Receptor Tyrosine Kinases in Osteosarcoma: 2019 update

Running Title: RTKs in Osteosarcoma

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Abstract

The primary conclusions of our 2014 contribution [1] to this series were:

- Multiple receptor tyrosine kinases (RTKs) likely contribute to aggressive phenotypes in osteosarcoma and therefore inhibition of multiple RTKs are likely necessary for successful clinical outcomes [2, 3].
- Inhibition of multiple RTKs may also be useful to overcome resistance to inhibitors of individual RTKs as well as resistance to conventional chemotherapies [2, 3].
- Different combinations of RTKs are likely important in individual patients.
- AXL, EPHB2, FGFR2, IGF1R, and RET were identified as promising therapeutic targets by our *in vitro* phosphoproteomic/siRNA screen of 42 RTKs in the LM7 and 143B highly-metastatic human osteosarcoma cell lines [4].

This chapter is intended to provide an update on these topics as well as the large number of osteosarcoma clinical studies of inhibitors of multiple tyrosine kinases (multi-TKIs) that were recently published.

AXL

AXL, from **a**ne**x**e**l**ekto the Greek word for uncontrolled, was originally identified as a transforming gene in chronic myelogenous leukemia. It is the primary member in the mesenchymal lineage of the TAM family of RTKs that also includes TYRO3 and MER. GAS6 is the primary ligand for the TAM RTKs. The initial evidence suggesting that AXL might be important in osteosarcoma was that *AXL* is the most highly upregulated (~40-fold) of the 637 measured cancer-related mRNAs in highly-metastatic subclones of the HuO9 human osteosarcoma cell line [5]. Osteosarcoma cell lines also had the second highest level of *AXL* mRNA of the 37 types of cancer cell lines included in the Broad Institute Cancer Cell Line Encyclopedia [1]. A phosphoproteomics study found abundant AXL phosphorylation in all four human osteosarcoma cell lines that were studied [6]. *AXL* expression may be higher in tumors than in those cell lines as its transcription is induced by hypoxia, at least in epithelial cancers [7]. In that regard, AXL was detected by immunohistochemistry in 30 out of 40 human osteosarcomas but in only 8 out of the 40 adjacent non-cancerous tissues [8]. Most importantly, high levels of *AXL* mRNA correlated with poor clinical outcomes in a study of 68 osteosarcoma patients [9]. Osteosarcoma cell lines also had the seventh highest level of

GAS6 mRNA of the human cancer cell lines included in the Broad Institute Cancer Cell Line Encyclopedia [1]. In contrast, *GAS6* mRNA is down-regulated in primary osteosarcoma biopsies and human osteosarcoma cell lines compared with both bone marrow derived stromal cells and osteoblasts [10]. Moreover, low levels correlated with poor clinical outcomes in that study of 83 osteosarcoma patients [10]. A high level of immunostaining for active phosphorylated AXL was also reported to correlate with poor clinical outcomes in osteosarcoma patients [11]. However, we (unpublished data) found that the anti-phospho-AXL antibody used in that study is not specific when used for immunohistochemistry.

Our *in vitro* phosphoproteomic/siRNA screen identified AXL as contributing to migration, invasion and non-adherent colony formation, but not to cell growth, by the highly-metastatic 143B human osteosarcoma cell line [4]. More recently, we found that *AXL* shRNA also inhibits migration, non-adherent colony formation, growth of sarcospheres generated from highly-metastatic human osteosarcoma cell lines [12]. Other investigators reported that *AXL* shRNA inhibits proliferation and induces apoptosis of the MG63 human osteosarcoma cell line [8] and GAS6 inhibits apoptosis and increases migration by the MG63 and U2OS human osteosarcoma cell lines [11]. All of those *in vitro* results are consistent with our finding that stable transfection of two different *AXL* shRNA constructs reduced tumor growth by ~70% and the number of metastases by ~90% by the 143B cell line in orthotopic murine xenografts [12]. A miR-199a-3p mimic down-regulates *AXL* mRNA and inhibits *in vitro* migration by the MG63 and U2OS human osteosarcoma cell lines [13]. Moreover, high levels of that miR correlated with better clinical outcomes in a study of 30 osteosarcoma patients [13]. The same group of investigators went on to show that overexpression of the lncRNA *DANCR* upregulates *AXL*, increases proliferation, migration, invasion, and expression of stemness genes by the HOS and 143B human osteosarcoma cell lines *in vitro*, and increases tumor growth and the number of metastases formed by the 143B cell line in subcutaneous murine xenografts [9]. Moreover, high levels of *DANCR* correlated with poor clinical outcomes in osteosarcoma patients [9].

Multiple small molecule inhibitors that target the ATP-binding domain of AXL are in development [14, 15]. Most, if not all, of them target multiple RTKs [14, 15]. More specific inhibition can be achieved by targeting the extracellular domain of AXL and the other TAM family RTKs with small molecules [16], neutralizing antibodies [17], decoy receptors [18], or nucleic acid aptamers [19]. However, the polypharmacology of the

more common inhibitors that target the intracellular ATP-binding domain may contribute to their potential clinical efficacy [2, 3]. For example, BGB324 (previously known as R428), which is often considered to be specific for AXL, also potently inhibits a number of other RTKs, including RET [16, 20]. Indeed, BGB324 inhibits growth in our *in vitro* 3D sarcosphere platform [21] by both AXL-dependent and AXL-independent mechanisms [12].

AXL and the other TAM RTKs can cause resistance to conventional chemotherapeutics and kinase inhibitors in many other cancers [15, 22, 23]. Molecular mechanisms responsible for that resistance include feedback loops that increase expression of the TAM RTKs or their ligand, GAS6, crosstalk with other kinases or other oncogenes, and induction of dormancy [15, 22-28]. AXL and the other TAM RTKs also repress innate immunity [29] and targeting their activity might therefore be especially useful in combination therapy with liposomal muramyl tripeptide, a macrophage activator approved for osteosarcoma therapy in Europe [30]. Activation of innate immunity by targeting AXL or the other TAM RTKs may also increase the efficacy of T cell-mediated immune checkpoint therapy [31, 32]. The discovery of T cell-mediated cancer immunotherapy received the 2018 Nobel Prize in Physiology or Medicine [33] and has also received considerable attention as a potential therapy for osteosarcoma [34, 35].

EPHB2

*EPH*s were originally discovered in an **E**rythropoietin-**p**roducing **h**epatocellular carcinoma cell line as a homologue of the viral oncogene v-*fps*. The 14 mammalian EPHs comprise the largest RTK family [36]. *EPHA3*, *EPHB2*, and *EPHB3* mRNAs were highly expressed in human osteosarcoma tissue samples when compared to primary human osteoblasts [37]. Proteomic studies showed that cell surface levels of EPHA2, EPHB2, and EPHB4 are respectively 12-, 43-, and 20-fold more abundant on five human osteosarcoma cell lines than on primary human osteoblasts [38] and found abundant EPHB2 phosphorylation in one of the four tested human osteosarcoma cell lines [6]. Our *in vitro* phosphoproteomic/siRNA screen detected higher levels of EPHA2, EPHA4, and EPHB2 in the highly-metastatic LM7 human osteosarcoma cell line than in its non-metastatic parental SAOS-2 cell line and identified EPHB2 as contributing to migration and non-adherent colony formation, but not to cell growth or invasion, by the LM7 cell line [4]. We confirmed the siRNA results

with *EPHB2* antisense experiments [4]. Other investigators showed that mRNAs encoding EFNA5 and EFNB1, two of the ligands that activate EPHB2 as well as a number of other EPH RTKs, are upregulated in human osteosarcomas and *EFNB1* mRNA level was prominent in samples from patients with poor clinical outcomes [39]. *EPHB2* is also highly expressed in *SYT-SSX2*-positive synovial sarcoma tissues and SYT-SSX2–induced stabilization of the microtubule network is blocked by soluble forms of the extracellular domain of EPHB2 that bind and inactivate its ligands [40]. Given that osteosarcomas arise from relatively immature members of the osteoblast lineage [41], it is intriguing that EPHB2 and the other EPH RTKs modulate differentiation at multiple steps in that lineage [36, 42, 43].

FGFR2

FGFRs were originally identified biochemically on fibroblasts and muscle cells as membrane **r**eceptors that bind **F**ibroblast **G**rowth **F**actors. All four of the *FGFRs* are amplified in human osteosarcomas [44-47]. Those amplifications can predict responsiveness to NVP-BGJ398, a fairly specific inhibitor of FGFR1-3, and are associated with a poor response to conventional osteosarcoma chemotherapy [45, 46]. A phosphoproteomics study found abundant FGFR1 phosphorylation in all four human osteosarcoma cell lines that were studied, and abundant phosphorylation of FGFR2 and FGFR4 in two of them [6]. A separate study found abundant FGFR1 phosphorylation in ~70% of human osteosarcomas but did not examine the other FGFRs [48]. Moreover, the intensity of total FGFR immunostaining in primary osteosarcomas correlated with metastasis and reduced survival [49]. Both FGFR1 and FGFR2 were identified as contributing to viability of human osteosarcoma cell lines in a kinome-wide siRNA screen [50]. Our *in vitro* phosphoproteomic/siRNA screen detected higher levels of FGFR2 and FGFR3 in the highly-metastatic LM7 human osteosarcoma cell line than in its non-metastatic parental SAOS-2 cell line and identified FGFR2 as contributing to migration and non-adherent colony formation, but not to cell growth or invasion, by the LM7 cell line [4]. We confirmed the siRNA results with *FGFR2* antisense experiments [4].

Signalling by FGFR2 can support stemness in many cancers, including osteosarcoma [51, 52]. An elegant study recently showed that FGFR2 signalling induces fibrogenic reprogramming in human osteosarcoma cell line-derived stem cells, which, in turn, induces growth of metastases in the lung microenvironment without affecting growth of the primary tumor [49]. Those results led to experiments in which nintedanib, an inhibitor

of FGFR1-3, reduced stemness and the fibrogenic reprogramming, and increased apoptosis, in the human osteosarcoma cell line-derived stem cells as well as in stem cells derived from all six of the primary human osteosarcomas that were studied [49]. Moreover, a preventive regimen of nintedanib blocked lung metastasis following tibial or tail vein injection of the Well5 human osteosarcoma cell line, and even more impressively, a therapeutic regimen of nintedanib caused regression of lung metastases [49]. A preventive regimen of another FGFR inhibitor, AZD4547, reduced metastasis from an orthotopic human osteosarcoma xenograft model [53]. PD173074, in combination with doxorubicin inhibited growth and stemness of the primary tumors in a murine syngeneic subcutaneous model, while neither agent had detectable effects as monotherapies [52]. It should however be noted that nintedanib, AZD4547, and PD173074 inhibit multiple tyrosine kinases with similar or greater potency than the FGFRs [54, 55].

IGF1R

IGF1R was originally identified biochemically as the type **1** membrane **r**eceptor that binds **I**nsulin-like **G**rowth **F**actor-I and -II. Amplification of *IGF1R* occurs in 14-31% of osteosarcomas, depending on the threshold used to define amplification [56, 57]. Those studies also found other genetic events predicted to activate IGF1R (amplifications of *IGF1* or *IGF2* and deletions of either *IGF2R, IGFBP3,* or *IGFBP5*) in an additional 4.5-19% of the osteosarcomas. *IGF1R* mRNA and IGF1R protein levels are substantially increased in human osteosarcomas compared with adjacent non-cancerous tissues [58] and a phosphoproteomics study found abundant IGF1R phosphorylation in three of the four human osteosarcoma cell lines that were studied [6]. *IGF1R* mRNA and IGF1R protein levels are substantially increased in human osteosarcomas compared with adjacent non-cancerous tissues [58]. Moreover, higher IGFIR protein levels in the tumors associate with poor clinical outcomes in both human [58, 59] and canine osteosarcomas [60]. At least eight miR's have been reported to inhibit proliferation and other *in vitro* measures of osteosarcoma aggressiveness in part by targeting IGF1R [61-68]. *IGF2* siRNA substantially reduced growth of the MG63 human osteosarcoma cell line in low-serum cultures [69] and exogenous IGF2 can induce dormancy in both human and murine osteosarcoma cell lines and thereby induce resistance to methotrexate, doxorubicin, and cisplatin [70]. Consistent with those *in vitro* findings, elevated IGF2 serum levels associate with decreased event-free survival in osteosarcoma patients [69] and *IGF2* mRNA tumor levels were reduced

post-chemotherapy in the five osteosarcoma patients who responded well to chemotherapy but were either unchanged or increased 13-fold in the two osteosarcoma patients who did not respond [70].

IGF1R is one of the most studied RTKs in osteosarcoma [71]. We therefore consider the identification of IGF1R as contributing to cell growth, migration, invasion, and non-adherent colony formation by the highly-metastatic LM7 human osteosarcoma cell line as validation of our *in vitro* phosphoproteomic/siRNA screen [4]. We confirmed the siRNA results with an IGF1R neutralizing antibody [4]. Other investigators found that stable transfection of *IGF1R* shRNA reduced adhesion, migration and invasion *in vitro* as well as the number of metastases and increased survival of mice following tail vein injection of the U2OS human osteosarcoma cell line [58]. A recent study showed that IGF1R upregulation is responsible for the increased *in vitro* measures of osteosarcoma aggressiveness that are induced by overexpression of CYR61/CCN1 [72]. We [73] and other investigators [74] found that picropodophyllin, which was originally described as an IGF1R inhibitor [75], reduced growth, migration, and non-adherent colony formation, and induced apoptosis, by multiple human osteosarcoma cell lines. However, subsequent studies showed that the effects of picropodophyllin are primarily due to microtubule destabilization, rather than inhibition of IGF1R [76, 77].

IGF binding proteins (IGFBPs) can inhibit IGF1R activity by sequestering IGFs [78]. In that regard, *IGFBP3*, *IGFBP4*, *IGFBP6*, and *IGFBP7* mRNA levels were down-regulated in primary osteosarcomas and in two osteosarcoma patient-derived xenografts compared with mesenchymal stem cells before and after osteogenic differentiation [37, 79]. Similarly, *IGFBP5* mRNA and IGFBP5 protein levels were substantially reduced in highly-metastatic human osteosarcoma cell lines compared with isogenic, but weakly-metastatic, cell lines and immunostaining for IGFBP5 was reduced in metastases compared with matched primary osteosarcomas from the same patients [80]. Low levels of *IGFBP4* mRNA correlated with poor clinical outcomes in the study of 83 osteosarcoma patients described above in the section on AXL [10]. Moreover, IGFBP5 overexpression induced apoptosis and inhibited primary tumour growth and metastasis by the highly-metastatic cell lines in orthotopic murine xenografts, and *IGFBP5* siRNA had the opposite effects [80].

An IGF1R neutralizing antibody inhibited primary tumor growth in subcutaneous xenografts of multiple human osteosarcoma cell lines [81, 82]. In a similar xenograft model, the combination of two neutralizing antibodies

that bind to different epitopes on IGF1R inhibited primary tumor growth more effectively than either agent as monotherapy [83]. Three different IGF1R neutralizing antibodies in combination with a mTOR inhibitor reduced primary tumor growth more effectively than either agent as monotherapy in multiple subcutaneous xenograft osteosarcoma models [84-86]. Nonetheless, multiple IGF1R neutralizing antibodies showed little clinical efficacy against osteosarcoma in phase II studies, either alone [87, 88] or in combination with a mTOR inhibitor [89, 90]. Targeting IGF1R along with other RTKs might be more effective as dual IGF1R/IR inhibitors resensitized doxorubicin-resistant and cisplatin-resistant subclones of human osteosarcoma cell lines *in vitro* [91, 92]. Moreover, the combinations of *IGF1R* siRNA and insulin receptor siRNA or neutralizing antibodies against IGF1R and HER2 were more effective in combination than alone at reducing *in vitro* growth of human osteosarcoma cell lines [69, 93]. A bispecific IGF1R/EGFR neutralizing antibody inhibited both tumor growth and the number of metastases from the 143B human osteosarcoma cell line in an orthotopic murine xenograft model [94]. Antibodies against either of those RTKs had less effect, either alone or in combination, and the authors suggest that the recruitment of Natural Killer (NK) cells by the bispecific antibody may account for its increased efficacy [94]. The EGFR neutralizing antibody used in that study stimulates NK cell-mediated cytotoxicity against the SJSA-1 human osteosarcoma cell line *in vitro* [95] but we are unaware of similar studies with the bispecific IGF1R/EGFR neutralizing antibody.

Identification of biomarkers that predict which osteosarcoma patients will respond robustly is another approach that could increase the clinical efficacy of IGF1R inhibitors [56, 96]. In the osteosarcoma clinical studies, however, responses to IGF1R neutralizing antibodies, either alone or in combination with the mTOR inhibitor, did not correlate with *IGF1R* mutations or amplifications or with levels of *IGF1R* mRNA or IGF1R protein [89, 97, 98]. However, nuclear immunostaining for IGF1R in the absence of cytoplasmic staining associated with 6-fold longer progression-free survival and 4-fold higher overall survival in a study of soft tissue sarcoma (n = 9), Ewing sarcoma (n = 3), and osteosarcoma (n = 4) patients treated with IGF1R neutralizing antibodies [97]. In that regard, a number of recent studies found that nuclear IGF1R can contribute to *in vitro* measures of aggressiveness in epithelial cancers [99-101].

RET

RET (**re**arranged during **t**ransfection) was originally identified as a transforming gene in lymphoma. Translocation-induced *RET* fusion genes are well known oncogenes in epithelial cancers such as thyroid and non-small-cell lung cancer [102, 103]. Although *RET* fusion proteins have not been identified in osteosarcoma [56], *RET* point mutations or overexpression can also be oncogenic in the absence of translocations [103, 104]. Our *in vitro* phosphoproteomic/siRNA screen detected higher levels of RET in the highly-metastatic LM7 and 143B human osteosarcoma cell lines than in their non-metastatic parental SAOS-2 and HOS-TE85 cell lines and identified RET as contributing to migration, and to a lesser extent non-adherent colony formation, but not to cell growth or invasion by the LM7 cell line [4]. We confirmed the siRNA results with *RET* antisense experiments [4]. Chen and colleagues reported that *RET* siRNA can also decrease migration, invasion and colony formation by other human osteosarcoma cell lines [105]. Most importantly, high levels of *RET* mRNA associated with poor clinical outcomes in studies of 68 and 19 osteosarcoma patients [105, 106].

Overexpression of the lncRNA MALAT1 upregulates *RET* in human osteosarcoma cell lines *in vitro*, at least in part, by inhibiting miR-129-5p [105]. MALAT1 overexpression increases, and MALAT1 knockdown decreases, proliferation, invasion and colony formation by multiple human osteosarcoma cell lines *in vitro* as well as tumor growth in subcutaneous or peritoneal murine xenografts [105, 106]. Moreover, MALAT1 expression correlated with *RET* expression and negatively correlated with expression of miR-129-5p and survival in the study of 68 osteosarcoma patients [105].

multi-TKIs

This section will focus on the multi-TKIs evaluated in clinical studies that included patients with osteosarcoma (Table 1). All eleven of those multi-TKIs can inhibit at least one of the RTKs identified in our original phosphoproteomic/siRNA screen [4]. For example, AXL and IGF1R were among the eight RTKs inhibited by imatinib in the HOS human osteosarcoma cell line, as assessed by phospho-RTK arrays [107]. Moreover, live cell, biochemical and proteomic profiling as well as X-ray crystallography revealed that, among many other RTK targets, sunitinib can potently inhibit AXL, EPHB2, FGFR2, IGF1R and RET; dasatinib can potently inhibit AXL, EPHB2, FGFR2 and RET; cabozantinib can potently inhibit AXL, EPHB2 and RET;

sorafenib can potently inhibit AXL, FGFR2 and RET; pazopanib can potently inhibit FGFR2, IGF1R and RET; cediranib can potently inhibit AXL and RET; axitinib and regorafenib can potently inhibit FGFR2 and RET; crizotinib can potently inhibit AXL; and apatanib can potently inhibit RET [2, 54, 103, 108-112]. The polypharmacology of the multi-TKIs likely contributes to their potential clinical efficacy [2, 3] but also can contribute to serious "off-target" toxicities [103, 113].

Cediranib, dasatinib, and sunitinib were among the most effective drugs in a screen that measured viability of monolayer cultures obtained from four primary canine osteosarcomas [114]. Sorafenib, the only other multi-TKI in Table 1 included in that screen, had no detectable effects on viability of cultures from any of the canine osteosarcomas. Those results led to dasatinib treatment of four canines with osteosarcoma following limb amputation and carboplatin chemotherapy, which is a standard-of-care chemotherapy for canine osteosarcoma [115]. In two of the four canines, initial results suggest that dasatanib led to stable disease or partial remission [115]. Many multi-TKIs are more effective against epithelial cancers in hypoxic conditions [116]. Similarly, gefitinib is substantially more potent against human osteosarcoma cell lines in low-serum cultures, and in the presence of doxorubicin or methotrexate (but not cisplatin), compared with cultures containing 10% serum without chemotherapeutics [117]. Since 3D cultures mimic the oxygen, nutrient, and drug gradients found in sarcomas and other solid tumors [41], it is therefore not surprising that multi-TKIs were one of the most effective drug classes in our screen of FDA-approved oncology drugs that measured effects on the *in vitro* growth of 3D sarcospheres in both the absence and presence of MAP (methotrexate, doxorubicin, and cisplatin) standard-of-care chemotherapeutics [118]. Moreover, six (cabozantinib, crizotinib, dasatinib, pazopanib, regorafenib, and sunitinib) of the nine multi-TKIs in Table 1 that were included in our screen were among the top hits in at least one of the three tested highly-metastatic human osteosarcoma cell lines [118]. The three other multi-TKIs in Table 1 that were included in our screen (axitinib, imatinib, sorafenib) had modest effects. Regorafenib was also the fourth most effective drug in a screen that measured viability of monolayer cultures of five human osteosarcoma cell lines [119].

To evaluate the potential clinical relevance of the *in vitro* screening results described in the previous paragraph, it is important to determine whether the drugs are effective *in vivo*. Imatinib reduced growth of primary osteosarcomas in a syngeneic murine model [107]. Moreover, preventive regimens of cediranib,

dasatinib, sorafenib, and sunitinib each had intermediate to high activity in multiple subcutaneous xenograft primary osteosarcoma models evaluated by the Pediatric Preclinical Testing Program [120], and crizotinib, pazopanib, and regorafenib reduced tumor growth in similar xenograft models [121-123]. However, none of those studies [107, 120-123] determined whether the multi-TKIs also block growth of osteosarcoma metastases – the life-threatening process in osteosarcoma. In contrast, a therapeutic regimen of sorafenib caused regression in a subcutaneous xenograft primary tumor model and reduced the number and size of lung metastases in mice after tail vein injections of the SJSA-1 and MMNG human osteosarcoma cell lines [124, 125] and a therapeutic regimen of pazopanib reduced the number of lung metastases in mice after subcutaneous injection of the LM8 murine osteosarcoma cell line and resection of the resultant primary tumor [126]. Similarly, a therapeutic regimen of sunitinib reduced primary tumor growth and the number of detectable metastases derived from intratibial injection of the 143B human osteosarcoma cell line in mice [127] but no effect was seen in response to dasatinib [128], imatinib [129], or sorafenib [130] as monotherapies in similar models. In the later studies however, combinations of doxorubicin with either sorafenib or imatinib were more effective than the monotherapies [129, 130]. Given the potential translational relevance [131], it is surprising that none of the multi-TKIs have been tested in animal models in combination with all three components of MAP chemotherapy. In other combinations, sorafenib either with the mTOR inhibitor everolimus or with the CDK inhibitor palbociclib blocked growth in a MNNG human osteosarcoma cell line subcutaneous xenograft primary tumor model and in a patient-derived osteosarcoma orthotopic xenograft model [125, 132, 133]. More importantly, the therapeutic regimen of sorafenib with everolimus inhibited the number and size of lung metastases more effectively than either agent as monotherapy following tail vein injection of the MNNG human osteosarcoma cell line [125]. To maximize clinical relevance, it will be important for future murine studies to focus on therapeutic rather than preventive regimens.

Although the available clinical trials are limited in size, some of multi-TKIs appear promising as monotherapies (Table 1). The most encouraging are the Phase II studies of apatanib [134, 135], regorafenib [136, 137], and sorafenib, both alone [138] and in combination with everolimus [139]. Those studies recently led to designation of regorafenib as a category 1 recommendation by the National Comprehensive Cancer Network for second-line therapy of osteosarcoma patients with relapsed/refractory

or metastatic disease (NCCN Guidelines Version 1.2020, Bone Cancer). Sorafenib alone and in combination with everolimus are included respectively as category 2A and 2B recommendations. Multi-TKIs in on-going clinical trials listed in ClinicalTrials.gov for osteosarcoma patients include apatinib plus gemcitabine and docetaxel (Phase II, NCT03742193), apatinib plus anti-PD1 (Phase II, NCT03359018), cabozantinib (Phase II, NCT02243605 and NCT02867592), dasatinib plus ifosfamide, carboplatin and etoposide (Phase II, NCT00788125), famitinib plus anti-PD1 (Phase I/II, NCT04044378), lenvatinib plus ifosfamide and etoposide (Phase I/II, NCT02432274), pazopanib plus topotecan (Phase II, NCT02357810), regorafenib (Phase II, NCT02048371 and NCT03277924), sunitinib plus anti-PD1 (Phase I/II, NCT03277924), and sunitinib plus losartan (Phase I, NCT03900793). In addition, the Pediatric MATCH (Molecular Analysis for Therapy Choice) screening trial (NCT03155620) includes osteosarcoma patients in sub-studies of ensartinib, erdafitinib, larotrectinib, ulixertinib, and vemurafenib. Future studies will be needed to determine whether the multi-TKIs are more effective in combination with other agents and whether a subset of osteosarcoma patients can be identified that will respond to individual multi-TKIs. For example, levels of RTKs or their ligands might serve as biomarkers to predict responsiveness to appropriate multi-TKIs [45-47, 56, 96].

Systemic toxicities are a major limitation regarding multi-TKI therapies. Strategies are therefore being developed to target multi-TKIs and other drugs to the involved tissue. For example, intranasal administration can directly target multi-TKIs to osteosarcoma metastases in the lung [140, 141]. Another potential approach is to target the multi-TKIs to the tumor and/or metastases following systemic administration. For example, a liposomal formulation of ponatinib inhibited primary tumor growth by the K7M2 murine osteosarcoma cell line in a subcutaneous syngeneic model more effectively than a ten-fold higher dose of free ponatinib without inducing the systemic toxicity caused by the free drug [142]. A high-dose but pulsatile (once every two weeks) regimen has also shown promise to increase efficacy and decrease toxicity of multi-TKIs in epithelial cancers [143, 144].

Much work, both pre-clinical and clinical, remains to be done to identify optimal multi-TKIs, optimal regimens, and the most responsive patients for each multi-TKI. We are nonetheless cautiously optimistic that multi-TKIs

will ultimately improve survival for osteosarcoma patients and/or will allow use of lower doses of conventional chemotherapeutics and thereby reduce their systemic toxicity.

Table 1. Clinical studies of multi-TKIs in osteosarcoma.

multi-TKI	Study Type	# of evaluable osteosarcoma patients / disease status	Outcomes (# of patients, %)	References
Apatanib	Case Report	1 / metastatic	Partial Response (1, 100%)	[145]
	Retrospective	2 / metastatic or recurrent	No objective response	[146]
	Retrospective	4 / refractory and progressive	Partial Response (2, 50%) Stable Disease (2, 50%)	[147]
	Observational	10 / refractory and metastatic	Partial Response (2, 20%) Stable Disease (5, 50%)	[148]
	Retrospective	22 / refractory and either local unresectable or metastatic	Partial Response (9, 41%7)	[149]
	Retrospective	27 / refractory and metastatic	Partial Response (7, 26%) Stable Disease (11, 41%)	[150]
	Phase II	11 / refractory and metastatic	Stable Disease (10, 91%)	[134]
	Phase II	37 / refractory and either locally advanced, unresectable, or metastatic	Partial Response (16, 43%) Stable Disease (8, 22%)	[135]
Axitinib	Phase I	2 / refractory	Stable Disease (2, 100%)	[151]
Cabozantinib	Phase I	2 / relapsed or refractory	No objective response	[152]
Cediranib	Phase I	4 / refractory	34% reduction in size of lung metastases (1, 25%)	[153]
Crizotinib	Phase I	7 / elapsed or refractory	Stable Disease (3, 43%)	[154]
Dasatinib	Phase I	5 / refractory	No objective response	[155]
	Phase II	46 / unresectable, recurrent, or metastatic	Clinical Benefit Response (CBR)* (6, 13%)	[156]
Imatinib	Phase II	10 / refractory or recurrent	No objective response	[157]
	Phase II	27 / metastatic or locally advanced	Clinical Benefit Response (CBR)** (5, 19%)	[158]
Pazopanib	Case report	1 / refractory and relapsed	No objective response	[159]
	Case report	2/ recurrent and metastatic	Partial Response (1, 50%) Stable Disease (1, 50%)	[160]
	Case report	3 / second recurrence	stabilization of serum alkaline phosphatase level (1, 33%)	[161]
	Case report	3 / refractory and metastatic	Stable Disease (2, 67%)	[162]
	Retrospective	6 / advanced, after 1-4 lines of therapy	Stable Disease (2, 33%)	[163]
	Case report	15 / refractory and metastatic	Partial Response (1, 7%) Stable Disease (8, 53%)	[164]
	Phase I	4 / recurrent or refractory	Stable Disease (1, 25%)	[165]
Regorafenib	Phase I	Not stated / refractory	Partial Response (1)	[166]
	Randomized Phase II	22 + 10 in placebo group who crossed over after progression / progressive and either advanced or metastatic, after ≥1 lines of therapy	Improved mean Progression-Free Survival (3.6 months vs 1.7 months w/ placebo group)	[136]
	Randomized Phase II	26 / progressive and metastatic, after 1-2 lines of therapy	Increased Stable Disease (7, 27% vs 0% w/ placebo)	[137]
Sorafenib	Case report	1 / refractory, progressive, and metastatic	Partial Response (1, 100%)	[167]
	Case report	4 / refractory and relapsed	Stable Disease (3, 75%)	[159]
	Case report	8 / metastatic (6 patients) or local (2 patients)	Partial Response (6, 75%)	[168]
	Case report, combo w/ denosumab	1 / relapsed and unresectable	Stable disease (1, 100%)	[169]
	Phase I	10 / refractory	No objective response	[170]
	Phase I, combo w/ bevacizumab and cyclophosphamide	2 / recurrent or refractory	Stable Disease (2, 100%)	[171]
	Phase II	35 / metastatic, relapsed, unresectable, and progressive	Progression-free survival at 6 months (10, 29%)	[138]
	Phase II, combo w/ everolimus	38 / progressive and either locally advanced, unresectable, or metastatic	Progression-free survival at 6 months (17, 45%)	[139]
Sunitinib	Case report	5 / refractory and relapsed	Partial Response (1, 20%) Stable Disease (1, 20%)	[159]
	Phase I	2 / refractory	Stable Disease (1, 50%)	[172]

* CBR: Dasatinib: Objective Response within 6 months or Stable Disease for ≥ 6 months
** CBR: Imatinib: Complete or Partial Response at 2 or 4 months or Stable Disease at 2 & 4 months

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