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Targets for Cancer Therapy in Childhood Sarcomas

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Abstract
Development of chemotherapeutic treatment modalities resulted in a dramatic increase in the survival of children with many types of cancer. Still, in case of some pediatric cancer entities including rhabdomyosarcoma, osteosarcoma and Ewing’s sarcoma, survival of patients remains dismal and novel treatment approaches are urgently needed. Therefore, based on the concept of targeted therapy, numerous potential targets for the treatment of these cancers have been evaluated preclinically or in some cases even clinically during the last decade. This review gives an overview over many different potential therapeutic targets for treatment of these childhood sarcomas, including receptor tyrosine kinases, intracellular signaling molecules, cell cycle and apoptosis regulators, proteasome, hsp90, histone deacetylases, angiogenesis regulators and sarcoma specific fusion-proteins. The large number of potential therapeutic targets suggests that improved comparability of preclinical models might be necessary to prioritize the most effective ones for future clinical trials.

Introduction
About 12’000 children under 20 years of age are diagnosed each year with cancer in the US alone. Fifty years ago, such a diagnosis was a sentence of death for most patients due to the lack of effective therapies at that time and despite surgical resection of the tumor. This changed dramatically with the discovery of different cytotoxic chemotherapeutic agents able to kill proliferating cells which resulted in the development of effective therapies for many of these cancer types. Intensification of treatment and optimized treatment modalities, together with more effective palliative drugs to fight side effects, resulted in a continuous improvement of outcome in the second part of the last century. In the period 1996-2002 the overall 5-year survival rate has reached 79% (1). For many of the pediatric cancer entities including Hodgkin lymphoma, retinoblastoma, Wilms tumor, and germ cell tumors, the survival rate nowadays even exceeds 90%.

However, for some other childhood cancer entities, chemotherapy still remains largely non-effective. Resistance to the drugs and metastatic spread represent the two most important mechanisms for therapy failure. Tumors belonging to this group include different types of sarcomas such as rhabdomyosarcoma, Ewing's sarcoma or osteosarcoma which reach an overall 5-year survival rate of 60-65% (2). Specific subgroups of these tumors with a tendency of early metastasis such as alveolar rhabdomyosarcoma even have a much poorer prognosis. In addition, intensive chemotherapeutic treatment can result in a variety of long-term sequelae in childhood patients, including impairment of growth and development, a variety of organ dysfunctions and subsequent secondary malignancies, preventing further intensification of therapy with these drugs (3). Therefore, based on the plateau which has now been reached with current treatment options there is an urgent need for alternative, more targeted treatment approaches.

This review will summarize some of the numerous recent developments in therapeutic approaches against childhood sarcomas with a special focus on rhabdomyosarcoma, Ewing’s sarcoma and osteosarcoma.
Targeted therapy
To overcome problems associated with the unspecificity of the current therapeutic approaches, the concept of “targeted therapy” has been developed. Per definition, such an approach is based on the application of drugs (more) specifically targeting tumor cells and sparing normal cells. Two general approaches can be distinguished:
The first approach is based on differences in the physical presence of molecular markers between cancer and normal cells. Cancer-specific markers could serve as targets for antibodies or antibody-like molecules linked to cytotoxic agents which, upon binding, are able to kill these cells. Furthermore, immunological techniques may allow priming of specialized immune cells to recognize cancer-specific structures.
The second approach is based on differences in the dependency on certain functional characteristics between cancer and normal cells, which could be therapeutically exploited. Four different dependencies have been defined (4). First, most cancer cells are dependent on the constant activity of only some of the underlying abnormalities (“oncogene addiction” or genetic dependence) (see also (5)). Second, in some cancer cells mutation of one pathway renders these cells dependent on another pathway (synergy dependence). Third, cancer cells are often dependent on the same signaling pathways as their normal counterparts (lineage dependence). Fourth, single tumor cells or the tumor as a whole can depend on activities coming from the host environment such as angiogenesis or growth factors produced by stromal cells (host dependence). Numerous studies demonstrated that inhibition of activities involved in these dependencies can either inhibit cell proliferation or induce apoptosis in tumor cells but leaves normal cells more or less unaffected, suggesting that cancer cells are to a much greater extent dependent on these functions than normal cells. Therefore, all these dependencies represent potential targets for a cancer cell specific treatment.
Based on these concepts, identification of dependencies for each tumor entity or maybe even each patient is a crucial prerequisite for the development of future targeted therapies. The process of defining novel targets includes several crucial steps such as target identification based on experimental data including gene expression or mutational analysis. However, the main work should be a careful preclinical validation both in vitro with clear effects on cell viability and tumor growth and in vivo using different animal models.
Technological advances during the past decade in the area of genome and transcriptome analysis, including sophisticated gene expression analysis as well as high throughput sequencing methods, accelerated identification of potential targets substantially and resulted in a plethora of potential specific targets in all kinds of cancer entities. Also in childhood sarcoma many potential targets have been defined and some of them have been preclinically evaluated. A comprehensive list of potential molecular targets for treatment of childhood sarcoma is given in table 1 and will be discussed in detail in the following paragraphs.

Receptor Tyrosine Kinases
Transmembrane receptor tyrosine kinases (RTKs) are important upstream elements of signaling cascades which regulate cell growth, proliferation and survival. Many different RTKs are implicated in tumorigenesis of numerous cancer types (6). Localization at the cell surface makes them accessible not
only for small molecule inhibitors, but also for inhibitory antibodies, highlighting them as key targets for cancer treatment. Importantly, in normal tissue activity of most RTKs is only mandatory during embryonic development and not essential during adulthood (7). A series of RTKs has been linked to the development of childhood sarcomas including IGF1R, PDGFR, c-met and c-kit (8). These RTKs have subsequently also been investigated as targets for therapy of these tumor types.

The IGF1R is one of the prototype targets in childhood sarcoma and has been extensively studied. Although no activating mutations are known for this RTK, a plethora of studies have linked aberrant activity of this kinase to different cancers (for a general review see (9)), for a sarcoma specific review see (10)). In sarcomas, overexpression of the IGF1R itself, deregulated expression of ligands/ligand binding proteins or constitutive activation of downstream effectors might play a role. Different studies have evaluated the effect of IGF1R inhibition on growth of childhood sarcomas cell lines and xenografts, both with inhibitory antibodies and small molecule inhibitors (11-21). In these studies, blockade of IGF1R activity has been shown to affect cell proliferation, survival and anchorage-independent growth in vitro and tumorigenesis, tumor invasion and metastasis in vivo. Since it also sensitizes cancer cells to chemo- and radiotherapy in vivo, IGF1R is one of the prime targets for treatment of these sarcomas. Indeed, the pre-clinical data initiated several clinical phase II trials which are currently ongoing and will evaluate the efficacy of IGF1R-targeting for pediatric sarcoma (Tab.2).

The significance of the other three RTKs mentioned as therapeutic targets is less well documented. Numerous studies reported c-met expression in rhabdomyosarcoma and osteosarcoma (22-24) and c-met activity has been linked to regulation of proliferation, metastasis and resistance to chemotherapy in these tumors (25-29). Furthermore, overexpression of HGF, the ligand for c-met, in an Ink4 null background leads to the development of RMS in mice (30). In contrast, only few studies evaluated c-met as target for therapy. SiRNA-based silencing of c-met by was found to inhibit proliferation, invasiveness, and anchorage-independent growth in vitro, and reduce tumor mass in a xenograft model of rhabdomyosarcoma (31). In a second study, the c-met inhibitor K252a was able to revert HGF dependent growth of osteosarcoma cell lines (25). However, based on promising findings obtained in numerous preclinical studies with c-met inhibitors for treatment of adult tumors (32), further evaluation of c-met in childhood sarcomas might be indicated.

PDGFR is expressed in all three types of childhood sarcomas discussed here (33-35) and c-kit expression has been found in Ewing’s sarcoma and osteosarcoma (36-38). Mutations were only found in the case of c-kit and in a small fraction of Ewing’s sarcomas (39). However, both PDGFR and c-kit are activated in these childhood sarcomas (37, 40) and PDGF was found to act as mitogen for osteosarcoma cells (41). Furthermore, expression of PDGF-AA was found to be associated with tumor progression in osteosarcoma (42). Based on these findings, preclinical studies evaluated the effects of PDGF and c-kit inhibitors on sarcoma cell growth. However, results from these studies are rather conflicting. Some studies demonstrated that imatinib, which inhibits both PDGFR and c-kit, is able to inhibit growth of Ewing’s sarcoma, osteosarcoma or rhabdomyosarcoma cells in vitro and as xenografts in vivo with a high IC50 of 6-15 μM (34, 36, 43-45). In contrast, IC50-concentrations of imatinib necessary to inhibit ligand induced phosphorylation of PDGFR and c-kit were in the range of 0.1-0.5 μM (44, 46). Only in one study using osteosarcoma cells, imatinib concentrations necessary for inhibiting PDGFR phosphorylation and inhibition of proliferation in vitro corresponded (41). However, in this case imatinib
had no effect on growth of xenografts. Furthermore, sunitinib, which also inhibits PDGFR and c-kit among a range of other kinases, inhibited growth of most childhood sarcomas as xenografts, but its effect was attributed to inhibition of angiogenesis (47). Despite these conflicting data, a phase II clinical study testing imatinib for the treatment of different tumors including Ewing’s sarcoma and osteosarcoma was carried out by the Children’s Oncology group. Imatinib demonstrated no activity as single agent, although drug concentrations were again well above the levels necessary for PDGF or c-kit inhibition (48). Taken together, these data suggest that the effects detected in vitro might be due to off target effects and that PDGF and c-kit might not be relevant targets for treatment of these childhood sarcomas. The use of siRNA-mediated silencing of target expression, which is far more specific than the use of small molecule inhibitors with an often ill-defined spectrum of activities, might be of help in future preclinical studies to demonstrate dependence of sarcoma growth on activity of PDGFR and c-kit receptor.

**Intracellular signaling molecules**

Transmembrane receptors transmit their signals via intracellular signaling cascades to downstream effectors such as transcription factors, cell cycle regulators, and regulators of translation and apoptosis. One central axis of signal transduction is represented by the PI3K-AKT-mTOR pathway which is indeed very frequently deregulated in cancer (49). On the one hand, many different membrane receptors transmit their signals via this route whereby the activation status of this cascade can reflect an aberrant activation of many different upstream receptor kinases. On the other hand, some members of this axis themselves belong to the group of proteins most frequently mutated in cancer, including PI3K (50) and its antagonist, the tumor suppressor PTEN (51). Therefore, potential inhibitors of this axis are expected to have a broad therapeutic utility in many tumors (52) and various components of this pathway are under current investigation as therapeutic targets in cancer (53).

In childhood sarcoma one research focus as target for therapy is mTOR. mTOR activation as assessed by its phosphorylation has been detected among all childhood sarcoma entities discussed here and was negatively correlated with rhabdomyosarcoma survival (54-56). Inhibition of mTOR by Rapamycin or derivatives thereof inhibited proliferation of childhood sarcoma cells in vitro (55, 57, 58) and also inhibited the growth of xenografts of rhabdomyosarcoma and osteosarcoma cell lines in vivo (58, 59). In vitro mechanisms of action has been reported to dependent on the p53/p21 status. Cells expressing wild-type p53 or p21 arrest in G1 whereas cells lacking functional p53/p21 undergo apoptotic cell death (57, 60). In vivo, part of the growth inhibitory effect seems to be caused by inhibition of angiogenesis via downregulation of mTOR-regulated expression of VEGF (59, 61). A similar link has also been found in other tumors (62) and may explain some discrepancies between moderate in vitro sensitivity and high in vivo responsiveness of some cell lines (58). Different clinical phase I/II studies with Rapamycin derivatives for the treatment of childhood solid tumors have been started (Tab.2). Initial data demonstrated good tolerability of Everolimus at doses effective for inhibition of mTOR (63). Interestingly, in rhabdomyosarcoma cells inhibition of mTOR signaling triggers a negative feedback loop which activates AKT via IGF1R signaling and acts protective against mTOR signaling (64, 65). Hence, a combined inhibition of mTOR and IGF1R activity resulted in an additive inhibition of cell growth for all sarcoma types discussed here (64) (P.Houghton et al, education book AACR 2008). These experiments
suggest that combining mTOR and IGF1R inhibitors may be superior for childhood sarcoma therapy compared to mTOR inhibitors alone.

**Cell cycle regulators**

Cell cycle progression is the basis for cancer cell proliferation, highlighting regulators of the cell cycle machinery as interesting targets for therapeutic intervention (for review see (66)). A central role play cyclin dependent kinases (CDKs) which are activated by association with cyclins and thereby regulate progression through cell cycle checkpoints. This process is counterbalanced by CDK inhibitors of the INK4a and Cip1/Waf1/Kip1-2 families which are frequent targets of genetic or epigenetic alterations. Consequently, CDK inhibitory drugs have been found to induce apoptosis in different tumor cells (67), thereby validating them as potential targets for cancer therapy.

Many studies have linked abnormal expression of different cell cycle regulators to childhood sarcomas. This includes overexpression of different CDKs and cyclins such as cyclin D1 (68, 69) or CDK2 (70) in Ewing’s sarcoma or cyclin D2 and cyclin D3 in Rhabdomyosarcoma (69). Furthermore, loss or low expression of different CDK inhibitors including p16INK4A, p21WAF1, p27KIP1 and p57KIP2 have been reported in these sarcomas (71-76). Since the frequency of deletions is generally much higher in cell lines compared to primary tumors (73, 77), some of these aberrations might be acquired in vitro. Therefore, careful evaluation of preclinical models is necessary to generate data useful in terms of translation to a clinical study.

Different CDK inhibitors including the Pan-CDK inhibitor flavopiridol, roscovitine, PD0332991 or merolins demonstrated to be effective against the growth of Ewing’s sarcoma, rhabdomyosarcoma and osteosarcoma cell lines in vitro and as xenografts in vivo (78-82). Importantly, flavopiridol has been shown to be only a weak substrate for proteins involved in cell detoxification such as P-glycoprotein (P-gp) (83) and was also effective against drug-resistant osteosarcoma and Ewing’s sarcoma cells expressing P-gp and multidrug resistance-associated protein 1 (78). A phase I trial has been carried out in paediatric sarcomas with flavopiridol assessing clinical safety (Tab.2) (84). On the other hand, in a clinical phase II study using flavopiridol for the treatment of different soft tissue sarcomas in adult patients, no objective responses were seen (85).

**Apoptosis Pathways**

Impaired apoptosis plays a key role in pathogenesis of cancer by allowing cancer cells to escape proapoptotic signals and thereby contributes to poor responses to chemotherapy. Different molecular mechanisms can explain an apoptotic block in tumor cells. Mutation or loss of pro-apoptotic proteins such as p53 or upregulation of anti-apoptotic proteins like Bcl-2 family members are very common in most cancers. Different strategies to promote apoptosis in cancer cells tailored to overcome these different blocking mechanisms are currently under investigation.

One strategy to antagonize the apoptotic block is to restore functional forms of mutated or lost pro-apoptotic proteins. In sarcoma cells restoration of functional p53 by adenoviral transduction has been found in different studies to enhance drug-induced apoptosis (86-88). However, transfer of such approaches to the clinics depends on the development of safe and efficient methods for gene transfer and therefore might not be available for patients in the near future.
Another strategy to induce apoptosis in cancer cells is to inhibit anti-apoptotic proteins. In childhood sarcomas, inhibition of the Bcl-2 family of proteins was investigated. Unfortunately, the small molecule Bcl-2 family protein inhibitor ABT-263, which is otherwise known to display cytotoxicity against different tumor types, demonstrated only limited activity against different sarcoma xenografts as single agent (89).

A third apoptosis promoting strategy is activation of the extrinsic apoptosis pathway through members of the TNF family of death-inducing ligands including TNF, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL). These proteins are able to induce apoptosis in different cancer entities. While TNF and FasL cause severe liver toxicity and are therefore not useful for clinical application, TRAIL does not have this drawback (90, 91) and is the most promising therapeutic agent within this group (90, 92) showing a good therapeutic window between normal and cancer cells, for so far unknown reasons (91). Among the different childhood sarcomas, sensitivity to TRAIL also differs. While many rhabdomyosarcoma and most Ewing’s sarcoma cell lines have been reported as TRAIL sensitive in vitro (93-97), most osteosarcoma cell lines are resistant to TRAIL as single agent (98-100). However, different combination treatments have been shown to sensitize TRAIL-resistant sarcoma cell lines to TRAIL-induced apoptosis by upregulation or activation of specific components of the extrinsic apoptosis pathway. Treatment with interferon-\(\gamma\) was found to upregulate caspase-8 in some TRAIL-resistant Ewing’s sarcoma cell lines thereby sensitizing these cells (101). Also, treatment with different chemotherapeutics appears to overcome TRAIL-resistance. Doxorubicin has been shown in different studies to overcome TRAIL-resistance in rhabdomyosarcoma cell lines (102, 103) and primary cultures (104). Similarly, doxorubicin, etoposide and cisplatin sensitized osteosarcoma cell lines, an effect which was correlated to upregulation of death receptors DR4 and DR5 (100). Casein kinases I and II are involved in regulating procaspase 8 activation upon binding of TRAIL to its receptor in rhabdomyosarcoma cells. Hence, application of inhibitors of casein kinase I or II potentiated the effect of TRAIL in these cells (105). Interestingly, in a TRAIL-resistant osteosarcoma cell line expressing mutant DR4 and wildtype DR5 an anti-DR5 antibody was able to induce apoptosis (106), although the difference between the antibody and TRAIL in this system could not be completely clarified.

These data exemplify the fact that there exist numerous molecular mechanisms responsible for resistance to apoptosis which might differ between and even within different cancer types. The results of single studies on a certain type of cancer might therefore not easily be generalized. In comparison to other cancer entities, childhood sarcomas are not well studied in this area and alternative mechanisms of cell death such as autophagy might also play an important role. Nevertheless, treatment with TRAIL might be a promising approach for the TRAIL-sensitive group of childhood sarcomas.

**Proteasome**

The proteasome is a protein complex involved in degradation of most intracellular proteins. The rationale of targeting the proteasome for cancer treatment is based on findings that transformed cells are more susceptible to proteasome inhibition than their related normal counterparts (for review see (107)). This effect is thought to be caused by abnormal accumulation of intracellular proteins to which cancer cells are more susceptible than normal cells. However, the exact mechanism behind this phenomenon is not clear. Factors that may play a role include faster accumulation of defective proteins
in cancer cells and accumulation of proteins involved in regulation of cell cycle and apoptosis to levels intolerable for tumor cells. The classical proteasome inhibitor Bortezomib has been approved by the FDA as anti-cancer drug for multiple myeloma and mantle cell lymphoma. Different preclinical studies evaluated the effect of proteasome inhibition on childhood sarcoma cells. Bortezomib was found in some studies to be effective against Rhabdomyosarcoma (108) and Ewing’s sarcoma (109) at nanomolar concentrations in vitro and in vivo and the proteasome inhibitor MG132 showed activity against osteosarcoma in vitro (110, 111). On the other hand, in a large study investigating its the effect in a range of different childhood tumors, Bortezomib displayed robust anti-proliferative effects only in vitro, it had only minor effects on xenograft growth in vivo (112). Furthermore, also single agent treatment of recurrent or metastatic sarcomas in adults in a phase II clinical study showed only limited efficacy (113). Nevertheless, Bortezomib might enhance the efficacy of both standard chemotherapeutic agents and targeted therapeutics in a variety of malignant cell types suggesting that it might be of value in combination treatments. Therefore, further investigation of proteasome inhibitors for childhood sarcomas therapy should also focus on combinations with other drugs.

**Hsp90**

Hsp90 is a molecular chaperone involved in correct folding of specific client proteins. Many of these client proteins are associated with cancer cell survival and proliferation, including EGFR, IGF1R, VEGFR, Her2, Raf, Akt, p53 and Hif-1α. Inhibition of hsp90 targets these proteins to proteasomal degradation, thereby inhibiting tumor cell growth. Since tumors frequently overexpress hsp90, it is implicated in cancer progression and has been proposed as target for cancer treatment (for review see (114)). As some of the client proteins are of relevance in childhood sarcomas (see above), inhibition of Hsp90 has been investigated in different studies. The most consistent results were found for rhabdomyosarcoma, for which several studies demonstrated an inhibitory effect upon hsp90 targeting with N-Allylamino-17-Demethoxygeldanamycin (17-AAG) on cell proliferation and migration in vitro (115) as well as on tumor growth in vivo (116). Similarly, for Ewing’s sarcoma one report demonstrated efficacy of 17-AAG against cell proliferation and xenograft growth alone or in combination with IGF1R inhibitors (117), whereas another study using Alvespimycin reported only effects on in vitro growth of Ewing’s sarcoma cell lines but not in xenografts (116). Also only moderate effects were found for osteosarcoma in single treatments, however in this case combination with cisplatin demonstrated to be synergistic (118). Taken together, although the biological basis for the responsiveness of some childhood sarcoma cell lines toward hsp90 inhibition is currently unknown, these data support further preclinical evaluations in some childhood sarcoma entities, especially rhabdomyosarcoma.

**Histone Deacetylases**

Acetylation and deacetylation of histones alters higher order chromatin structure by influencing histone interaction with DNA. Histone acetylation is regulated by the balance of histone acetyltransferases (HAT) and histone deacetylases (HDAC). Highly acetylated nucleosomes are generally associated with transcriptionally active chromatin, whereas hypoacetylated chromatin regions are often found transcriptionally inactive. A tight equilibrium between histone acetylation and deacetylation, controlled by
HATs and HDACs, appears to be essential for normal cell growth. Aberrant deacetylation of chromatin by HAT inactivation or upregulated HDAC activity has been linked to tumorigenesis, due to abnormal transcriptional silencing of tumor suppressors (119). Based on these facts, drugs reversing aberrant histone deacetylation have been evaluated for their effects on cancer cells. HDAC inhibitors (HDACI) have been found to induce numerous anti-proliferative effects including apoptosis, cytostasis and differentiation on different types of cancer cells. Importantly, these effects are much less pronounced in normal cells (120). HDACIs are therefore considered promising agents for cancer therapy and in different clinical trials, objective clinical responses to HDACI treatments have already been detected (121).

Among the different childhood sarcomas, Ewing’s sarcoma seems especially adequate for treatment with HDACIs. Expression of the fusion protein EWS-FLI1, on which Ewing’s sarcoma cells depend, has been shown to be negatively influenced by HDACIs (122). Furthermore, EWS-FLI1 was found to deregulate histone acetylation by both repressing HAT activity and upregulating HDAC activity by, at least in part, interacting with p300 and thereby inhibiting its HAT activity (123). These data suggest that deregulation of histone acetylation might play a specific role in tumorigenesis of Ewing’s sarcoma. Indeed, a series of preclinical studies demonstrated activity of HDACIs against Ewing’s sarcoma in vitro and in vivo (122-125). Anti-proliferative effects were also found for osteosarcoma in vitro and in vivo (125-127), and against rhabdomyosarcoma in vitro (125, 128). Mechanisms of growth inhibition are not fully understood and may differ in each combination of reagent and tumor cells. However, although clinical anti-cancer activity was found in single treatment approaches, it was generally suggested that HDACIs might produce the best clinical effects in combination with other agents (121). In this respect, HDACIs were reported to sensitize osteosarcoma and Ewing’s sarcoma cells to death receptor mediated apoptosis via different mechanisms (124, 129, 130). Furthermore, combination of HDACIs with an inhibitor of DNA methylation (5-aza-2’-deoxycytidine) was found to act synergistic in Ewing’s sarcoma cells (131) and combination with genistein was synergistic in osteosarcoma cells (127). However, in some drug-resistant osteosarcoma or Ewing’s sarcoma cells the cyclic tetrapeptide subfamily of HDACIs only displayed an effect on cell growth in combination with inhibitors of drug efflux systems (132), suggesting that resistance mechanisms to HDACI have to be taken into account.

Angiogenesis

To grow to sizes beyond 1-2 mm³, tumors depend on growth of new blood vessels supplying the tumor with oxygen and nutrients. Angiogenesis therefore is a key factor for progression of all solid cancers (133). Due to the near universality of a potential application as well as due to the low mutation rate of the targeted endothelial cells, strongly decreasing the development of resistances, targeting of the signaling involved in induction of angiogenesis became a major focus of targeted cancer therapy. Among the many different positive and negative regulators of angiogenesis (134), VEGF and its receptors have emerged as key stimulatory molecules for induction of angiogenesis in many tumors (135) and became important targets for tumor therapy. Several approaches for VEGF inhibition exist, including neutralization of the ligands and their receptors by antibodies, and blocking VEGFRs signaling by small molecule inhibitors. Although single agent treatment showed low activity against most cancers studied (with exception of some hypervascular tumors including renal cell carcinoma and hepatocellular...
carcinoma), anti-VEGF treatment provides significant clinical benefit in combination with conventional chemotherapy for many adult tumors.

Different studies explored VEGF-based antiangiogenic therapy in childhood sarcomas. VEGF is a critical regulator of angiogenesis in Ewing’s sarcoma (136, 137) and in osteosarcoma, and VEGF expression levels could be correlated with clinical parameters including metastatic phenotype and overall survival (138, 139). Different experimental approaches to inhibit the VEGF signaling axis including anti-VEGFR or anti-VEGF antibodies, kinase inhibitors targeting the VEGFR or VEGF-trap showed broad inhibitory action in preclinical models of childhood sarcomas (137, 140-142). There is also growing evidence that apart from endothelial cells the sarcoma cell themselves might be responsive to targeting the VEGF axis. In both rhabdomyosarcoma and osteosarcoma simultaneous expression of VEGF and VEGFR was detected (143-145). In rhabdomyosarcoma, an autocrine VEGF signaling loop has been detected activating proliferation of tumor cells. Its inhibition reduces tumor cell proliferation in vitro (144). Similar autocrine loops have already been found in a range of other tumors (146, 147). In such cases, anti-VEGF treatment in vivo might be effective not only by inhibiting growth of endothelial cells in the stroma but also by directly affecting tumor cell proliferation. Indeed, targeting of the VEGFR1 specifically on human cancer but not on mouse endothelial cells with an anti-human VEGFR1 antibody, suppressed growth of VEGFR1-positive breast cancer xenografts in the mouse (148).

Phase I trials demonstrated that therapy with a VEGF-targeting antibody (Avastin; Bevacizumab) is well tolerated in children (149, 150). Further clinical trials with Avastin (phase II) or the small molecule VEGFR inhibitor Cediranib (phase I) are currently ongoing for treatment of childhood sarcoma (Tab. 2). However, compared to adult patients, such antiangiogenic treatment in children may need more attention to long term side effects. Angiogenesis in adults is relatively rare in normal tissues (with exception of wound healing and female reproductive cycle), therefore side effects of anti-VEGF treatments are rather moderate compared with other therapies (151). In contrast, in children anti-angiogenic therapies might exert effects on growing tissues such as growth plates and possibly other tissues (140).

**Cancer specific fusion proteins**

A characteristic of different childhood sarcomas is the presence of specific chromosomal translocations leading to expression of chimaeric fusion proteins, including EWS/FLI1, EWS/ERG and others in Ewing’s sarcoma and PAX3(7)/FKHR in alveolar rhabdomyosarcoma (152). Ewing’s sarcoma and rhabdomyosarcoma cells have been found to be addicted to the activity of these fusion proteins (153, 154), suggesting that blocking this activity could be a possible therapeutic strategy. Moreover, these proteins are strictly cancer cell specific and therefore represent ideal targets for targeted therapy. Until antisense oligonucleotides and siRNA-based approaches will be clinically established, immunotherapeutical approaches may be best suited to target these fusion proteins for therapy. Although established tumors usually escape patient immunosurveillance, the immune system is potentially able to recognize and reject tumors (155) and there is preliminary evidence that different manipulations of the immune system can exert a clinical benefit in selected settings (156-158). Since the
final effector against tumor cells is in most models a CD8+ T cell, the majority of cancer immunotherapy efforts are devoted to stimulating cellular immune responses.

In the case of childhood sarcoma, initial approaches used different vaccination strategies for induction of tumor specific responses. However, in accordance with many other studies showing only limited efficacy of single agent tumor vaccines to induce regression of established tumors (159), vaccination with breakpoint spanning peptides did not alter clinical outcome of childhood sarcoma patients (160). Such approaches might therefore only be considered as a consolidation approach following conventional therapy. In this respect, a pilot study of immunotherapy following chemotherapy in patients with Ewing’s sarcoma and rhabdomyosarcoma could demonstrate an increase in 5-year overall survival following immunotherapy with autologous T cells and dendritic cells pulsed with breakpoint spanning peptides (161). Nevertheless, the current general view is that transfer of ex vivo selected tumor-specific T cells with high avidity and in an already activated state, in combination with depletion of potentially inhibitory host lymphocytes, might be a more effective approach (159). The breakpoint region of PAX3/FKHR has already been demonstrated to be immunogenic and allowed ex vivo generation of a human cytotoxic T lymphocyte line specific for a breakpoint fusion peptide using autologous dendritic cells (162), suggesting that a trial for such an approach might be applicable for therapy of rhabdomyosarcoma.

An alternative to immunological approaches might be pharmacological inhibition of the fusion proteins. Unfortunately, the fusion proteins in case of Ewing’s sarcoma and rhabdomyosarcoma are composed of parts of transcription factors which might not be easily druggable by small molecule inhibitors. However, recent findings demonstrating that PAX3/FKHR activity is regulated by phosphorylation (163) suggest that the activity of such fusion proteins might be inhibited indirectly by inhibition of important posttranslational modifications such as phosphorylation. Since most transcription factors might be regulated by phosphorylation (164), this strategy might also be applicable to other fusion proteins. Small molecule inhibitors for upstream kinase(s) of these key molecules might therefore be used for treatment of the respective tumors.

Perspective

In the last few years many novel drugs against childhood sarcomas have been tested in preclinical studies. Many of these compounds might not be used as stand alone drugs but in combination with a second, specifically targeting or conventional drug, thereby further increasing the number of potential therapeutic approaches. In sharp contrast to the large number of potential novel therapies, the incidence of these tumors is per se rather low. Therefore, the number of childhood sarcoma patients eligible for evaluation of novel, experimental therapies is small and only a small subset of pre-clinically efficient drugs might be tested also in a clinical setting. Thus, the most promising of these should be prioritized based on available preclinical data. However, for such a selection process some important aspects have to be taken into account. First, the variability of cell lines and xenograft models used to model the tumor types of interest in the different studies complicates a comparison of effectiveness and toxicity of the different compounds. Therefore, from a translational point of view improvements in comparability need to be implemented in future studies. A first step towards this direction has been undertaken by the National Cancer Institute (NCI) which has brought together a consortium of investigators using a standardized panel of cell lines and xenograft models from a range of childhood tumors to test the most
promising novel treatment approaches (Pediatric Preclinical Testing Program; PPTP) (165). Another approach might be the use of well characterized compounds as internal standards in tests of novel compounds.

A second important attribute of preclinical models, beside their comparability, is their validity to predict the performance of a compound in the clinics. In case of conventional chemotherapeutic drugs \textit{in vitro} cell line and xenograft models have been found to be predictive in some but not all cases (166, 167). Since conventional chemotherapeutics act on unspecific molecular components such as DNA or cytoskeleton, the efficacy of these compounds might rely to a lesser extent on very accurate representation of the specific tumor characteristics compared to very specific targeting compounds. It is obvious that cell line and xenograft models represent only approximations of the primary tumor and are not able to reproduce all tumor characteristics in detail, particularly not the influence of a specific (micro)environment. However, it is also clear that highly specific compounds require at least expression and regulation of the target of interest to be as similar as possible in model systems and primary tumors of interest. Unfortunately, many of the cell lines used as model systems \textit{in vitro} and as xenografts \textit{in vivo} have been in culture for many years and might have acquired changes during this time both at the genomic and the transcriptomic level. Such effects might vary from lab to lab, depending on culture conditions, time in culture and number of passages. Additional problems include cross-contamination with other cell lines (168) or misclassification (169). It is therefore reasonable to assume that at least some of the cell line models used might not represent the characteristics of the primary tumor cells in all details required and that such differences impair validity of the model systems. For example, the cannabinoid receptor 1 (CB1), which is a potential target for therapy in a range of tumor types (170), is expressed very consistently and at high levels in translocation-positive alveolar rhabdomyosarcoma (tpARMS) biopsy samples (171, 172). However, expression of this protein varies substantially among the commonly used tpARMS cell lines (unpublished observation in datasets from authors (171) as well as in dataset from the PPTP set of cell lines (169)), suggesting that only tpARMS cell lines strongly positive for CB1 represent potential preclinical models to test compounds targeting CB1. This case exemplifies the importance of a careful molecular characterization of preclinical models for testing of very specific compounds.

A potential alternative might be the usage of primary patient material as xenografts in the mouse for preclinical treatment trials. For that, small pieces of primary tumor material sampled by biopsy or surgery would first be transplanted into a set of mice for amplification. The amplified material then could be stored in liquid nitrogen or used for secondary transfers into a second set of mice used for treatment studies. However, also this approach requires a representative number of patients for a given tumor type.

Nevertheless, only a small fraction of novel compounds has been tested in the clinics so far. Many additional drugs including vast numbers of combinations are ready for clinical testing and will eventually help to improve outcome of childhood cancer. It is therefore reasonable to assume that, in addition to improved survival rates achieved with conventional chemotherapeutics in the last 30 years, a further increase in survival rates for many childhood malignancies lies ahead of us with hopefully simultaneous decrease of side effects.
### Table 1. Therapeutic targets in rhabdomyosarcoma, Ewing’s sarcoma and osteosarcoma

<table>
<thead>
<tr>
<th>Groups of therapeutic targets</th>
<th>Tumor type</th>
<th>Tumor type</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhabdomyosarcoma</td>
<td>Ewing’s sarcoma</td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td></td>
<td>PDGFR [44,47-48]</td>
<td>c-Kit [36,43,44,47-48]</td>
<td>IFN-α, IFN-β [185]</td>
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<tr>
<td></td>
<td>CD99 [184]</td>
<td>PDGFR [34,41,47-48]</td>
<td>c-Met [35,58]</td>
</tr>
<tr>
<td></td>
<td>Smad4 [181]</td>
<td>IFN-α, IFN-β [185]</td>
<td>Mdm2, p21 [197]</td>
</tr>
<tr>
<td></td>
<td>Relnoic acid [182]</td>
<td>mTor [55,58]</td>
<td>Targeta discussed in the text are written in bold.</td>
</tr>
<tr>
<td><strong>Intracellular signaling molecules</strong></td>
<td>mTor [57-59]</td>
<td>mTor [58]</td>
<td>p53 [86,88]</td>
</tr>
<tr>
<td></td>
<td>PDK-1/AKT [175]</td>
<td>Src [178]</td>
<td>Bcl-2 [89]</td>
</tr>
<tr>
<td></td>
<td>Src [179]</td>
<td>Lyn [186]</td>
<td>TRAIL [93,102-105]</td>
</tr>
<tr>
<td></td>
<td>Smad4 [181]</td>
<td>Src [175,198]</td>
<td>Mdm2, p21 [197]</td>
</tr>
<tr>
<td><strong>Cell cycle</strong></td>
<td>CDK4/CDK6 [82]</td>
<td>CDKs [78-81]</td>
<td>CDKs [78]</td>
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<td></td>
<td>CDKs [78-81]</td>
<td>CDKs [78]</td>
<td>CDKs [78]</td>
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<tr>
<td><strong>Apoptosis</strong></td>
<td>p53 [86,88]</td>
<td>p53 [87]</td>
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<tr>
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<td>Bcl-2 [89]</td>
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<td>Bcl-2 [89]</td>
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<td>Survivin [183]</td>
<td>TRAIL [94-97,101]</td>
<td>TRAIL [96-100,106]</td>
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<td><strong>Proteasome</strong></td>
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<td>[109,112]</td>
<td>[110-112]</td>
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<td><strong>Histone Deacetylases</strong></td>
<td>[116-117]</td>
<td>[116]</td>
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<td>[116]</td>
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<td>[116]</td>
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<td><strong>Angiogenesis</strong></td>
<td>VEGF [144]</td>
<td>VEGF [137]</td>
<td>VEGF [142]</td>
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<td><strong>Cancer specific fusion proteins</strong></td>
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<td>EWS/FLI1 [160-161]</td>
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<td>Patient age (y)</td>
<td>Drug</td>
<td>Target</td>
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<td>EGFR</td>
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<td>RMS/Ewing</td>
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<td>Cediranib</td>
<td>VEGFR</td>
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*a* source used: clinicaltrials.gov
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