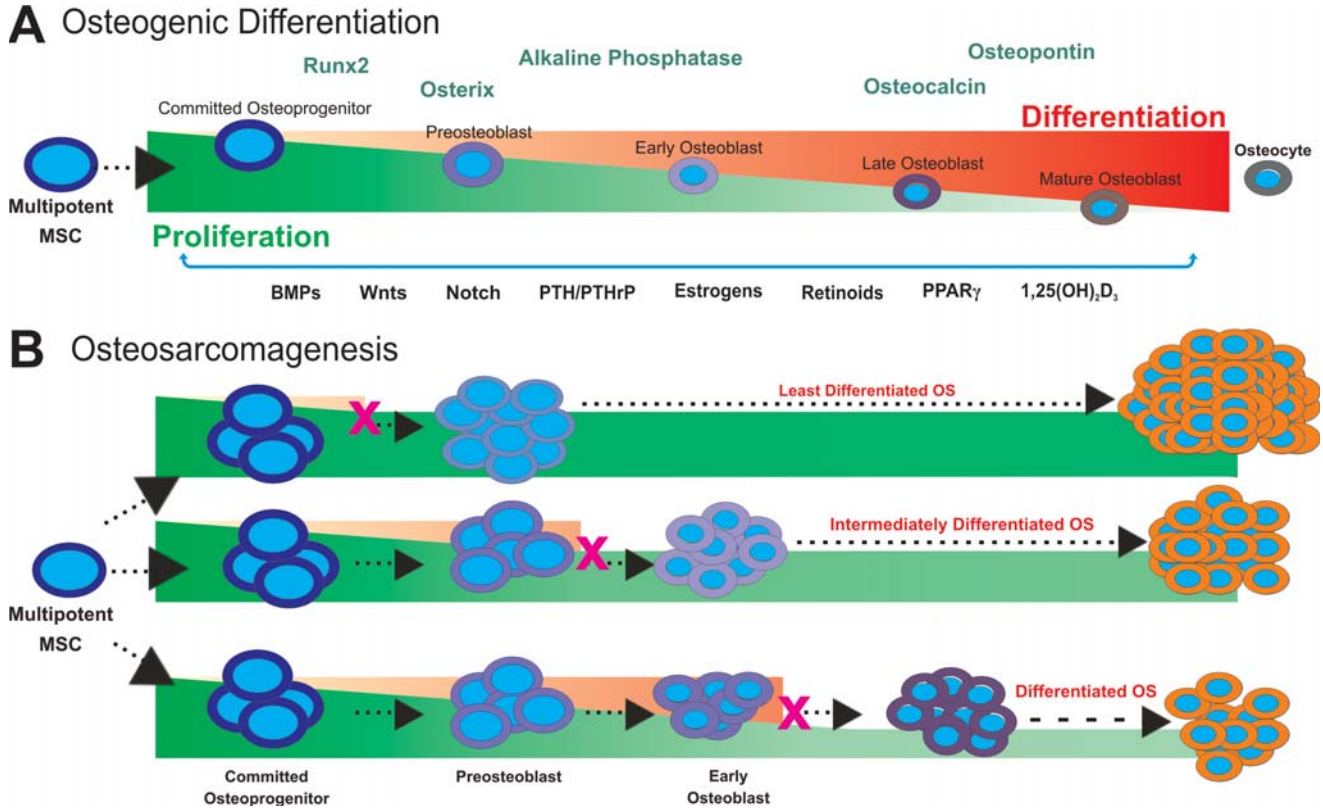


**Fig. (1). Osteosarcoma is a differentiation disease. (A)** Osteogenic differentiation of mesenchymal stem cells (MSCs). Multipotent bone marrow stromal cells have the capacity to differentiate into bone, muscle, tendon and adipose tissue, and are shown progressing along the osteogenic differentiation cascade. Osteogenic differentiation is a tightly regulated process which can be monitored by using early, middle and late markers. Early markers of the osteogenic differentiation cascade include the essential osteogenic transcription factor Runx2. Early/middle osteogenic markers include alkaline phosphatase (ALP) and Osterix. Osteocalcin and osteopontin are late markers of bone formation. MSCs can be direted along the osteogenic pathway by stimulation from osteogenic regulators including BMPs, Wnts, Notch, PTH/PTHrP, Estrogens, Retinoids, PPARγ and $1,25(OH)_2D_3$. **(B)** Defects in osteogenic differentiation may lead to OS development. Alterations in MSC differentiation include activation of oncogenes (eg, MDM2, c-Myc, CDK4), inactivation of tumor suppressor genes (eg, p53, RB, $p16^{INK4A}$) and epigenetic changes. If these genetic or epigenetic changes block progression to terminally differentiated osteoblasts or osteocytes, tumorigenic precursors may be formed, maintaining proliferative capacity and increasing susceptibility to OS development. Alterations disrupting the tightly regulated balance between proliferation and differentiation may lead to a tumorigenic phenotype, with defects at early stages leading to the development of more aggressive and undifferentiated OS.

tase (ALP), Osterix, Runx2, osteopontin (OPN), osteocalcin (OCN) and collagen [270,272,294,309,313-318]. Dysregulation in the balance between proliferation and terminal differentiation of MSCs, indicated by the overexpression or absence of these markers, may lead to the development of tumors (Fig. **1**).

Undifferentiated osteoprogenitors and OS cells are similar in that they are both highly proliferative, resistant to apoptosis and express many of the same osteogenic markers. Normally, gradual telomere shortening over many replication cycles leads to senescence as a cell approaches terminal differentiation. However, in the case of OS, an alternative lengthening of telomere (ALT) pathway can prevent the loss of telomeres with cells instead remaining in a highly proliferative, stem cell-like state [319]. This ability to evade senescence allows OS cells to self-renew and continually respond to growth factors in the same manner as early osteoprogenitors [320]. Furthermore, aggressive OS phenotypes more closely resemble early progenitors, while less aggressive phenotypes share similarities with more differentiated MSCs [272,312]. The expression of ALP, an early/middle marker of osteogenesis, is decreased in OS cells when compared to a committed osteoblastic line [317,321]. Conversely, human OS cells demonstrate increased basal levels of CTGF, a marker upregulated in the earliest stages of osteogenic differentiation [322]. Late markers such as OPN and OCN are highly expressed in mature osteoblasts but only expressed at minimal levels in OS cell lines and tumors [294,313,323,324]. These findings strongly suggest that OS cells arise from MSCs that are unable to undergo terminal differentiation, with increasing degress of differentiation associated with an improved prognosis.

To better understand defects in osteogenesis that can promote OS tumor growth, Runx2 and Wnt can be investigated. Runx2, a transcription factor of the runt family, is a master regulator of osteoblastic differentiation and thought to be linked to various human cancers including OS when its expression is altered [325-327]. When associated with $p27^{KIP1}$ and hypophosphorylated Rb, Runx2 normally induces exit from the cell cycle at the G1 checkpoint, leading to terminal osteoblastic differentiation [327]. Furthermore, Runx2 regulates bone morphogenetic protein (BMP)-induced dif-

ferentiation along the osteogenic cascade. In OS, Runx2 is significantly underexpressed and therefore leads to uncontrolled proliferation and poor differentiation, contributing to a malignant phenotype [270]. Additionally, decreased $p27^{KIP1}$ expression levels are associated with poor differentiation and high-grade tumors [327]. Therefore, a thorough understanding of the Runx2 pathway and aberrations may provide valuable insight into the development of OS, its prognosis and the development of novel therapies.

The Wnt pathway has also been studied in the context of various cancers. Typically, the Wnt glycoprotein binds to the Frizzled receptor and LRP5/6 co-receptors [318,328,329] which inhibits downstream phosphorylation and degradation of β-catenin, facilitating nuclear translocation, proliferation and differentiation [316]. During osteoblastic differentiation, Wnt3a suppresses osteogenic differentiation and promotes proliferation in adult MSCs [330]. Certain variations in this pathway have been implicated in OS [331,332]. Overexpression of β-catenin, for example, is associated with osteoprogenitor proliferation and an increased metastatic potential of OS cells. Furthermore, LRP5 overexpression by OS cells is correlated with poorer prognoses and decreased survival [333]. Similar to the Runx2 pathway, it is evident that aberrations in the Wnt signaling pathway can lead to increased proliferation and decreased differentiation of immature osteoblast progenitors.

### Potential Therapeutic Agents for MSC Differentiation Defects

Since defects in differentiation may lead to the development of OS, there is great promise in identifying therapeutic targets with the ability to induce terminal osteoprogenitor differentiation, decrease aberrant cell proliferation and promote apoptosis. Such therapies may also circumvent the need for highly toxic and often resistance-inducing chemotherapy regimens. Recent studies have investigated a variety of therapeutic approaches to promote osteogenic differentiation including the use of nuclear receptor agonists and growth factors [272,312,334-339].

Super proteins of the nuclear receptor family, including PPARy, retinoids and estrogens, have been investigated as potential OS differentiation agents. PPARy agonists have been shown to prevent proliferation, increase susceptibility to apoptosis and induce differentiation with in OS cells (1:15,153,59)[272,302,313,315]. 9-cis retinoic acid and all-trans retinoic acid also inhibited growth while promoting differentiation of OS cells, demonstrating a strong synergistic effect when combined with PPARy agonists [336,337]. Furthermore, various estrogen receptor antagonists are able to inhibit proliferation, increase osteoblast differentiation and promote apoptosis in OS cells [340].

1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) is another nuclear receptor agonist that has shown promise in promoting differentiation along the osteogenic cascade [339]. $1,25(OH)_2D_3$ induces expression of p21, a downstream target of the p53 tumor suppressor, which in turn induces differentiation of osteoprogenitors and also triggers apoptosis. Since p53 is often absent or mutated in OS cells, $1,25(OH)_2D_3$ may be used as an exogenous promoter of p21 activity and cellular differentiation, preventing tumorigenesis and promoting apoptosis [338].

Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) have demonstrated the ability to promote differentiation in MG63 OS cells [334]. After forming heterodimers, these molecules bind to a G-protein transmembrane receptor. Acting through the MAPK/PKA/PKC pathway, these ligands have been shown to elevate expression of markers of osteogenic differentiation including ALP and type I collagen [334,341]. Furthermore, transfection of MG63 OS cells with an inhibitor of the PTH/PTHrP pathway resulted in decreased levels of ALP and type I collagen, indicating incomplete differentiation of the OS cells [334]. PTH also appears to regulate c-fos, an oncoprotein whose overexpression can lead to poor differentiation and aggressive malignancy, further demonstrating the therapeutic potential of this hormone [342-344].

Finally, bone morphogenetic proteins (BMPs) appear to be involved in osteogenic differentiation, and may prove to be valuable therapeutic agents. The highly osteogenic BMPs, BMP2, 4, 6 and 9 induce the expression of ALP, OCN and OPN differentiation markers in MSCs [294,311,313,323, 345-348]. However, in OS cells, these osteogenic BMPs alone are unable to promote terminal differentiation with no increase on ALP, OCN or OPN levels and conversely may promote tumor growth [317]. Since OS cells likely contain differentiation defects regulated by upstream BMPs, exogenous administration of these proteins may promote cell proliferation rather than overcoming the underlying aberrations, facilitating tumor growth [270]. However, when combined with Runx2 overexpression, BMPs appear to induce osteogenic differentiation and inhibit tumor growth, bypassing downstream defects [270,317].

### Potential Therapeutic Agents for OS Signaling Pathway Defects

Because of the cytotoxicity and serious adverse effects associated with current standard-of-care chemotherapy regimens, there is a critical need to develop novel OS therapies that specifically target tumor cells without harming normal, healthy tissue. Several recent studies have sought to identify molecular targets in OS cells (Table **2**), which hopefully will lead to the development of new treatments leading to improved survival and quality of life in OS patients.

As mentioned earlier, defects within the insulin-like growth factor (IGF) signaling axis have been observed in OS cells [299]. Insulin-like growth factor binding protein 5 (IGFBP5), which is significantly underexpressed in OS cells, is also being investigated as a potential therapy [299]. Adenoviral-mediated overexpression of IGFBP5 leads to decreased cell proliferation, migration and invasion while promoting apoptosis *in vitro*, and furthermore inhibits tumor growth and pulmonary metastases *in vivo* in mice. Moreover, our lab has recently demonstrated that the N-terminal domain of IGFBP5 has anti-proliferative and pro-apoptotic effects, whereas the C-terminal domain inhibits cell migration and invasion. Similar results were demonstrated in an orthotopic xenograft model. The effects of the N-terminal domain may be explained by a hydrophobic stretch of amino acids that binds directly to IGF molecules and prevents

**Table 2. Molecular Targets of OS Therapies Under Investigation**

| Target | Therapy | Outcome | Mechanism | Ref. |
|---|---|---|---|---|
| IGF-1R | Ganitumab (monoclonal antibody inhibitor of IGF-1R) | Decreased OS cell proliferation *in vitro* | Prevents binding of IGF-1 and IGF-2 ligands to IGF-1R | Beltran *et al*. 2009 |
| | | Decreased OS tumor growth *in vivo* | | Beltran *et al*. 2011 |
| IGF-1 (and IGF-2) | Overexpression of IGFBP5 | Decreased OS cell proliferation, migration and invasion *in vitro* | (i) N-terminal IGFBP5 binds to IGF molecules and prevents interaction with IGF-1R | Su *et al*. 2011 |
| | | Decreased tumor growth and pulmonary metastases *in vivo* | (ii) C-terminal IGFBP5 binds to ECM and decreases metastatic potential | Luther *et al*. 2012 |
| mTOR (in AKT signaling pathway) | Rapamycin | Decreased OS tumor growth *in vivo* | (i) Increases length of G1-phase and decreases S-phase | Gazitt *et al*. 2009 |
| | | | (ii) Leads to decreased expression of Cyclin D1 and mTOR | |
| VEGF | AZD2171 (VEGF inhibitor) | Decreased tumor growth *in vivo* | Inhibits angiogenesis | Maris *et al*. 2008 |
| | PEDF | Decreased OS differentiation, proliferation and metastasis *in vitro* | (i) Inhibits angiogenesis by suppressing VEGF | Takenaka *et al*. 2005 |
| | | Decreased OS tumor growth and pulmonary metastases *in vivo* | (ii) Promotes Fas apoptotic cascade | Ek *et al*. 2007 |

ligand interaction with IGF-1R, thereby preventing proliferation and promoting apoptosis [300]. The C-terminal domain, on the other hand, appears to bind directly to the extracellular matrix (ECM), modulating cell-to-cell and cell-to-ECM interactions as a result. Overall, there appears to be great promise in identifying molecular targets within the IGF signaling axis in the development of more effective and less toxic OS therapies.

Various studies have investigated the binding of various growth factors. Downstream of receptor activation, the AKT signaling pathway is thought to induce cell proliferation and possibly tumor growth in osteosarcoma [349,350]. mTOR is a critical element of this signaling cascade, and rapamycin inhibitors of this protein have shown promising results [302]. By increasing the length of the G1-phase and decreasing the S-phase, rapamycin leads to lower expression levels of cyclin D1 and mTOR [351]. These effects have demonstrated diminished OS tumor growth in a mouse model as well as in pediatric clinical trials [258].

There is strong evidence supporting the significant role of angiogenesis in supporting tumor growth, and this has led researchers to investigate the notion that inhibition of vascular growth factors may be an effective therapeutic approach for OS. Vascular endothelial growth factor (VEGF) inhibits apoptosis and promotes angiogenesis [352]. An inhibitor of the VEGF receptor, AZD2171, has shown promising results by inhibiting OS tumor growth in an *in vivo* model [352]. In contrast to VEGF, pigment epithelium-derived growth factor (PEDF) inhibits angiogenesis and is expressed at much lower levels in many tumors [353]. By suppressing the activity of VEGF and promoting the Fas apoptotic cascade, recombinant PEDF overexpression leads to OS antitumor effects through decreased differentiation, proliferation and metastases [354].

Osteoclast activity has also been demonstrated as an important component of OS tumorigenesis. Receptor activator of the nuclear factor kappa-B ligand (RANKL) is a key promoter of osteoclast development and activity [355]. Inhibitors of RANKL may serve as potential therapeutic agents with antitumor properties. Denosumab is a monoclonal antibody to RANKL has been studied in breast cancer patients to prevent bone metastases [356]. Future studies using Denosumab or similar RANK pathway inhibitors may reveal potential applications in OS.

## Augmenting Chemotherapy and Improving Drug Delivery

There are several molecules under investigation that may improve OS patient outcomes when administered in conjunction with chemotherapy. One such agent is interferon-γ, which has been demonstrated to augment the effect of chemotherapy on OS tumors [357]. Interferon- γ can upregulate Fas receptors and caspase 8, both of which are involved in the extrinsic apoptotic cascade. The sensitization induced by interferon-γ may therefore be advantageous when administering apoptosis-inducing drugs such as doxorubicin or cisplatin to OS cells with an intact p53 pathway.

Interleukins (ILs) have also been studied as potential agents to augment the immune response during highly toxic chemotherapy regimens [118]. Specifically, IL-2 has been shown to induce differentiation of natural killer (NK) cells, with elevated numbers of NK cells strongly correlated with improved patient outcomes [118,358]. Following surgical resection of the primary tumor and also during administra-

tion of aggressive chemotherapy, administration of IL-2 has demonstrated the ability to maintain sufficiently high levels of NK cells, potentially leading to improved patient prognoses [358].

New drug delivery techniques are currently being investigated to improve the efficacy of standard-of-care therapeutic agents. For patients with pulmonary metastases, researchers have experimented with aerosolized forms of chemotherapy agents, such as cisplatin. This route of delivery has great promise for delivering maximum local concentrations of drug while limiting systemic side effects. Aerosolized cisplatin is currently in early clinical trials [359]. In order to control and optimize timing of drug release, achieve maximum potency and minimize harm to surrounding tissue, substances such as liposomes, lipoproteins, microcapsules and other synthetic polymers are being studied. Currently undergoing clinical testing, these agents may likely prove to be effective in treating drug-resistant or inaccessible tumors that normally require high doses of toxic chemotherapy with devastating effects on healthy tissue and quality of life [359]. Finally there have been limited studies investigating combination stem cell therapy and chemotherapy [360,361].

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

While tremendous progress has been made in the treatment of osteosarcoma over the past half century, there is a critical need for the development of novel therapies to improve patient survival. Patients with localized osteosarcoma have benefitted greatly from improvements in medical treatments as well as surgical and imaging techniques, but therapies for metastatic OS have not improved patient outcomes significantly over the past few decades. Limb-salvage surgery is now performed in the vast majority of OS patients, improving patient satisfaction from a functional, psychological and cosmetic standpoint. Nonetheless, pediatric patients often undergo multiple revisions throughout their lifetimes, and improvements in prosthetic construction and biologically fixed prostheses as well as the use of bisphosphonates may improve endoprosthetic fixation and ultimately patient satisfaction. The various clinical trials described hold great promise in improving patient survival and limiting the toxicity of standard-of-care cytotoxic chemotherapy. Cisplatin-free regimens containing carboplatin and methotrexate-free regimens may soon become the standard of care based upon the results of recently completed clinical trials. L-MTP-PE, now approved for use in Europe for non-metastatic high-grade OS, may soon be incorporated into regimens in the US. Other agents, including inhaled GM-CSF, HER-2 inhibitors, IGF pathway inhibitors, VEGF-inhibitors and mTOR inhibitors are currently under investigation in clinical trials and warrant further investigation before their incorporation into OS regimens. Finally, promising preclincial studies involving the IGF pathway, the mTOR/AKT signaling pathway and targets of MSC differentiation such as PPARγ, retinoids, $1,25(OH)_2D_3$ PTH/PTHrP and the BMPs will be the basis on which future OS clinical trials are based.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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