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Review

Targeting receptor tyrosine kinases in osteosarcoma and Ewing sarcoma: Current hurdles and future perspectives

Emmy D.G. Fleuren [a,*], Yvonne M.H. Versleijen-Jonkers [a], Otto C. Boerman [b], Winette T.A. van der Graaf [a]

[a] Department of Medical Oncology, Radboud University Medical Centre, Nijmegen, The Netherlands
[b] Department of Nuclear Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands

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ABSTRACT

Osteosarcoma (OS) and Ewing sarcoma (ES) are the two most common types of primary bone cancer, which mainly affect children and young adults. Despite intensive multi-modal treatment, the survival of both OS and ES has not improved much during the last decades and new therapeutic options are awaited. One promising approach is the specific targeting of transmembrane receptor tyrosine kinases (RTKs) implicated in these types of bone cancer. However, despite encouraging in vitro and in vivo results, apart from intriguing results of Insulin-like Growth Factor-1 Receptor (IGF-1R) antibodies in ES, clinical studies are limited or disappointing. Primary resistance to RTK inhibitors is frequently observed in OS and ES patients, and even patients that initially respond well eventually develop acquired resistance. There are, however, a few remarks to make concerning the current set-up of clinical trials and about strategies to improve RTK-based treatments in OS and ES.

This review provides an overview concerning current RTK-mediated therapies in OS and ES and discusses the problems observed in the clinic. More importantly, we describe several strategies to overcome resistance to RTK inhibitors which may significantly improve outcome of OS and ES patients.

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Abbreviations: OS, osteosarcoma; ES, Ewing sarcoma; RTK, receptor tyrosine kinase; IGF-1R, Insulin-like Growth Factor-1 Receptor; TKI, tyrosine kinase inhibitor; IR, Insulin Receptor; ALK, Anaplastic Lymphoma Kinase; EGFR, Epidermal Growth Factor Receptor; PDGFR, Platelet-Derived Growth Factor Receptor; VEGFR, Vascular Endothelial Growth Factor Receptor; EFS, event-free survival; OSV, overall survival; CR, complete response; CBR, clinical benefit response; PR, partial response; MR, minor response; SD, stable disease; PD, progressive disease; IHC, immunohistochemistry; SPECT, single photon emission computed tomography; PET, positron emission tomography; CT, computed tomography

* Corresponding author at: Department of Medical Oncology, Radboud University Medical Centre, P.O. Box 9101, 452, 6500 HB Nijmegen, The Netherlands. Tel.: +31 24 3618897; fax: +31 24 3540788.
E-mail address: Emmy.Fleuren@radboudumc.nl (E.D.G. Fleuren).

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1. Introduction

Osteosarcoma (OS) and Ewing sarcoma (ES) are the two most common types of primary bone cancer, predominantly affecting children and adolescents. The majority of these sarcomas are high-grade tumors with advanced disease at presentation. Moreover, primary metastatic disease at presentation is not a rare event. Current curative treatment regimens consist of surgery, radiotherapy (in the case of ES) and intensive chemotherapeutic schedules. Despite this multi-modal approach, survival in ES and OS patients is still disappointing, with an overall 5-year survival rate of 60–65%. Additionally, treatment is accompanied by many early and late side effects [1,2]. This urges the need for more specific therapeutic approaches to target OS and ES.

One promising approach is the specific targeting of transmembrane receptor tyrosine kinases (RTKs) implicated in these bone sarcomas. RTKs are high affinity cell-surface receptors consisting of an extracellular ligand-binding domain, an intracellular catalytic kinase domain and a single transmembrane domain separating these two regions. In the absence of ligand, RTKs are in an unphosphorylated and monomeric state, and the conformation of their kinase domains is inactive. Ligand binding to the extracellular domain leads to activation of the receptor by inducing conformational changes that induce receptor dimerization, resulting in increased kinase activity and rapid autophosphorylation of tyrosine residues. This creates binding sites for several intracellular signaling proteins, which are recruited to the cell membrane and in turn activate multiple downstream signaling pathways such as phosphatidylinositol 3 (PI3)/Akt kinase and extracellular signal regulated kinase (Erk) [3,4].

RTKs are key mediators in regulating normal cellular processes such as cell growth, proliferation and survival. In addition to their pivotal role in normal physiology, several RTKs have been implicated in the development and progression of numerous cancers, including OS and ES. Aberrant RTK signaling is in most cancers the result of gene amplification, mutations and/or protein overexpression [4]. Consequently, several molecular therapies have been and are still being developed to target these constitutively activated receptors. These strategies include the development of monoclonal receptor-blocking antibodies targeting the extracellular RTK domain, small molecule inhibitors targeting the intracellular catalytic RTK domain (TKIs) and ligand neutralizing agents.

For several reasons, progress in the application of RTK-directed therapy in the clinical setting is hampered for OS and ES. First of all, single agent activity is either not yet established or has only proven to be timely, and secondly, pharmaceutical companies envisage these rare bone sarcomas not as their primary goal of interest [5].

In this review, we will focus upon the RTKs involved in OS and ES, including the Insulin-like Growth Factor-1 Receptor (IGF-1R), Insulin Receptor (IR), Anaplastic Lymphoma Kinase (ALK), Epidermal Growth Factor Receptor (EGFR), Platelet-Derived Growth Factor Receptor (PDGFR), c-KIT, MET and Vascular Endothelial Growth Factor Receptor (VEGFR) and will summarize the activity of currently available inhibitors to these RTKs in the pre-clinical and clinical settings. More importantly, we will also discuss existing problems such as primary and acquired resistance mechanisms, and demonstrate potential strategies to overcome resistance and improve therapeutic outcome.

2. RTKs in OS and ES

2.1. IGF-1R

IGF-1R is one of the most intensively studied targets in OS and ES and the activity of this receptor is regulated by the presence and binding of the IGF-1 and IGF-2 ligands. In addition, the interaction between IGFs and IGF-1R is influenced by the presence of IGF binding proteins (IGFBPs). Consistent protein expression of IGF-1R was demonstrated in both OS and ES cell lines and various tumor samples, but no mutations have been identified in OS. In ES, only one of 47 tumor samples showed a nonsynonymous mutation in a region with low functional impact [6–11]. In OS, increased IGF-1R expression correlated with tumor metastasis and a poor prognosis in one study although another study reported no correlation with outcome [11,12]. In ES, high IGF-1R expression was either associated with more favorable clinical outcomes or did not influence patient outcome [8,9]. Nevertheless, numerous in vitro and in vivo studies demonstrated that both OS and ES cells are highly dependent on IGF-1R signaling, and activation of IGF-1R by IGF-1 stimulated OS cell growth and metastatic behavior in vivo [13–15]. Since the peak incidence of OS and ES coincides with the burst of growth hormone (GH) and IGF-1 release during puberty, a causal relationship has been suggested [6]. IGF-1R blocking with monoclonal antibodies or TKIs resulted in marked anti-tumor activity in several OS and ES xenografts and significant anti-tumor activity was observed in some OS and ES patients as well using IGF-1R antibodies, with relatively limited and manageable side effects [16–32]. Notably, in phase I studies the overall response rate with IGF-1R antibodies appeared higher in ES than in OS or other sarcoma subtypes (Table 1). Subsequent phase II studies focused therefore predominantly on ES. Unfortunately, despite some spectacular clinical responses, the majority of both OS and ES patients are primary resistant to IGF-1R-targeted therapy thus only a small proportion benefits from IGF-1R antibodies [16]. Even if initially good anti-tumor effects are observed, prolonged therapy response is not guaranteed as responses to IGF-1R inhibitors are in general short-lasting in ES and OS due to secondary resistance mechanisms. Consequently, the further development of several IGF-1R inhibitors has been put on hold [33].

2.2. IR

The IR shares a high degree of structural homology (±60%) with IGF-1R, but the ligand binding kinetics and function of these receptors are different. Two isoforms of IR have been identified: IR-A and IR-B, and insulin is the main ligand for both isoforms. Although IR is predominantly known for its major role in glucose metabolism, recent studies have demonstrated a role for IR in cancer as well [34]. It was shown that, in addition to IGF receptors, IR-A is also able to bind IGF-2 thereby serving as a second physiologic receptor for this ligand [35]. IR-A was also shown to heterodimerize with IGF-1R, and these hybrid receptors can stimulate cell motility in response to IGF-1, IGF-2, and insulin [36,37]. More importantly, co-targeting of IGF-1R and IR-A with the TKI BMS-536924 appeared to be more effective than IGF-1R targeting alone in inhibiting growth of OS cell lines, confirming the oncogenic role of IR signaling in bone sarcomas [38]. OSI-906, a dual IGF-1R/IR TKI, was also effective in 3 out of 4 OS cell lines in vitro (IC_{50} <0.1 μM) [39]. In ES patients, IR was abundantly expressed although high IR mRNA expression was significantly associated with more favorable clinical outcomes [8]. IR targeting may however still be of interest in ES. The dual IGF-1R/IR inhibitor GSK1904529A for instance inhibited IGF-1R and IR kinase activities in ES cell lines with similar potencies, resulting in proliferation inhibition. Oral administration of GSK1904529A also decreased growth of human tumor xenografts in mice. It was hypothesized that the anticancer properties of GSK1904529A may be mediated in part through IR-A inhibition [40]. Moreover, the ability to inhibit both IGF-1R and IR kinase activities potentially provides a

Table 1
Clinical results of currently available RTK inhibitors for the treatment of OS and ES.

Drug	Type inhibitor	Targets	Study type	Clinical results[a]
Figitumumab	Antibody	IGF-1R	Phase I	ES: 1/15 CR, 1/15 PR, 6/15 SD, 7/15 PD [21]
			Phase I/II	ES: 16/122 PR, 25/122 SD[b], 81/122 PD (phases I and II); OS: 11/11 PD (phase I) [23]
R1507	Antibody	IGF-1R	Phase I	ES: 2/9 PR, 2/9 SD, 5/9 PD [19]
			Phase I	ES: 4/6 SD, 2/6 PD; OS: 2/3 SD, 1/3 PD [16]
			Phase II	ES: 1/115 CR, 10/115 PR, 8/115 PR (unconfirmed), 18/115 SD[c], 78/115 PD [24]
Dalotuzumab	Antibody	IGF-1R	Phase I	ES: 1/6 SD (mixed response), 5/6 PD [29]
Cixutumumab	Antibody	IGF-1R	Phase I/II	ES: 3/35 PR, 5/35 SD, 27/35 PD (phase II); OS: 3/3 PD (phase I) [25]
			Phase II	OS: 1/11 SD [32]
			Phase II	ES: 1/18 PR, 5/18 SD [30]
Ganitumab	Antibody	IGF-1R	Phase I	ES: 1/12 CR, 1/12 PR (unconfirmed), 10/12 PD [27]
			Phase II	ES: 1/18 PR, 7/18 SD[b], 10/18 PD [28]
AVE1642	Antibody	IGF-1R	Phase I	ES: 1/8 (bone/soft tissue tumors) PR, 4/8 SD[d] or PD; OS: 1/8 (bone/soft tissue tumors) PR (unconfirmed), 4/8 SD[d] or PD [31]
Crizotinib	Small molecule inhibitor	MET and ALK	Phase I	ES: 4/4 PD; OS: 3/7 SD, 4/7 PD [49]
			Phase I/II	Initiated: NCT01182896 (incl. OS)
Gefitinib	Small molecule inhibitor	EGFR	Phase I	ES: 1/3 PR, 2/3 PD; OS: 6/6 PD [56]
Imatinib	Small molecule inhibitor	PDGFR, c-KIT and Bcr/Abl	Phase II	ES: 1/24 PR, 23/24 PD; OS: 10/10 PD [75]
			Phase II	ES: 1/13 SD, 12/13 PD; OS: 2/26 SD, 5/26 CBR, 19/26 PD [77]
			Phase II	ES: 2/2 PD [76]
Dasatinib	Small molecule inhibitor	PDGFR, c-KIT, Bcr/Abl and SRC	Phase II	Initiated: NCT00464620 (incl. ES)
Sunitinib	Small molecule inhibitor	PDGFR, c-KIT, Bcr/Abl, VEGFR and FLT-3	Phase I	ES:2/2 PD; OS: 1/2 SD, 1/2 PD [78]
Cediranib	Small molecule inhibitor	VEGFR and c-KIT	Phase I	ES: 1/3 PR, 2/3 PD; OS: 1/4 MR, 3/4 PD [102]

[a] Clinical responses were defined as described in the referred articles. Abbreviations: CBR = clinical benefit response, CR = complete response, PR = partial response, MR = minor response, SD = stable disease (for at least 8 weeks), PD = progressive disease (no response).
[b] No minimum time for SD reported.
[c] SD reported from 5.6 weeks.
[d] SD was reported in 4 not further specified soft tissue or bone tumor patients.

therapeutic advantage over anti-IGF-1R monoclonal antibodies since more and more evidence is pointing towards an oncogenic role for IR in ES and OS. Of note, IR inhibition by GSK1904529A resulted in only minimal effects on glucose homeostasis at efficacious doses [40]. At present, the dual IGF-1R/IR inhibitor OSI-906 has been tested in two ES patients and was well tolerated, although no anti-tumor activity was observed [41]. A study combining OSI-906 with the EGFR inhibitor erlotinib reported stable disease (SD) in several tumor types, including cases of ES [42].

2.3. ALK

ALK is a member of the IR superfamily with high homology to leukocyte tyrosine kinase (LTK). The ligands thought to be involved in ALK activation are pleiotrophin (PTN) and midkine (MK) [43]. In normal tissues, ALK expression is confined to discrete regions of the developing nervous system, but several studies have demonstrated a role for ALK in cancer [43,44]. ALK expression was demonstrated in several ES cell lines and multiple ES tumor samples, and weak expression was observed in one extraskeletal OS as well [45–47]. Although no data is available concerning ALK expression and clinical outcome in OS, a recent study showed a trend towards a worse event-free survival (EFS) and overall survival (OSV) in ES patients with highest ALK expression. Sporadic ALK translocations and several exon deletions in the ALK RTK domain were identified as well, although the significance of these aberrations is still unknown [46]. The small molecule ALK-inhibitor NVP-TAE684 affected ES cell viability in vitro (IC_{50} 0.15–0.79 μM) and the multi-targeted ALK/IGF-1R/IR (GSK1838705A) TKI demonstrated comparable anti-tumor effects in ES cell lines (EC_{50} 0.14–1.03 μM), although this was ascribed to an anti-IGF-1R effect and ALK was left out of consideration for ES in that study [46,48]. The dual ALK/MET TKI crizotinib (PF-02341066) also affected ES cell growth in vitro (IC_{50} 1.22–3.59 μmol/L) [46]. A phase I trial assessing the safety and activity of crizotinib for pediatric patients with refractory solid tumors reported SD in 3/7 OS patients though no objective responses were observed in the four included ES patients. Crizotinib was tolerated well [49]. Another clinical trial testing crizotinib in children with relapsed or refractory solid tumors, including OS, has been initiated recently, but no results are available at present (NCT01182896).

2.4. EGFR

The EGFR is a member of the EGFR family, and its activity is regulated by several ligands including EGF, transforming growth factor α (TGF-α) and amphiregulin [50]. Expression of EGFR has repeatedly been described in human OS and constitutive EGFR phosphorylation was demonstrated in OS cell lines as well. In addition, EGFR copy number gain is a common event in OS [51]. Inhibition of EGFR signaling in vitro resulted in OS growth arrest and apoptosis, making it an attractive target for the further development of therapeutic approaches [52]. However, in high grade OS, EGFR expression showed favorable clinical outcomes instead of a poor prognosis, and tumors with lack of or very weak EGFR expression had the poorest prognosis [53]. EGFR expression was demonstrated in several ES cell lines as well, although no EGFR mutations have been reported in ES at present [54–56]. The TKIs gefitinib (EGFR) and vandetanib (VEGFR/EGFR/RET) demonstrated a modest suppression of ES cell proliferation in vitro. This effect was, however, non-specific and ES cells were therefore not considered highly sensitive to EGFR inhibition,

although this study included only two ES cell lines [54]. In an ES xenograft, gefitinib had little effects on tumor growth [55]. However, a phase I study using gefitinib showed a partial response (PR) in 1/3 ES patients. Although in non-small cell lung cancer (NSCLC) specific EGFR TK domain mutations correlated with responsiveness to gefitinib, no mutations were found in the EGFR gene in the responding ES patient [56,57]. Another phase I study using both cediranib (VEGFR/c-KIT inhibitor) and gefitinib demonstrated one confirmed PR in an OS patient out of two not further specified primary bone sarcoma patients [58].

2.5. PDGFR

The PDGFR occurs in two isoforms (α and β) and can be activated by PDGFs (A, B, C and D). These four PDGFs bind PDGFRs with different specificity, and currently PDGFR-α/PDGF-A, PDGFR-α/PDGF-C and PDGFR-β/PDGF-B are the three most well known complexes with functional evidence [59]. PDGFR expression was observed in a variety of human solid tumors, including OS and ES [60–65]. Although correlations between PDGFR expression and outcome are not always as consistent, several studies indicate that PDGFR-α and PDGF-A correlate to a poor prognosis in OS [60,62,63]. PDGF was found to act as a mitogen in OS cells and expression of PDGF-A was associated with tumor progression in human OS samples [62,66]. In the majority of human ES samples, both PDGFR-α and PDGFR-β are activated and PDGFR-β was shown to contribute towards tumor proliferation and metastasis, supporting the rationale for inhibiting these RTKs in ES [64,65]. At present however, no activating mutations in the PDGFR-α (exon 12 and 18) or PDGFR-β (exon 12) genes were reported in ES or OS [63,65,67]. PDGFR-β silencing however reduced spontaneous growth and metastasis in an ES xenograft model [68]. The most well known drug to inhibit PDGFR signaling is imatinib, an oral multi-targeted inhibitor of several kinases, including PDGFR, c-KIT and Bcr/Abl. Imatinib inhibited growth of OS and ES cell lines in vitro, as well as in in vivo xenografts. Drug concentrations necessary to achieve anti-tumor activity in these bone sarcoma models were however rather high (IC_{50} 6–15 μM), while IC_{50}-concentrations necessary to inhibit ligand-induced phosphorylation of PDGFR were much lower (0.1–0.5 μM). This suggests that the observed anti-tumor activity may not be achieved through PDGFR inhibition, but through an off-target effect [1,60,69]. Other drugs used for inhibiting PDGFR signaling are dasatinib and sunitinib, which are also multi-targeted TKIs. In addition to PDGFR and c-KIT, dasatinib also inhibits SRC, and sunitinib also inhibits VEGFR and FLT-3. In vitro, dasatinib demonstrated superior anti-proliferative and anti-migratory activities in ES cell lines compared to imatinib, possibly through an additive effect of anti-SRC activity [70,71]. In vivo however, dasatinib demonstrated only limited activity in OS and ES xenografts [72]. Interestingly, an in vitro screening process predicted dasatinib to be effective for a dog with spontaneous OS (IC_{50} 151 nM), and indeed, the canine patient remains without evidence of recurrent disease 24 months following initial diagnosis [73]. Sunitinib showed in vivo anti-tumor efficacy in various OS and ES xenografts, but this was the result of an anti-angiogenic effect instead of PDGFR inhibition [74]. The problem with PDGFR inhibition is that most PDGFR-targeting drugs are aimed at multiple other kinases as well, making it difficult to specifically study the effect of PDGFR inhibition. The use of other, more specific approaches, such as siRNA-mediated silencing of target expression, may be a more appropriate approach to tackle this problem. In clinical trials, imatinib demonstrated little or no activity in children with relapsed or refractory ES and OS. Only one PR was seen among 24 ES patients [75]. In a phase II clinical trial of imatinib in c-KIT and/or PDGFR-α-expressing tumors, no response was observed in 2/2 ES patients [76]. Another phase II imatinib trial demonstrated SD in 2/26 and a clinical benefit response (CBR) in 5/26 OS patients. In this trial, 1/13 ES patients had SD [77]. One clinical trial using dasatinib in advanced sarcomas, including ES, has been initiated, but no results have been published [NCT00464620]. Concerning sunitinib, a phase I trial in pediatric patients with refractory solid tumors (including 2 OS and 2 ES patients) reported no objective responses, although one OS patient had SD [78].

2.6. c-KIT

c-KIT is a RTK that binds to stem cell factor (SCF). c-KIT overexpression was found in both OS and ES, and was shown to be activated in ES [65,69,79]. In ES cells and xenografts, a central role for c-KIT and its ligand SCF concerning survival and metastasis has been indicated [80]. In ES patient samples, however, no significant association between c-KIT expression and clinical outcome was observed, but it was thought that targeting this receptor could have a therapeutic effect in a select group of patients [81]. In OS, the effect of c-KIT expression on patient outcome is not as consistent; two studies demonstrated no significant correlation between c-KIT expression and patient outcome, while in another study OS patients with higher c-KIT protein expression were significantly more likely to experience local disease recurrence and had a significantly lower survival time than patients with lower c-KIT expression [82–84]. Similar to PDGFR, c-KIT can be targeted by imatinib, dasatinib and sunitinib. In addition, the pan-VEGFR kinase inhibitor cediranib was recently shown to have similar activity against c-KIT as well [85]. Although c-KIT positive gastrointestinal stromal tumors (GISTs) showed response to imatinib, c-KIT positive ES cell lines were insensitive to imatinib. In addition, expression of other imatinib-sensitive tyrosine kinases, including PDGFR and ABL did not result in anti-tumor activity in ES by imatinib, at least not to clinically achievable imatinib concentrations [86]. Several studies in GIST demonstrated that imatinib is efficacious in the majority of patients harboring activating c-KIT mutations, however, no c-KIT mutations in exon 9, 11 or 17 were found in OS, and only 2/71 ES patients harbored activating c-KIT mutations within exon 9 but none in exons 11, 13 or 17 [67,83,84].

2.7. MET

MET or hepatocyte growth factor receptor (HGFR) is essential for embryonic development and wound healing. The hepatocyte growth factor (HGF) is the only known ligand for MET. Expression of MET was observed in both benign and malignant bone tumors [87]. In addition, numerous studies reported MET expression in OS, and MET activity was associated with proliferation, metastasis and chemotherapy resistance in these tumors [88–90]. K252a, a specific MET inhibitor, was able to revert HGF dependent growth of OS cell lines, emphasizing its oncogenic role [91]. Crizotinib inhibited OS growth and osteolysis/matrix production in a xenograft model [92]. Very recently, MET has been implicated in ES as well. Cytoplasmic MET expression was demonstrated in virtually all ES samples ($n = 50$), membranous MET expression in 34% of samples and vascular MET was observed occasionally. Most importantly, in 20 patients membranous MET expression in primary tumors correlated significantly with a worse OSV compared to non-membranous expression. MET RTK domain aberrations were demonstrated in 3/33 ES samples, including one missense mutation and two exon deletions. The ALK/MET inhibitor crizotinib (IC_{50} 1.22–3.59 μmol/L) and the MET-inhibitor cabozantinib (IC_{50} 2.69–8.27 μmol/L) affected ES cell viability in vitro [46]. Although several strategies to block MET are currently under development and tested in clinical trials for the treatment of a variety of adult tumors, including the direct MET inhibitor ARQ 197 in clear cell and alveolar soft part sarcomas, results from only one study aiming at MET inhibition are available in bone sarcomas [49,93]. This is, however, not a specific MET inhibitor but the dual ALK/MET inhibitor crizotinib, as described in Section 2.3.

2.8. VEGFR

There are three different types of VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3), which can be activated by their ligands the VEGFs (A, B, C and D). These receptors and ligands are key mediators in regulating

angiogenesis in many tumors, including OS and ES [1,94]. VEGF expression was observed frequently in OS and ES and expression levels correlated with clinical parameters including metastatic phenotype and OSV [95–97]. Numerous preclinical studies demonstrated that targeting of vascular mechanisms may be effective in OS and ES [98,99]. At present, the most common methods to block VEGFR signaling are the use of antibodies directed against VEGF (bevacizumab (Avastin)), or by directly blocking VEGFR with TKIs (e.g. cediranib and sunitinib). Although originally developed as a pan-VEGFR kinase inhibitor, recent research demonstrated that cediranib has similar activity against c-KIT as well [85]. Cediranib demonstrated anti-tumor activity against both OS and ES xenografts [100]. In a phase I trial with bevacizumab, 3/5 ES patients had SD but no response was observed in 3/3 OS patients [101]. A phase I trial using cediranib demonstrated however objective responses in pulmonary metastasizes of both ES and OS patients; 1/3 ES patients had a PR and 1/4 OS patients had a minor response (MR) [102]. One confirmed PR was reported in an OS patient using both cediranib and gefitinib [58].

A summary of the clinical results of RTK inhibitors currently tested in OS and ES patients is given in Table 1.

3. Hurdles with current RTK therapies

Although several RTKs have been identified as potential targets in OS and ES and numerous preclinical studies demonstrated promising results for RTK-targeted therapies, the results from clinical trials have not met the high expectations as mentioned in the sections above. A major problem concerning almost all RTK inhibitors, is that only a very small population of OS and ES patients benefits from RTK-targeted therapy, which cannot be explained by RTK protein expression levels or the lack of activating RTK mutations alone.

Since OS and ES are relatively rare tumors, clinical trials often comprise small patient numbers and the small subpopulation benefiting from RTK inhibitors is often not enough to stimulate pharmaceutical companies to further develop their novel drugs. In addition, initially good anti-tumor responses do not guarantee prolonged therapy responses as acquired resistance to RTK inhibitors is observed frequently in OS and ES patients. Consequently, several studies concluded that the administration of a single RTK inhibitor is not sufficient for bone sarcoma treatment, and the further development of various RTK inhibitors is put on hold. There are, however, a few remarks to make concerning the current set-up of clinical trials and about strategies to improve RTK-based treatments in OS and ES.

4. Predictive response biomarkers

4.1. Screening for a therapeutic target

At present, practically all clinical RTK inhibitor trials are performed upon a heterogeneous population of (bone sarcoma) patients. However, more and more evidence is emerging that the introduction of a priori selection of patients presenting a predictive response biomarker would be more successful and more cost-effective by excluding primary resistant patients [33,103]. The search for a suitable predictive biomarker remains however a challenge. Although screening for the presence of a therapeutic target would be a logical first step towards predicting response, mere target expression is not sufficient. IGF-1R expression levels for instance as assessed by conventional immunohistochemistry (IHC) or Western Blot (WB) could not accurately predict therapy response in in vivo bone sarcoma models [17,18,104,105]. This reflects problems seen in the clinic, because IGF-1R expression is observed in virtually all ES patients (272/290 primary tumor samples), but only a small proportion shows IGF-1R antibody response [8,9]. Similar findings are observed for most other RTKs implicated in OS and ES, as described in Section 2.

In practically all studies however total protein expression levels were considered, mostly with IHC on archival formalin-fixed paraffin embedded (FFPE) material. The specific screening for phosphorylated RTKs may provide more accurate information concerning the dependency of tumor cells on a particular pathway, and may consequently correlate better with therapy response. Although at present no studies are published in OS or ES concerning this approach, active EGFR and Her2/neu were more predictive for survival than total EGFR overexpression and Her2/neu overexpression in breast cancer patients [106]. Screening for phosphorylated RTKs in patient samples requires however standardization of tumor fixation, since the detection of these specific proteins is very delicate and depends on various factors including time between surgery and fixation. In addition, the subcellular RTK localization could also be informative for RTK-pathway activity. Membranous RTK expression is linked to a poor prognosis in various cancer types, including membranous MET in ES, implying a functional role of the receptor at this subcellular localization. This suggests that patients with apparent membranous RTK expression may benefit from drugs directed against these RTKs [26,107]. For antibody-mediated therapies, it seems even more appropriate to specifically screen for membranous RTK expression, since antibodies target the extracellular RTK ectodomain. The specific determination of membranous RTK expression levels remains however a challenge. Although FACS is a reliable method to screen for membranous RTK expression in cell lines, this method cannot be applied on patient samples since single cell suspensions are required. IHC can give some information concerning membranous expression levels in tumor samples, though this method is at best semi-quantitative and high cytoplasmic expression levels may prevail the level of specific membranous staining. With an in vivo screening approach however, it was recently demonstrated that membranous IGF-1R expression (and accessibility) could distinguish between high, modest and non-IGF-1R antibody responsive bone sarcoma xenografts, while conventional methods could not [26]. Another study correlated nuclear IGF-1R expression with IGF-1R antibody response, whereby nuclear expression may reflect IGF-1R pathway activation, further underscoring the significance of subcellular target localization [108,109].

Although activating mutations in EGFR and c-KIT RTK domains have been linked to gefitinib and imatinib responses in NSCLC and GIST, respectively, only a negligible number of c-KIT mutations are reported in OS and ES at present and in the one ES patient showing a PR to gefitinib no EGFR mutations were found [56,84]. Also for IGF-1R, no functional mutations were found in OS and ES and the few ALK and MET RTK domain aberrations found in ES cell lines could not be linked to in vitro sensitivity to ALK/MET inhibitors [46]. Although more research in larger OS and ES patient populations is required, current findings suggest that the presence of activating mutations is not sufficient to predict RTK inhibitor response in OS and ES. It is however very important to keep in mind that tumors with constitutive RTK activation as a result of activating RTK domain mutations need to be targeted with TKIs, while constitutive activation as a result of RTK/ligand overexpression can also be inhibited by antibodies. When a RTK domain aberration responsible for constitutive RTK activation induces (conformational) changes preventing TKI binding, therapeutic strategies should be aimed at inhibiting signaling pathways downstream of that RTK.

4.2. In vivo screening methods

As mentioned earlier, most biomarker screenings are performed on a single biopsy, often archival FFPE material. However, RTK expression levels determined from one biopsy not necessarily represent target expression in the whole tumor since both OS and ES can be very heterogeneous and metastases not necessarily express similar RTK levels as corresponding primary tumors [110]. Another major draw-back with conventional techniques is that information concerning target accessibility cannot be obtained. Physiological factors, including tumor interstitial pressure, blood vessel density, vascular permeability and diffusion

capacity are of great importance in determining whether or not an inhibitor reaches its target [111–113]. This is especially important when dealing with bone sarcomas, since these tumors may severely impede antibody diffusion due to a calcified bone matrix as seen in OS, or large necrotic areas in ES [114]. Obviously, an inhibitor unable to reach its target cannot achieve anti-tumor activity. Consequently, various parts within one tumor can respond different to a certain (RTK-targeted) therapy due to either varying target expression or physiological factors and different lesions within one patient can also show differences in drug sensitivity [115,116]. This urges implementation of in vivo screenings instead of conventional methods. Proof of principle of such a method was recently described in OS and ES mouse models using a radiolabeled anti-IGF-1R antibody (^{111}In-R1507) with immuno-single photon emission computed tomography (SPECT). This study showed that while IGF-1R expression as assessed by conventional IHC was not able to predict response to IGF-1R antibody therapy, specific membranous IGF-1R expression and target accessibility as assessed by ^{111}In-R1507 immuno-SPECT could clearly distinguish between high, modest and non-responsive bone sarcoma xenografts [26]. The development of in vivo markers for response prediction to other RTK inhibitors is warranted.

Another, more general way to determine primary resistance in vivo are 2-deoxy-2-[^{18}F]-fluoro-D-glucose (^{18}F-FDG) and/or 3′-deoxy-3′-[^{18}F]-fluorothymidine ([^{18}]F-FLT) positron emission tomography (PET) scans, although these scans are more commonly used to monitor treatment response [117]. ^{18}F-FDG is a glucose analog that internalizes in cells with enhanced glucose requirement, thereby reflecting cellular glucose metabolism. ^{18}F-FLT-uptake reflects the proliferative activity of cells. ^{18}F-FDG-PET scans are routinely available and have already been tested in multiple studies for predicting and monitoring chemotherapy response in OS and ES [118–123]. In addition, ^{18}F-FDG-PET-scans should be combined with computed tomography (CT) scans for ES patient follow-up, since ^{18}F-FDG-PET/CT was superior to PET alone in terms of sensitivity, specificity, and accuracy, mainly for the detection of new lesions [124,125].

^{18}F-FDG-PET response prediction and monitoring for RTK inhibitors is gaining more and more attention for bone sarcomas. Recently, ^{18}F-FDG-PET/CT scans were used to monitor IGF-1R antibody (cixutumumab and AMG-479) monotherapy response and combined cixutumumab and temsirolimus (RTK-pathway inhibitor) responses in ES patients with promising results so far [27,115,126]. ^{18}F-FDG-PET could also be a helpful tool to assess response to RTK inhibitors in OS, as illustrated in a phase II trial with the multi-kinase inhibitor sorafenib (Section 5.4) [127]. These results warrant further studies to elucidate the exact predictive role of ^{18}F-FDG-PET for RTK-targeted therapies in larger clinical trials.

^{18}F-FLT-PET is an emerging new technique for the staging of bone sarcomas and little information is available concerning its usefulness to monitor treatment response. However, since ^{18}F-FLT-PET significantly correlated with tumor grade in several bone sarcomas, including OS and ES, this technique may very well be used to monitor treatment response because the degree of tumor proliferation can reflect the effectiveness of a RTK-targeted therapy [128]. Indeed, ^{18}F-FLT-PET was very recently pointed out to be a promising method to monitor temsirolimus-based treatment response in OS xenografts [129]. Further (pre)clinical research concerning the predictive value of ^{18}F-FLT-PET to RTK-inhibitors is warranted.

5. Improving RTK-targeted therapies

5.1. Dealing with primary resistance

One way to actually improve therapeutic outcomes is to understand and deal with primary resistance mechanisms. Recently, it was demonstrated in two ES models that F(ab′)$_2$ fragments of the IGF-1R antibody R1507 have superior tumor penetrating and IGF-1R-targeting properties as compared to intact R1507 molecules, suggesting that IGF-1R therapies in ES may be improved by using smaller therapeutic compounds [26,130]. Indeed, an ES model unresponsive to multiple IGF-1R antibodies showed an improved anti-tumor response when treated with the IGF-1R-targeting TKI BMS-754807 [131]. Although the exact therapeutic value of these and other small (IGF-1R-targeting) compounds remains to be evaluated, these findings do suggest that smaller compounds such as TKIs (e.g. BMS-754807/OSI-906), or single-chain antibodies/affibodies, may be better candidates for future ES (and OS) treatment rather than conventional antibodies [41,131–133]. Also for other RTKs implied in ES and OS, TKIs, and not antibodies, may be the drugs of choice.

In addition, if a patient proves to be primary resistant to the selected RTK inhibitor, it is worthwhile to screen for expression of other RTKs involved in ES and OS. In primary IGF-1R-resistant bone sarcoma cell lines for instance, overexpression of EGFR, TGFβR2, and MET was demonstrated, providing possible alternative therapeutic targets [22].

5.2. Dealing with acquired RTK resistance

Another important aspect for improving therapeutic outcomes is gaining more insight in the biology behind acquired RTK inhibitor resistance, and anticipate upon this. Although the exact mechanisms underlying this phenomenon remain unclear, some progress has been made lately, particularly concerning IGF-1R-targeting. A recent study in ES and rhabdomyosarcoma cell lines showed that ribosomal protein S6 (rpS6) and the RTK macrophage-stimulating 1 receptor (MST1R) were potential modifiers of IGF-1R inhibition by showing that MST1R knockdown restored BMS-536924 efficacy in highly drug-resistant cell lines, which correlated with inhibition of rpS6 phosphorylation [134]. In a rhabdomyosarcoma model, chronic loss of IGF-1R expression was demonstrated during BMS-754807 treatment, resulting in a decreased dependency of the IGF-1R pathway for growth. Consequently, other receptors, including PDGFR-α and AXL, were upregulated and cells subsequently relied on downstream signaling of these receptors [135]. In addition, several recent studies demonstrated that efficacy of and resistance to anti-IGF-1R therapies in ES are dependent on IR signaling, and also in osteoblasts disruption of IGF-1R was shown to enhance insulin signaling and action [136,137]. Similar results have been published in other tumor types [138]. Furthermore, since primary IGF-1R-resistant bone sarcoma cell lines demonstrated overexpression of other RTKs, including EGFR, TGFβR2, and MET, this supports the idea of dependency on alternate RTKs during acquired resistance [22]. In addition, p-Akt and p-mTOR upregulation was observed in an ES patient that relapsed after initial response to an IGF-1R antibody [139]. Upregulation of these intracellular pathways may have been the result of upregulation of other RTKs, although this was not further elucidated in that study.

Although acquired resistance mechanisms to other RTK inhibitors, such as EGFR and PDGFR inhibitors, are less studied in OS and ES, some indications concerning these mechanisms can be found in research of other cancer types. Particularly the ability of one RTK to compensate for another to maintain tumor cell viability is emerging as a common resistance mechanism. In NSCLC for instance, acquired resistance to EGFR-inhibitors is often accompanied by overexpression and activation of VEGFR and IGF-1R. Amplification and strong activation of MET and AXL were linked to acquired EGFR resistance as well [50,140,141]. Combined treatments of a MET inhibitor and the EGFR-inhibitor cetuximab were additive, indicating the significance of combination therapies [142]. In GISTs, it is well established that the leading cause for acquired resistance to imatinib is secondary mutations in the c-KIT or PDGFRA kinase domain [143]. In addition, c-KIT downregulation was accompanied by AXL overexpression and MET overexpression, thereby replacing c-KIT as the predominant RTK in imatinib-resistant GIST cells [144]. Furthermore, since 65% of imatinib-resistant GIST patients demonstrate a good response to sunitinib, this implies that one or more of the additional targets (VEGFR, FLT and RET) are upregulated in some imatinib-resistant patients [143].

5.3. Rationale for combined therapies

The abovementioned findings highlight the importance of monitoring treatment response for the early detection of acquired resistance and provide a rationale for combining RTK-targeted therapies. In addition, they urge the further development of RTK inhibitors, even for those compounds to which the majority of OS and ES patients are primary resistant. For instance, although PDGFR and/or c-KIT targeting by imatinib did not show promising results in a clinical bone sarcoma trial, PDGFR was found to be upregulated in IGF-1R-resistant sarcoma cells [135]. This suggests that IGF-1R-resistant bone sarcoma patients might benefit from other RTK inhibitors, including PDGFR-targeting compounds. This also provides a rationale for combining PDGFR-inhibitors with IGF-1R-inhibitors when resistance occurs.

Combined RTK-targeted therapies can be implemented with different strategies. One option is to start with a single RTK inhibitor and closely monitor treatment response and RTK expression levels, and start an alternate RTK-targeted therapy when the first signs of acquired resistance occur. With conventional techniques, this however requires repetitive biopsies, which may be difficult to perform. Another way is to directly start targeting several RTKs at once, which already proved to be very effective in preclinical studies. Based on cross-talk between IGF-1R and EGFR pathways, combination studies were performed in sarcoma cell lines to target both pathways in vitro, and enhanced inhibitory effects were observed [22]. In addition, IGF-1R and IR-A co-targeting appeared to be more effective than IGF-1R-targeting alone in inhibiting the growth of OS cell lines, and an IGF-1R inhibitor combined with imatinib synergistically augmented anti-tumor effects in ES cell lines [38,145]. Interestingly, the combination of two IGF-1R antibodies targeting distinct epitopes led to an enhanced anti-tumor response as well in an OS cell line and OS xenograft model [146].

5.4. Novel multi-kinase inhibitors

In this light, another attractive strategy to target OS and ES appears to be the use of new multi-kinase inhibitors, some of which already proved to be very effective in preclinical studies. Sorafenib (Nexavar) for instance, a multi-kinase inhibitor targeting VEGFR, PDGFR, RET, FLT3 and c-KIT, demonstrated in vitro and in vivo activities against several sarcomas, including OS and ES [147–150]. Combination studies with other drugs indicated that sorafenib has a tolerability profile conducive to be combined with other agents [151]. A recent phase II study using sorafenib in 35 patients with relapsed and unresectable high-grade OS after failure of standard multimodal therapy demonstrated clinical anti-tumor activity, including three PRs, two MRs and twelve SDs with an acceptable toxicity profile [127]. Other studies showed modest clinical activity of sorafenib in patients with advanced refractory soft-tissue sarcomas (STS) or solid tumors [152,153]. When combined with everolimus, sorafenib showed even greater anti-tumor effects in preclinical OS models [154]. Amuvatinib (MP-470) is a multi-kinase inhibitor targeting among others PDGFR, c-KIT, MET and AXL, and has very recently shown anti-proliferative activity in OS cell lines. Combination with chemotherapy demonstrated synergistic effects and in the clinic amuvatinib was well tolerated [155,156]. Linifanib (ABT-869) is a multi-targeted TKI that targets Fms-like tyrosine kinase-3 (FLT-3), c-KIT, VEGFR and PDGFR. In ES xenograft models, linifanib therapy inhibited tumor growth and prolonged survival, which was ascribed to PDGFR-β and c-KIT pathway inhibition [157]. A phase I study reported SD in 1/5 not further specified sarcoma patients and linifanib was well-tolerated [158]. Very recently, the novel oral multikinase inhibitor lenvatinib (E7080), targeting among others VEGFR, FGFR, PDGFR and c-KIT, showed anti-tumor activity in bone sarcomas. Although lenvatinib did not significantly affect OS cell proliferation in vitro, lenvatinib was able to inhibit tumor cell migration and invasion at clinically achievable concentrations inhibiting its known targets (FGFR and PDGFR) [159]. In addition, in vivo studies demonstrated that 3/5 OS xenografts were sensitive to lenvatinib, which was mainly ascribed to angiogenesis inhibition [160]. In ES, the effects of lenvatinib remain to be investigated. A phase I dose escalation study demonstrated that lenvatinib has a manageable toxicity profile encouraging future clinical studies in OS and ES patients [161]. In addition, a phase I study using the VEGFR2-3, PDGFR, RET and c-KIT inhibitor regorafenib (Bay-73-4506) demonstrated a PR in 1/2 OS patients [162]. Very recently, a study using the VEGFR1-3, PDGFR and c-KIT inhibitor pazopanib

Table 2
Pre-clinical and clinical results of new emerging multi-RTK inhibitors for the treatment of OS and ES.

Drug	RTK targets	Pre-clinical results		Clinical results[a]
		In vitro[b]	In vivo[c]	
Sorafenib (Nexavar)	VEGFR, PDGFR, RET, FLT3 and c-KIT	ES: 4/4 partially sensitive (IC_{50} 3.2–7.6 μM) [147] ES: 2/4 partially sensitive, 2/4 little activity (IC_{50} 2.4–3 μM and >5 μM); OS: 4/4 little activity (IC_{50} >5 μM) [149] OS: 7/7 partially sensitive (IC_{50} 2.4–4.8 μM) [148]	ES: 3/5 intermediate, 2/5 low; OS:3/4 intermediate, 1/4 low [147] OS (high grade): 2/2 intermediate-high [148] ES: 1/1 response (response monitored by contrast ultrasound) [150]	OS (high grade): 3/35 PR, 2/35 MR, 12/35 SD, 18/35 PD [127] ES: 4/4 SD[d] or PD; OS: 10/10 SD[d] or PD [153]
Amuvatinib (MP-470)	PDGFR, c-KIT, MET and AXL	OS: 2/3 partially sensitive, 1/3 little activity (IC_{50} 1.3–3.6 and 35–100 μM, resp.) [155]	None	None initiated
Linifanib (ABT-869)	FLT-3, c-KIT, VEGFR and PDGFR	ES: 2/2 partially sensitive (IC_{50} 1.25–2 μM) [157]	ES: 2/2 growth delay ES (metastatic): 2/2 prolonged survival [157]	None initiated
Lenvatinib (E7080)	VEGFR, FGFR, PDGFR and c-KIT	OS: 1/1 little activity (IC_{50} 34.8 μM) [159]	OS: 3/5 high, 1/5 intermediate, 1/5 low [160]	None initiated
Regorafenib (Bay-73-4506)	VEGFR2-3, PDGFR, RET and c-KIT	None	None	OS: 1/2 PR, 1/2 PD [162]
Pazopanib (Votrient)	VEGFR1-3, PDGFR and c-KIT	OS: 1/1 little activity (IC_{50} >10 μM) [164]	OS: 1/1 growth delay [164] OS: 1/1 reduction rate and size of pulmonary metastasis [165] ES: 1/2 intermediate, 1/2 low [166]	ES: 2/2 PD; OS: 1/1 SD [167] Initiated: NCT01759303 (OS metastatic to the lung)

[a] Clinical responses were defined as described in the referred articles. Abbreviations: CBR = clinical benefit response, CR = complete response, PR = partial response, MR = minor response, SD = stable disease (for at least 8 weeks), PD = progressive disease (no response).

[b] In this table, only cytotoxic and anti-proliferative effects were taken into account. Since there is a substantial variation concerning sensitive/resistant demarcation between different studies, we used the following criteria: IC_{50} ≤1 μM sensitive; IC_{50} 1–10 μM partially sensitive; and IC_{50} >10 μM little activity. Since this demarcation remains arbitrary, IC_{50} values of all studies are given.

[c] When possible, in vivo responses were defined as described by Houghton et al. [176]. In short, the mean relative tumor volume (RTV) of the treatment group (T) was divided by the mean RTV of the control group (C): T/C. Criteria: T/C ≤15% high response; T/C ≤45% but >15% intermediate response; and T/C >45% low response. Best anti-tumor responses are depicted (e.g. in the case of repeated experiments/use of more than 1 dose/etc.). If these values are not given in the referred article, tumor response is described as in the article.

[d] No objective responses observed, but SD was reported in 14 not further specified solid tumor patients.

(Votrient) was initiated for the treatment of OS metastatic to the lung (NCT01759303). Pazopanib treatment resulted in tumor growth delays and reduced the rate and size of pulmonary metastasis in in vivo OS models, showed some activity in in vivo ES models and was effective in patients with metastatic non-adipocytic soft-tissue sarcoma [163–166]. A phase I pediatric study reported SD in 1/1 OS patient but no objective response in 2/2 ES patients treated with pazopanib [167]. Pazopanib combined with topotecan demonstrated greater antitumor efficacy compared to each single agent in among others an OS model, supporting its future combination with chemotherapeutics [164].

A summary of new emerging multi-kinase inhibitors for OS and ES treatments is given in Table 2.

With multi-targeted kinase inhibitors it may however be more difficult to initially assess primary resistance, since this would involve screening for the presence, and preferably also the accessibility, of multiple targets. Another important aspect to pay attention to when developing multi-kinase inhibitors is toxicity, although most recently developed multi-kinase inhibitors demonstrated manageable toxicity profiles. Combinations of several kinase inhibitors could however be hampered by intolerability, as illustrated by clinical experience when combining sorafenib or pazopanib with temsirolimus [168,169].

Since most mono- and multi-RTK inhibitors exert their effects by inhibiting tumor growth instead of killing tumor cells, attempts have been made to combine RTK-targeted therapies with chemotherapy to gain optimal anti-tumor response. Several RTK inhibitors, including but not limited to IGF-1R and EGFR inhibitors, were previously shown to sensitize both OS and ES cells to various types of chemotherapy which may significantly potentiate anti-tumor activity [69,170,171]. Multiple studies demonstrated synergistic effects when RTK-inhibitors were combined with chemotherapy in bone sarcoma models [145,155]. In the clinic, gefitinib enhanced the bioavailability of irinotecan and this combination resulted in a PR in 1/3 ES patients [172]. Of note, although bevacizumab monotherapy seemed not very promising for OS and ES treatments as described in Section 2.8, various very recently published clinical studies showed promising results when combined with chemotherapy, particularly in ES. A pilot combination study reported a PR and complete response (CR) in the two included refractory ES patients, another combination study reported a CR in the sole included primary metastatic ES patient and a third combination study reported SD in 1/1 refractory ES patients and a SD and PD in the two included refractory OS patients [173–175]. Although the exact benefit of the addition of bevacizumab to chemotherapy regimens remains unclear because these studies were not conducted in a randomized controlled fashion, the results in these very poor-prognosis patients are encouraging.

6. Conclusions

In the past years, various RTK-inhibitors have been developed and tested in OS and ES. Although very effective in the pre-clinical setting, several problems occurred concerning RTK-targeted therapies in the clinic. The frequently observed primary and acquired resistance to these inhibitors led to the suspension and even termination of the further development and testing of some of these compounds. However, there are several strategies to improve the effectiveness and therapeutic outcomes of RTK inhibitors. Prior to RTK-targeted therapy, patients should preferably be screened for biomarkers – if available – and response should be monitored frequently and adequately. In addition, it is vital to elucidate the underlying mechanism of constitutive RTK activation for determining the right treatment strategy. While monoclonal antibody therapy can be helpful when ligand/membranous receptor overexpression is the leading cause for aberrant signaling, TKIs are more appropriate when (activating) TK domain mutations or intracellularly active RTKs (e.g. fusions) are present. Moreover, TKIs might even be more effective than antibodies because of better tumor penetration and subsequent enhanced antigen-targeting. Since more and more evidence is emerging that the simultaneous targeting of one or more RTKs is a more effective approach than targeting a single RTK, more research should be focused on rational combinations of several mono- or multi-targeted RTK inhibitors. Preclinical in vitro and in vivo studies are essential for providing more insight in the best combinations and should be completed prior to the initiation of clinical trials.

Altogether, implementation of these strategies may significantly improve future therapeutic outcomes of OS and ES patients.

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