Review

Molecular profiling of childhood cancer: Biomarkers and novel therapies

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ABSTRACT

Background: Technological advances including high-throughput sequencing have identified numerous tumor-specific genetic changes in pediatric and adolescent cancers that can be exploited as targets for novel therapies.
Scope of review: This review provides a detailed overview of recent advances in the application of target-specific therapies for childhood cancers, either as single agents or in combination with other therapies. The review summarizes preclinical evidence on which clinical trials are based, early phase clinical trial results, and the incorporation of predictive biomarkers into clinical practice, according to cancer type.
Major conclusions: There is growing evidence that molecularly targeted therapies can valuably add to the arsenal available for treating childhood cancers, particularly when used in combination with other therapies. Nonetheless the introduction of molecularly targeted agents into practice remains challenging, due to the use of unselected populations in some clinical trials, inadequate methods to evaluate efficacy, and the need for improved preclinical models to both evaluate dosing and safety of combination therapies.
General significance: The increasing recognition of the heterogeneity of molecular causes of cancer favors the continued development of molecularly targeted agents, and their transfer to pediatric and adolescent populations.

Contents

1. Introduction

Childhood cancers are rare, representing less than 1% of new cancer diagnoses, however, they are still the leading cause of disease-related death in children in industrialized countries such as the US [1]. Children's malignancies are generally treated with a combination of cytotoxic chemotherapy, radiation, surgery, and occasionally bone marrow transplant, which have markedly improved the overall 5-year survival rate over the past decades from 50 to 60% for cases diagnosed during the 1970s, to over 80% today [2].

The greatest contribution to better outcomes comes from advances in our understanding of the genetics of cancer [3–5], and the discovery of molecular biomarkers and incorporation of novel targeted agents has led to improved outcomes for cancer patients, and decreases in both short- and long-term toxicities [6]. Some biomarkers are routinely used for diagnostics (such as Ki-67 staining as proliferation index) [7], risk-stratification (*MYCN* amplification in neuroblastoma) [8] and monitoring (S100-beta in melanoma) [9]. Others are used to direct the use of targeted therapy, such as the fusion tyrosine–kinase protein BCR–ABL for the use of imatinib in chronic myeloid leukemia (CML) and Philadelphia chromosome positive (Ph+) acute lymphoblastic leukemia (ALL) [10,11] or *anaplastic lymphoma kinase* (*ALK*) mutations (especially EML4–ALK rearranged cancers) for the use of crizotinib, the ALK inhibitor approved for treatment of non-small cell lung cancer (NSCLC) [12, 13]. In summary, the genetic heterogeneity now recognized to underpin pediatric and adolescent cancers may be exploited by agents targeting these specific molecular/genetic lesions. A principal challenge is to now further expand the number of molecular targets investigated, and integrate conventional practices with the best target-matched therapies. The aim of this review is to give an overview of recent advances in the application of "druggable" biomarkers, and the use of target-specific therapies for childhood cancers.

2. Relevant biomarkers in childhood malignancies and novel therapies

The role of germ-line mutations in explaining susceptibility to childhood cancer and genetic predisposition to familial malignancies is well established [14], whereas the roles of somatic, acquired mutations have become a central subject of study in more recent years. Cancer biomarkers are now increasingly used to characterize human tumors and to explain the heterogeneity that exists between different tumors. Such heterogeneity is reflected by the wide range of sub-classifications (diagnostic markers) and risk-stratifications (prognostic markers) existing for many cancer types, as well as by the increasing number of molecules able to forecast the response of patients to personalized therapies (predictive markers) [15].

The following sections represent an overview of relevant markers and matched therapies in pediatric tumors. A full list of the molecular inhibitors reviewed is available in Table 1, while a schematic summary of cancer pathway inhibition is shown in Fig. 1. Table 2 reports the frequency of mutation/overexpression of some biomarkers relevant in childhood cancer.

2.1. Leukemias

Leukemias are the most common type of childhood cancers, accounting for one-third of all cancers diagnosed in children. Among them, ALL accounts for approximately three-quarters of all childhood leukemia diagnoses, while acute myelogenous leukemia (AML) is 5 times less frequent. Chronic myeloid leukemia (CML) and chronic lymphocytic leukemia are very rare in the pediatric population [16]. Several chromosomal abnormalities have been identified in pediatric leukemias, including translocations such as t(9;22) BCR–ABL, t(12;21) TEL–AML1, t(8;21) AML1–ETO and t(15;17) PML–RARA [17].

Table 1
List of targets in childhood cancer discussed in this review with matching relevant targeted therapies.

Activity	Target	Agent	Activity	Target	Agent
Monoclonal antibodies	ERBB2	Pertuzumab	Tyrosine kinase inhibitors	ALK	Crizotinib
		Trastuzumab			NVP-TAE684
	EGFR	Cetuximab		BCR–ABL	Imatinib
		Nimotuzumab			Dasatinib
	VEGFR	Bevacizumab		c-KIT	Dasatinib
	IGF-1R	Cixutumumab			Sorafenib
		Figitumumab			Pazopanib
		Teprotumumab		MET	Foretinib
		Robatumumab			Cabozantinib
		Alpha IR-3			SU11274
		AMG 479			K252a
		R1507		EGFR/ERBB2	Erlotinib
		EM164			Gefitinib
		IMC-A12			Lapatinib
		SCH 717454			Afatinib
Ser/Thr kinase inhibitors	PI3K	XL147			Canertinib
		NVP-BEZ235			AEE788
		NVP-BKM120		JAK/STAT3	AZD1480
		GDC-0941			Ruxolitinib
		IC87114			AG-490
		PI103		PDGFRA	Imatinib
	AKT	Perifosine			Dasatinib
		SF1126			Sorafenib
		GSK690693			Sunitinib
		MK-2206			Pazopanib
	Aurora kinase	Alisertib			Foretinib
		Tozasertib		PDGFRB	Dasatinib
		AT9283			Sorafenib
	BRAF	Sorafenib			Sunitinib
		Vemurafenib			Pazopanib
		Selumetinib			CP-673,451
	mTOR	Rapamycin		VEGFR	Sunitinib
		Temsirolimus			Dasatinib
		PI103			Sorafenib
		PP242			Vandetanib
Hedgehog pathway inhibitors	SHH	Vismodegib			Cediranib
		SEN450			Pazopanib
		PF-5274857		PLK1	BI2536
	SMO	LDE225			BI6727
γ-Secretase inhibitors	Notch	RO4929097			LFM-A13
Histone deacetylase inhibitors	HDAC	Vorinostat			GW843682X
		Panobinostat		SYK	Fostamatinib
		Entinostat			C-61
		Valproic acid		TrkB	Lestaurtinib
		Romidepsin			AZ64
		Trichostatin A			AZ623
Topoisomerase inhibitors	TOP1	Topotecan		FLT3	Lestaurtinib
		Irinotecan			Linifanib
		Namitecan			Sorafenib
		Gimatecan			Midostaurin
		EZN-2208			AC220
		Genz-644282			SU11657
	TOP2	Etoposide		IGF-1R	BMS-754807
		(R+)XK469			NVP-AEW541
MAP kinase pathway inhibitors	MAP2K1–2	AZD6244	Chaperone inhibitors	Hsp90	Geldanamycin
		Sorafenib			Tanespimycin
	KRAS	Tipifarnib			Ganetespib
	MEK	Selumetinib			AT13387
		U0126			NVP-AUY922
	ERK	U0126			Alvespimycin
PARP inhibitors	PARP	Olaparib			SNX-2112
		Veliparib			17-AAG
		ABT-888			17DMAP-GA
		AG-014700			17AEP-GA

2.1.1. Tyrosine kinase inhibitors

The tyrosine kinase inhibitor imatinib (Gleevec) is a rare example of an approved first-line targeted treatment in adults and children, which is indicated for the treatment of Philadelphia chromosome-positive CML. Imatinib used in combination with cytotoxic chemotherapy was also demonstrated to dramatically improve the 3-year event-free survival of children and adolescents with Ph + ALL [11]. Although

BCR–ABL kinase domain mutations have been reported in Ph + ALL patients relapsing after imatinib, this may occur less frequently than in adults treated with imatinib [18].

Following the success of imatinib, a number of other tyrosine kinase inhibitors have emerged as potential therapies in pediatric leukemias. Dasatinib is an oral multi-BCR–ABL and Src family inhibitor (also active against c-KIT, platelet derived growth factor alpha/beta (PDGFRA/B)
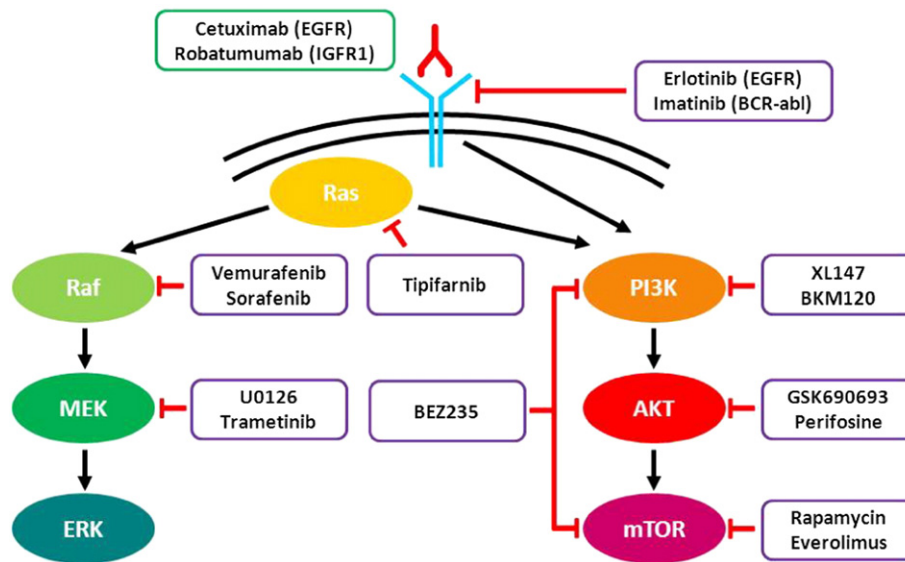
Fig. 1. Schematic representation of two main pathways aberrantly activated in pediatric tumors and corresponding targeted therapies. Molecular inhibitors and monoclonal antibodies are circled in purple and green, respectively.

and vascular endothelial growth factor (VEGF)/VEGFR, but not epidermal growth factor receptor (EGFR)/ERBB2) that was recently granted approval for adult Ph-CML [19]. Dasatinib showed encouraging results in a phase I trial in pediatric CML patients, with 6/8 evaluable patients achieving partial or complete cytogenetic responses [20], and is currently in phase II study (NIH trial NCT01460160). Sorafenib is a small molecule that inhibits several tyrosine (VEGFR and PDGFR) and serine/threonine kinases (MAP kinases), and has been approved for the treatment of renal cell and hepatocellular carcinoma [21]. In a phase 1 study of single-agent sorafenib, two acute myeloid leukemia (AML) patients with *FLT3* internal tandem duplication achieved dramatic reductions in bone marrow blasts, and proceeded to bone marrow transplantation [22]. Sorafenib is currently being evaluated for incorporation into standard chemotherapy regimens in a Children's Oncology Group multi-center study [22]. Other tyrosine kinase inhibitors directed against FLT3 such as AC220 and midostaurin (PKC412) are in phase I or I/II trials for relapsed or refractory

Table 2
Frequency (%) of positivity by immunohistochemistry or mutation of relevant biomarkers in childhood cancers. References are listed in Supplemental document (S1).

Target	Childhood cancer	IHC (%)	Reference	Mutation (%)	Reference
ALK	Neuroblastoma	23.5% (stage 1–2) 77% (stage 3–4)	[I]	10.4%	[II]
	Rhabdomyosarcoma	53% (ARMS) 23% (ERMS)	[III]	16% (deletion)	[IV]
	Ewing sarcoma	69%	[V]	16%	[V]
	Glioblastoma	82% (in cell lines)	[VI]	–	
BRAF	Low-grade glioma	–		15% (V600E)	[VII]
cMET	Glioblastoma	29% (pediatric and adult cohort)	[VIII]	–	
	Rhabdomyosarcoma	35%	[IX]	–	
	Ewing sarcoma	86%	[V]	9%	[V]
EGFR	Glioma	80%	[X]	17% (deletion)	[XI]
	Neuroblastoma	95%	[XII]	infrequent	[XII]
	Osteosarcoma	59%	[XIII]	–	
	Wilms tumor	38%	[XIV]	–	
	Rhabdomyosarcoma	47%	[XV]	13%	[XVI]
ERBB2	Medulloblastoma/ATRT	57.5%	[XVII]	–	
	Rhabdomyosarcoma	33%	[XV]	–	
	Wilms tumor	39.1%	[XVIII]	–	
	Osteosarcoma	18.8%	[XIX]	–	
MGMT	Glioblastoma	11%	[XX]	–	
PDGFRA	DIPG	36%	[XXI]	5%	[XXII]
	Glioblastoma	18%	[XXIII]	14%	[XXII]
	Neuroblastoma	100%	[XXIV]	–	
	Ependymoma	29.2%	[XXV]	–	
	Rhabdomyosarcoma	>40%	[XXVI]	–	
PDGFRB	Rhabdomyosarcoma	>40%	[XXVI]	–	
	Glioma	78.9%	[X]	0%	[XI]
	Ewing sarcoma	79%	[XXVII]	0%	[XXVIII]
	Astrocytoma	31%	[XXIX]	–	
	Neuroblastoma	72.5%	[XXX]	–	
PIK3CA	Neuroblastoma	92%	[XII]	Infrequent	[XII]
PTEN	Osteosarcoma	–		20% (deletion)	[XIII]
	Neuroblastoma	100%	[XII]	39% (deletion)	[XII]
SPARC	Osteosarcoma	96.3%	[XXXI]	–	
VEGF	ALL	21%	[XXXII]	–	
	AML	38%	[XXIV]	–	

– indicates not studied.

pediatric leukemia (NCT01411267 and NCT00866281, respectively), while SU11657 is in preclinical development [23]. Overall, primary pediatric AML samples with *FLT3* or *KIT* mutations were significantly more sensitive to SU11657 than wild-type AML samples [23].

In 2011, the JAK/STAT inhibitor ruxolitinib was approved for the treatment of intermediate or high-risk myelofibrosis [24]. However, recent results demonstrated its *in vivo* activity in Ph-ALL xenograft models, when administered in combination with the mammalian target of rapamycin (mTOR) inhibitor rapamycin [25]. Fostamatinib is an experimental drug targeting spleen tyrosine kinase (SYK), and is in clinical trial for rheumatoid arthritis (NCT01242514), autoimmune thrombocytopenia (NCT00706342) and lymphoma (NCT00798096). Dietary fostamatinib was reported to reduce the burden of leukemic blasts in mice injected intrafemorally with primary B-ALL samples [26]. Recently, a nanoscale liposomal formulation of another selective SYK inhibitor C61 exhibited potent anti-leukemic activity against patient-derived ALL xenografts *in vivo* [27]. The same authors described the *in vivo* chemosensitizing and apoptosis-promoting activity of LFM-A13, a dual-function inhibitor of Bruton's tyrosine kinase and polo-like kinase 1 (PLK1), against pediatric ALL [28].

2.1.2. Serine/threonine kinase inhibitors

A second class of molecular inhibitors that has been employed in the treatment of pediatric leukemias is one directed against serine/threonine kinases such as MAP kinase, phosphatidylinositol 3′-kinase (PI3K) and Aurora kinase. The MAP kinase pathway is often activated in pediatric malignancies [29] and other inhibitors have been developed to target this specifically. Among them, the farnesyl transferase inhibitor tipifarnib was tested in a phase I clinical trial of pediatric patients with advanced, heavily pretreated leukemia, where no objective responses were observed in 23 evaluable patients [30]. GDC-0941 and IC87114, two inhibitors of PI3K directed against the alpha and delta subunits, respectively, reduced granulocyte macrophage-colony stimulating factor (GM-CSF) hypersensitivity in a mouse model of juvenile myelomonocytic leukemia [31]. However the pan-PI3K inhibitor XL147 (SAR245408) significantly improved event-free survival in only 2/7 pediatric leukemia xenografts, and produced no objective responses [32].

Aurora kinase inhibitors have produced more encouraging results in childhood leukemia in the preclinical setting. The pan-Aurora kinase inhibitor AT9283 produced target kinase inhibition [33] and inhibited the growth and survival of pediatric leukemia cell lines [34], although a significant correlation between AT9283 and target protein expression could not be established [34]. Similarly, the Aurora kinase A (AURKA) inhibitor alisertib (MLN8237) produced complete responses in 6/6 ALL xenograft models, although there was again no correlation between *AURKA* copy number or expression [35]. Objective responses were subsequently demonstrated in 3 additional ALL xenografts with low *AURKA* expression [36]. AT9283 reached phase I/II clinical trial for pediatric relapsed or refractory acute leukemia (NCT01431664), which is still in progress.

2.1.3. Histone deacetylase (HDAC) and heat shock protein 90 (Hsp90) inhibitors

Small molecules directed against other targets, including histone deacetylases (HDAC) and heat shock protein 90 (Hsp90), have been evaluated in the preclinical setting. Vorinostat (SAHA), an HDAC inhibitor approved for the treatment of refractory cutaneous T-cell lymphoma, produced no objective responses in 8 ALL xenografts [37]. No objective responses were similarly obtained in 6 pediatric leukemia patients in a phase I clinical trial [38]. Vorinostat is now being evaluated in combination with the demethylating agent decitabine and chemotherapy in relapsed ALL patients (NCT01483690). Hsp90 inhibitors reduce the stability of associated client proteins, and may thereby exert anti-oncogenic effects. Tanespimycin (17-AAG), a member of the geldanamycin family of antibiotics, rendered a pre-B ALL patient-derived cell line significantly more sensitive to imatinib, and reduced the IC50

values for a number of other chemotherapeutic agents [39]. However ganetespib, another Hsp90 inhibitor, showed no significant activity as a single agent towards 4 JAK-mutated ALL xenografts [40].

2.1.4. DNA topoisomerase inhibitors

Among the DNA topoisomerase 1 (TOP1) inhibitors, topotecan is one of the best characterized and one of the first to enter clinical trials. Topotecan is a derivative of the natural alkaloid camptothecin, and is currently approved for the treatment of adult ovarian and cervical cancer, as well as small cell lung cancer [41]. Topotecan has also been successful in clinical trials against relapsed pediatric ALL [42] and AML [43] in combination with a variety of chemotherapy agents. Irinotecan is another TOP1 inhibitor that has been approved for the treatment of colorectal cancer [44]. A panel of 6 patient-derived pediatric ALL cell lines was shown to be more sensitive to irinotecan than topotecan, based on measured IC50 values [45]. Recently, a combination of irinotecan, the TOP2 inhibitor etoposide and cytarabine entered a phase I clinical trial for pediatric refractory or relapsed ALL and AML (NCT01239485). Etoposide in combination with clofarabine and cyclophosphamide has previously been tested in phase I and II studies of refractory or relapsed ALL or AML, resulting in complete remissions (with or without platelet count recovery) in 16/25 (64%) patients in the phase I trial [46], and in 11/25 (44%) patients in the phase II trial [47]. This treatment regimen therefore produced encouraging response rates, and sustained remission in refractory or relapsed patients [47].

2.1.5. Proteasome inhibitors

The proteosome inhibitor bortezomib exerts anti-tumor activity by affecting a number of physiological processes, including preventing degradation of pro-apoptotic factors. Bortezomib treatment as a single agent showed encouraging preclinical results, significantly increasing event-free survival in 4/7 ALL xenograft models [48], but produced no objective clinical responses in a phase I clinical trial of 12 refractory or relapsed ALL or AML patients [49]. Because of bortezomib's distinctive mechanism of action, there was interest in combining this with standard anti-leukemia agents, and synergistic or additive interactions were demonstrated with range of chemotherapeutic agents in T-ALL and pre-B ALL cell lines *in vitro* [50]. In a phase I trial, bortezomib added to standard therapy for relapsed pediatric ALL induced complete responses in 6/9 patients [51], with 16/22 (73%) relapsed ALL patients achieving complete remission in the subsequent phase II trial, which met the pre-defined criteria for early trial closure [52]. The study authors recommended evaluating the addition of bortezomib in randomized studies on childhood pre-B ALL protocols [52].

2.1.6. Immunotherapy

Understanding the role of the immune system in tumor surveillance has led to novel approaches for cancer treatment, such as unconjugated and conjugated monoclonal antibodies (mAbs), and chimeric antigen receptors (CARs) [53]. Hematopoietic differentiation antigens represent a major tumor antigen class for the treatment of leukemia and lymphoma, which may be relevant to both adult and pediatric diseases. In a phase II window trial, 36/87 (41.4%) pediatric B-cell non-Hodgkin's lymphoma patients responded to the anti-CD20 mAb rituximab as a single agent [54], producing at least objective responses at an index site, and no other signs of progression. Combining rituximab with chemotherapy produced a 60% response rate in 12/20 patients with relapsed and refractory pediatric B-cell non-Hodgkin's lymphoma or mature B-cell leukemia [55]. In contrast, the anti-CD52 mAb alemtuzumab induced complete remission in only 1/13 (7.6%) relapsed ALL patients, and was not recommended for further use as a single agent [56]. However, in combination with busulfan and fludarabine, alemtuzumab improved the engraftment rate in pediatric patients undergoing hematopoietic stem cell transplantation [57]. As unconjugated antibodies have been approved to treat a number of adult malignancies [58],

there may be scope for further translation of these reagents to pediatric oncology.

Payload-delivery mAbs conjugated with cytotoxic agents, including antibody–immunotoxin and antibody–drug conjugates, and radio-immunoconjugates, could all improve the potency of unconjugated mAbs, particularly in immunocompromised patients [59,60]. Gemtuzumab ozogamicin is an anti-CD33 mAb conjugated to a derivative of the cytotoxic antibiotic calicheamicin, and has been comprehensively studied in adults. In a phase I study as a single agent, 8/29 (28%) pediatric AML patients with relapsed or refractory disease achieved complete remission, with or without platelet count recovery [61]. Higher proportions of AML patients (40% and 55%) achieved complete remissions in a larger subsequent dose de-escalation study where gemtuzumab ozogamicin was combined with standard chemotherapy [62]. In relapsed and refractory pediatric ALL patients, the anti-CD22 immunotoxin moxetumomab pasudotox produced complete responses in 4/17 (24%) patients [63], and the anti-CD22 calicheamicin conjugate inotuzumab ozogamicin produced complete responses in 3/5 relapsed pre-B ALL patients, all of which expressed CD22 [64]. There is further scope for testing other conjugated mAbs in pediatric leukemia, as the anti-CD19 mAb–drug conjugate SAR3419 delayed progression in 4/4 pre B-ALL and 3/3 infant mixed lineage leukemia xenograft models as a single agent [65].

T-cell engaging antibodies represent new therapeutic approaches for treating leukemia and lymphoma, with the bi-specific T-cell engaging (or BiTE®) antibody blinatumomab now being tested in pediatric leukemia patients, following encouraging results in adults [66]. Blinatumomab is a fusion of two single chain antibodies to CD19 and CD3, and thereby brings malignant B cells into close proximity with CD3-positive T cells [66]. Blinatumomab treatment of 3 pediatric patients with refractory pre-B ALL rapidly induced complete remissions, which was maintained in one case for over 23 months [67]. Preliminary data from the first phase I clinical trial of blinatumomab indicate complete remissions in 11/34 (32%) relapsed/refractory pre-B ALL patients [68], although the associated side effect profile, which can include cytokine release syndrome, requires careful management [66]. The phase II NCT01471782 clinical trial to investigate the pharmacokinetics, pharmacodynamics and safety of escalating doses of blinatumomab in pediatric and adolescent patients with relapsed/refractory pre-B ALL is ongoing, and estimated to complete in 2016.

Chimeric antigen receptors (CARs) represent an exciting new form of targeted therapy that confers the targeting specificity of mAbs to cytotoxic T cells, the first of which were reported some 25 years ago [69]. Second and third generations of CARs have been developed by the addition of one or two co-stimulatory molecules [70], with the goal of improving the *in vivo* persistence of the transferred T cells [71]. B-cell malignancies are particularly amenable to CAR therapy, as mAbs are available to target B-cell surface antigens, and temporary loss of mature B-cells is an acceptable on-target, off-tumor effect [70]. A recently reported trial of CD19-targeting CAR T-cells showed the feasibility and safety of this approach [72], while two pediatric patients with relapsed B-ALL treated with CD19-specific CAR T-cells were reported to achieve remission [73]. Numerous trials of T cells engineered with CD19-directed CARs are currently undergoing in ALL patients (NCT00608270, NCT01593696, see also [70] for other examples).

2.1.7. Leukemia summary

Over the past 50 years, outcomes for pediatric leukemia patients have dramatically improved by varying the schedules and doses of established cytotoxic drugs, albeit at the cost of severe toxicity [53,74]. However, there are encouraging signs that this situation is changing, largely through the exploitation of targets such as protein kinases known from adult oncology [74]. The presence of tissue restricted antigens on hematopoietic cells also provides numerous options for targeting B-cell malignancies in children, with acceptable on-target side effects, and the accessibility of leukemic cells within the circulation makes leukemias particularly suited to antibody-based treatment strategies [53,70]. Evolution of resistant disease may be avoided by the use of less selective kinase inhibitors, and of targeted agents that can access sanctuary sites such as the CNS [18].

2.2. Neuroblastoma

Neuroblastoma originates from primordial neural crest cells and is the most common extra-cranial malignant childhood tumor. It is a heterogeneous malignancy classified into low-, intermediate-, or high-risk categories based upon clinical and biological features correlating with prognosis. Low- and intermediate-risk patients have an overall survival exceeding 90%, while newly diagnosed high-risk neuroblastoma and patients whose disease recurs show survival rates of 50% and less than 10%, respectively [75].

2.2.1. MYCN

MYCN is a member of the MYC transcription factor family, with *MYCN* gene amplification being strongly associated with poor outcome in neuroblastoma. *MYCN* amplification is present in approximately 20% of neuroblastomas and nearly half of high-risk cases [8]. As such, MYCN represents a prime therapeutic target in neuroblastoma, and small molecules have been developed to target MYC family members as well as MYC DNA binding functions, transcription and protein–protein interactions. 10058-F4, a novel drug that inhibits MYC–MAX interactions, produced modest survival benefit in a MYCN-dependent neuroblastoma mouse model (*TH-MYCN*) [76]. Recently, the bromodomain and extra terminal (BET) family adaptor proteins were shown to localize to *MYC* promoters [77], with BET proteins binding acetylated histones associated with open chromatin and transcriptional activation [78]. Use of the small molecule BET inhibitor JQ1 induced *MYC* down-regulation [77, 79] and improved survival of mice bearing *MYCN*-amplified BE(2)-C neuroblastoma or primary human neuroblastoma xenografts, and of *TH-MYCN* transgenic mice [80]. A JQ1 derivative has now entered phase I/II clinical trial for the treatment of *MYCN*-amplified cancers, including neuroblastoma (NCT01587703). Myc was also shown to cause DNA damage through induction of replication stress, with the resulting replication arrest being partially overcome by Chk1 inhibition [81]. *CHK1* transcript levels were subsequently found to be significantly higher in *MYCN*-amplified *versus* non-amplified neuroblastomas, and neuroblastoma cell lines were more sensitive to CHK1 inhibition than controls [82]. CCT244747, a highly selective, orally active CHK1 inhibitor, showed single-agent activity in the *TH-MYCN* transgenic mouse neuroblastoma model, as evidenced by significantly reduced tumor weights after 7 days of treatment [83]. Future clinical studies will now determine whether agents targeting MYCN activity or downstream cellular processes could improve outcomes in *MYCN*-amplified neuroblastoma patients.

2.2.2. Differentiating agents and immunotherapy

In children with high-risk neuroblastoma, treatment with the differentiating agent 13-cis-retinoic acid reduces the risk of recurrence after high-dose chemotherapy and stem cell transplant [84]. Fenretinide, a synthetic retinoid, was shown to induce apoptosis in neuroblastoma cells *in vitro* [85] and, as a consequence, entered phase I and II clinical trials for the treatment of refractory or recurrent neuroblastoma. However, the best reported response was stable disease, potentially due to the scarce bioavailability of this molecule [86,87]. Novel fenretinide formulations with improved bioavailability have been evaluated in pre-clinical studies [88] and are currently in phase I clinical trials in recurrent or refractory neuroblastoma patients (NCT00295919).

In the last decade, the addition of disialoganglioside (GD2) immunotherapy to retinoid maintenance therapy for high-risk neuroblastoma has constituted a major advance, where typically 50% of this group of patients die from their disease [89]. Disialoganglioside GD2 is a surface glycolipid antigen normally found on neurons, peripheral pain fibers

and skin melanocytes that is also abundantly expressed on neuroblastoma cells. Clinical use of the murine anti-GD2 mAb 3F8 was frustrated by severe side effects, and the development of human anti-mouse antibodies [89]. A subsequent phase III trial of a chimeric human–murine anti-GD2 mAb ch14.18 in high-risk neuroblastoma patients who had previously received intensive multi-model therapy, demonstrated significantly improved event-free and overall survival in the immunotherapy group, and met the criteria for early stopping [90]. To improve the side-effect profile associated with ch14.18, a humanized anti-GD2 mAb was developed with a single point mutation (K322A) to reduce complement-dependent lysis [91]. In summary, anti-GD2 antibodies are likely to provide a major shift in the treatment of minimal residual disease in high-risk neuroblastoma patients, once these are commercially available [90].

2.2.3. PI3K/AKT/mTOR inhibitors

The PI3K/AKT/mTOR pathway is one of the most potent pro-survival signaling cascades [92] and its aberrant activation is a common event in high-risk neuroblastoma patients [93]. Perifosine is an alkylphospholipid inhibitor of the PI3K/AKT pathway, which when used as a single agent, significantly improved the survival of mice bearing human neuroblastoma xenografts (AS, NGP, BE2, and KCNR) [94], and showed synergistic effects in combination with etoposide [95]. Results from these studies paved the way for a phase I clinical trial of perifosine in refractory or recurrent pediatric solid tumors (NCT00776867). Preliminary results indicate encouraging perifosine activity in neuroblastoma patients, in the absence of significant toxicity [96].

Rapamycin (sirolimus) is a natural macrolide antibiotic with immunosuppressive and antiproliferative properties. Its anti-cancer activity derives from its ability to bind the cytosolic protein FKBP12, inhibit the mTOR complex 1, and as a consequence, inhibit VEGF and angiogenesis [97]. Temsirolimus, a derivate of rapamycin, is an approved drug for the treatment of renal cell carcinoma, and has been tested as a single agent in phase I and II trials for pediatric solid tumors [98,99]. After 12 weeks of temsirolimus treatment, 6/19 (32%) neuroblastoma patients showed stable disease, supporting further exploration of temsirolimus in combination with other therapies [99].

Recent in vivo studies reported the anti-proliferative ability of four novel drugs active against the PI3K/AKT/mTOR pathway, namely SF1126, XL147, PI103 and NVP-BEZ235 [32,100–104]. The peptidic pro-drug inhibitor SF1126 was demonstrated to interfere with the pAKT–MDM2 axis, and to markedly inhibit the growth of neuroblastoma xenografts [100]. SF1126 is also in phase II trial for adult solid tumors (NCT00907205). XL147, which is in phase I and II trials for adult cancers (NCT01587040), significantly improved event-free survival in 6/6 neuroblastoma xenografts, but produced no objective responses [32]. PI103 is a dual PI3K/mTOR inhibitor that was demonstrated to cooperate with TNF-related apoptosis-inducing ligand (TRAIL) to synergistically induce apoptosis in neuroblastoma cells (SH-EP, LAN-5 and CHP-212) [101] and to delay growth of MYCN-amplified SK-N-BE(2) neuroblastoma xenografts [102]. Another dual PI3K/mTOR inhibitor NVP-BEZ235 showed increased sensitivity towards MYCN-amplified neuroblastoma cells in vitro, and reduced tumor angiogenesis through destabilization of MYCN protein levels in vivo, thereby significantly improving the survival of tumor-bearing TH-MYCN mice [103]. Synergistic interactions were subsequently demonstrated between NVP-BEZ235 and the lysosomotropic agent chloroquine in vitro, triggering lysosome-mediated apoptosis through lysosome enlargement and the generation of reactive oxygen species (NVP-BEZ235) and lysosomal membrane permeabilization (chloroquine) [104]. NVP-BEZ235 is yet to enter clinical trial for neuroblastoma, with phase I trials involving adult patients currently ongoing. As chloroquine is in clinical use, this could be feasibly be combined with NVP-BEZ235 for neuroblastoma therapy [104], following preclinical testing in vivo.

2.2.4. Tyrosine kinase and HDAC inhibitors

Some 8% of all neuroblastoma cases and over 12% of high-risk patients harbor alterations in the tyrosine kinase ALK sequence [105,106], and 23.5% of stage 1–2 and 77% of stage 3–4 patients show high-level ALK immunoreactivity [107]. Crizotinib, an ALK and MET inhibitor, entered a phase I/II clinical trial as single agent for pediatric refractory solid tumors, producing several complete responses and prolonged stable disease in both ALK-positive and unknown ALK status neuroblastoma patients [108]. A combination phase I trial of crizotinib and conventional chemotherapy is currently ongoing (NCT01606878). Among other ALK inhibitors, LDK378 demonstrated greater potency over crizotinib, and anti-tumor activity in crizotinib-resistant adult tumors [109]. A pediatric phase I clinical trial with LDK378 as a single agent is also underway (NCT01742286).

Several drugs synthesized to specifically inhibit other tyrosine kinases have also been tested in clinical trials for neuroblastoma. The ERBB2/EGFR inhibitors erlotinib and gefitinib have completed phase I clinical trials in combination with temozolomide [110] or topotecan and cyclophosphamide [111], respectively, with both trials reporting prolonged stable disease in some neuroblastoma patients. A phase II trial of erlotinib in combination with pertuzumab (a mAb against ERBB2) was terminated due to high toxicities (NCT00947167), while a phase II trial of gefitinib in combination with irinotecan reported partial responses in only 5/19 (26%) high-risk neuroblastoma patients [112]. Canertinib (CI-1033), a pan-ERBB inhibitor, reduced the growth and size of SK-N-SH neuroblastoma xenografts to a greater extent than erlotinib [113].

Neurotrophin receptors of the tyrosine kinase (Trk) family play important roles in neuroblastoma etiology, with TrkB expression being associated with unfavorable outcomes and MYCN amplification. A phase I trial of lestaurtinib (a FLT3 and TrkB inhibitor) supported its safe use as a single agent in children with refractory neuroblastoma, warranting further evaluation [114]. Similarly, Iyer and colleagues reported significant improvements in the survival of mice bearing SH-SY5Y xenografts when lestaurtinib was combined with topotecan plus cyclophosphamide [115]. Two other TrkB inhibitors AZ64 and AZ623 have also been tested in neuroblastoma xenografts. AZ64 enhanced the response to irinotecan [116], while AZ623 inhibited tumor growth, and prolonged the inhibition of tumor regrowth when combined with topotecan [117]. The same authors found that the VEGFR inhibitor, vandetanib (ZD6474), in combination with 13-cis-retinoic acid, reduced tumor vascularity and induced apoptosis in neuroblastoma xenografts [118]. Vandetanib also reduced the growth of SK-N-SH neuroblastoma xenografts by 85% as a single agent [119]. VEGFR is also the target of cediranib (AZD2171), an investigational drug in phase III clinical trial for a number of adult malignancies including glioblastoma (NCT00777153), NSCLC (NCT00245154) and colorectal cancer (NCT00384176). Two separate in vivo studies demonstrated that cediranib delayed the growth of neuroblastoma xenografts as a single agent [120], but reduced the event-free survival of NB-EBc1 neuroblastoma xenografts associated with cyclophosphamide treatment alone when used in combination [121].

Polo-like 1 (PLK1) is another kinase recently identified as a potential therapeutic target in neuroblastoma [122]. BI2536 and BI6727, two PLK1 inhibitors in clinical trial for adult malignancies (NCT00526149 and NCT01022853, respectively), were shown to be cytotoxic towards neuroblastoma cells (NB12, NB88R2, and NB122R), and BI2536 significantly inhibited the growth of neuroblastoma xenografts as a single agent, and in combination with irinotecan [122]. The PLK1 inhibitor GW843682X also blocked the proliferation of a panel of pediatric cancer cell lines in vitro, including IMR-5 and SMS-KCN MYCN-amplified neuroblastoma cells [123].

PDGFR inhibitors are a family of novel drugs that have been widely tested in neuroblastoma cell lines and mouse models. For example, dasatinib showed cytostatic and anti-migratory activity in vitro [124] but only partial tumor growth inhibition in vivo [125]. More encouraging results have come from in vivo testing of pazopanib, sorafenib and

sunitinib. Pazopanib, approved for renal cell carcinoma and soft tissue sarcomas in adults [126], showed high anti-tumor and anti-angiogenic activity when administered as metronomic chemotherapy in combination with topotecan in SK-N-BE and SH-SY5Y neuroblastoma xenografts [127]. Sorafenib inhibited the growth of neuroblastoma xenografts by targeting both cell proliferation and angiogenesis [128]. Sunitinib, a PDGFR (and VEGFR) inhibitor approved for renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor [129], showed anti-tumor activity both in early stage tumor formation and in progressive metastatic neuroblastoma in SK-N-BE and SH-SY5Y xenografts [130].

Other tyrosine kinase inhibitors with activity against neuroblastoma are those directed against the JAK/STAT axis and Aurora kinase. AZD1480, a JAK/STAT3 inhibitor [131], was recently demonstrated to show anti-tumor activity, and to decrease expression of STAT3 and downstream targets in KCNR and SH-SY5Y neuroblastoma xenografts [132]. Alisertib, an AURKA inhibitor, disrupted mitotic spindle assembly and the mitotic checkpoint in neuroblastoma cell lines (NB-1643, NB-EBC1, CHLA-90 and CHLA-136), and induced maintained complete responses in a panel of 7 neuroblastoma xenograft models [35]. These results provided the preclinical rationale for alisertib to enter phase II clinical trials, either as a single agent (NCT01154816, for neuroblastoma and sarcomas), or in combination with temozolomide and irinotecan (NCT01601535, for neuroblastoma). Thus far, the only reported result for neuroblastoma patients indicated stable disease as the best response to alisertib single-agent therapy [133]. In IMR-32 neuroblastoma cells, alisertib showed additive cytotoxic effects in combination with the HDAC inhibitor vorinostat [134]. This supported the inclusion of vorinostat in clinical trials for refractory or recurrent pediatric solid tumors including neuroblastoma. Either as a single agent [38] or combined with the proteasome inhibitor bortezomib [135], vorinostat was demonstrated to be safe, and produced stable disease or complete response in some neuroblastoma patients. Other HDAC inhibitors including romidepsin and entinostat have been tested in KCNR neuroblastoma xenografts, resulting in tumor growth inhibition and apoptosis [136, 137].

2.2.5. DNA topoisomerase inhibitors

Topoisomerase poisons such as irinotecan have been successfully used to treat neuroblastoma patients [112] and proved active in phase I and II trials in combination with temozolomide [138,139]. The combination of irinotecan with temozolomide is considered standard therapy in relapsed high-risk neuroblastoma patients [140]. Recent studies have reported the NF-κB pathway and the B-MYB/MYCN axis as targets of topotecan in neuroblastoma cells [141,142] and clinical trials have shown encouraging responses to topotecan in neuroblastoma patients. For example, topotecan and etoposide administered together induced partial or complete remissions in 17/36 (47%) patients [143], with the addition of cyclophosphamide inducing partial or complete responses in 19/31 (61%) relapsed and 8/11 (72%) newly diagnosed neuroblastoma patients [144]. In a more recent trial, topotecan in combination with cyclophosphamide produced significantly better progression-free survival than topotecan as a single regimen [145]. Etoposide has also been employed in numerous trials for refractory and relapsed neuroblastoma, with encouraging results [146], while preclinical studies have examined other TOP1 and TOP2 inhibitors including namitecan, gimatecan and the investigational drugs EZN-2208, and (R+)XK469 [147–151]. Namitecan demonstrated antitumor activity in SK-N-AS neuroblastoma xenografts, inducing apoptosis and tumor shrinkage, and a significant enhancement of platinum agent activity [147], whereas gimatecan produced significant regression of SK-N-DZ neuroblastoma xenografts in vivo [148]. This latter result supported the superior activity of gimatecan versus irinotecan and topotecan, in terms of producing growth arrest in 5 human neuroblastoma cell lines (SK-NDZ, BE(2)M17, LAN-1, RNGA, SK-N-BE(2)c) [149]. Similarly, EZN-2208, a PEGylated version of irinotecan, was superior to standard irinotecan in inducing tumor regression and preventing tumor relapse in a panel of

neuroblastoma mouse xenografts [150], while (R+)XK469 induced growth inhibition of neuroblastoma xenografts and produced disease stabilization in a case-report of a neuroblastoma patient [151].

2.2.6. Polyamine inhibitors

Polyamines are multifunctional polycations that are indispensible for cancer cell survival, influencing a range of biological processes including DNA synthesis and stability, replication, transcription and translation, ribosome biogenesis, and protein phosphorylation [152]. Ornithine decarboxylase 1 (ODC1), the first and rate limiting enzyme in polyamine biosynthesis is a well characterized Myc and MYCN target gene, and high ODC1 levels were significantly associated with reduced event-free survival in neuroblastoma patients [153]. Difluoromethylornithine (DFMO), a suicide inhibitor of ODC1, demonstrated inhibition of tumor growth in TH-MYCN mice and synergized with chemotherapy in treating established tumors [154]. Clinical trials employing DFMO as a single agent or as part of combination treatment for neuroblastoma are ongoing (NCT01059071; NANT 2012-01). In addition to DFMO, a number of other polyamine depletion agents have been identified [152], although their clinical potential remains to be determined.

2.2.7. Hsp90 inhibitors

A final category of targeted therapies tested on neuroblastoma models are those directed against Hsp90, including geldanamycin, SNX-2112 and ganetespib. Geldanamycin treatment depleted key anti-apoptotic proteins in SK-N-SH neuroblastoma cells [154], and both geldanamycin and SNX-2112 demonstrated marked single-agent activity, and synergistic [154] or greater than additive activity with cisplatin [155] in BE(2)-C [154] or SK-N-SH and IMR-32 cells, respectively [155]. However, ganetespib demonstrated limited activity as a single agent against NB-1643 neuroblastoma xenografts in vivo [40].

2.2.8. Neuroblastoma summary

Neuroblastoma accounts for 8–10% of all childhood cancers, but is responsible for more than 15% of childhood cancer deaths [156]. In order to improve this latter statistic, many targeted agents are being tested in neuroblastoma patients, and their clinical trial outcomes are eagerly awaited. To date, ALK inhibitors show promise in the patient subset whose tumors bear ALK alterations, and substantial efforts are being made to target MYCN and its downstream targets. The use of anti-GD2 antibodies in combination with immunomodulators represents a major advance in the treatment of high-risk neuroblastoma, and the control of minimal residual disease [89,90].

2.3. CNS tumors

Pediatric brain tumors represent the second most common neoplastic disease of childhood. The main categories of childhood CNS tumors are gliomas, including low- and high-grade astrocytomas and ependymomas, and embryonal tumors, such as medulloblastoma, CNS primitive neuroectodermal tumors and atypical teratoid/rhabdoid tumors (AT/RTs) [157]. Of all childhood cancers, malignant brain tumors are the most significant in terms of mortality and morbidity, and would greatly benefit from more targeted therapeutic approaches with less long-term toxicities [158].

2.3.1. Temozolomide

Temozolomide is an oral DNA damaging (alkylating) agent approved for the treatment of astrocytoma and melanoma in adult patients [159]. Testing for epigenetic silencing of O-6-methylguanine-DNA methyltransferase (MGMT, encoding a DNA repair protein) predicts which adult patients will receive the greatest benefit from temozolomide treatment [160]. Randomized trials in adult glioblastoma multiforme patients have shown a small but significant survival advantage when temozolomide was added to radiation therapy [161]. Similar studies have not been performed in the pediatric setting, but given the poor prognosis

of high grade gliomas, and the results demonstrated in adult patients, the use of temozolomide has become standard of care.

Temozolomide has shown anti-tumor activity in pediatric low grade gliomas, where single-agent temozolomide induced disease stabilization in children with progressive low-grade glioma [162]. However, no significant activity was demonstrated for temozolomide in the most aggressive glioma of childhood, diffuse intrinsic pontine glioma (DIPG) [163]. Moreover, temozolomide in combination with the MGMT inhibitor O-6-benzylguanine (O6-BG), although safe [164], did not achieve the target response rate for activity in pediatric patients with recurrent or progressive gliomas [165]. To allow dose escalation of temozolomide while minimizing bone marrow toxicity, a phase I trial (NCT01269424) has been initiated for adult glioblastoma multiforme patients. Here, O6-BG will be combined with infusion of autologous hematopoietic stem cells transduced with a lentiviral vector encoding a mutant form of MGMT P140K resistant to O6-BG, which may reduce the bone marrow side-effects of temozolomide. Favorable results using a similar approach have already been reported for 3 glioblastoma multiforme patients, who all exceeded the median expected survival [166]. A similar phase I trial enrolling patients aged 1–21 years with recurrent or progressive brain tumors, or DIPG, is now underway in Australia (ACTRN12612000535875). These trials will provide valuable indications as to whether gene therapy approaches can improve outcomes in CNS tumor patients treated with temozolomide.

2.3.2. Poly(ADP-ribose) polymerase (PARP) inhibitors

Inhibitors of poly(ADP-ribose) polymerase (PARP) prevent the repair of single-stranded DNA breaks, and may produce synthetic lethality in cells with DNA repair defects, or other forms of DNA damage. Temozolomide in combination with the PARP inhibitor AG-014700 proved active in medulloblastoma xenografts, with sustained tumor regression being achieved in the D425Med MGMT-negative model [167]. The PARP inhibitor olaparib also sensitized pediatric high-grade glioma, medulloblastoma and ependymoma cell lines to radiotherapy *in vitro*, albeit at micromolar concentrations [168]. Clinical trials involving pediatric brain tumor patients are now combining radiotherapy and/or temozolomide with the PARP inhibitors veliparib (NCT01514201) or ABT-888 (NCT00946335).

2.3.3. Sonic hedgehog (SHH) inhibitors

The secreted SHH ligand is a morphogenic protein that signals through the smoothened (SMO) receptor, inducing proliferation of neuronal precursor cells. As medulloblastoma has been classified into Wnt, sonic hedgehog (SHH), Group 3 and Group 4 subgroups, according to molecular features [169], SHH antagonists represent exciting novel prospects for treating a sub-set of medulloblastoma patients. LDE-225 (erismodegib) is a potent and selective SMO antagonist that also led to acquired resistance during treatment of medulloblastoma xenografts [170]. However, LDE-225 used in combination with the PI3K inhibitor NVP-BKM120, or the dual PI3K–mTOR inhibitor NVP-BEZ235, markedly delayed the development of resistant disease, and may provide an efficient way to treat refractory and relapsed medulloblastoma patients [170]. Vismodegib (GDC-0449) is another SHH inhibitor that has induced tumor regression in mouse medulloblastomas and xenografts [171–174], and dramatic anti-tumor effects in a relapsed medulloblastoma adult patient [175]. More recent preclinical *in vivo* studies highlighted potential roles for novel SHH inhibitors such as SEN450 and PF-5274857 in treating gliomas and medulloblastoma [176,177]. SEN450 showed anti-proliferative activity as a single agent, and further reduced tumor size after temozolomide pretreatment in an adult glioblastoma xenograft model [176]. PF-5274857 effectively penetrated the blood–brain barrier in mice bearing medulloblastoma xenografts, and produced better survival rates than vehicle-treated mice [177]. Vismodegib is now in phase I (NCT00822458) and phase II (NCT00939484, NCT01239316) clinical trials for medulloblastoma treatment, and in phase II trial for DIPG (NCT01774253). Temozolomide and LDE-225

are currently in phase III trial for the treatment of relapsed medulloblastoma (NCT01708174).

2.3.4. PI3K/mTOR inhibitors

Several trials have been reported or are ongoing for the use of rapamycin and other derivatives for childhood brain tumors. Rapamycin in combination with erlotinib induced prolonged disease stabilization in 2/19 (10.5%) low-grade glioma patients [178], while everolimus as a single agent produced tumor volume reductions in 65–79% tuberose sclerosis patients with subependymal giant cell astrocytoma [179]. After obtaining encouraging results in a pre-clinical medulloblastoma mouse model, temsirolimus entered phase I and II trials for pediatric solid tumors [98,99], where it was well tolerated as a monotherapy, and induced disease stabilization in 7/17 (41%) high-grade glioma patients [99]. Other molecules evaluated for the treatment of pediatric CNS tumors and active against the PI3K/mTOR pathway include perifosine, XL147 (SAR245408) and torkinib (PP242). Perifosine was tested in a PDGFRA-overexpressing mouse model of high-grade brainstem glioma as either a monotherapy, or in combination with radiation, but did not significantly improve survival relative to either no treatment, or radiation alone [180]. Similarly, XL147 was tested against 8 CNS tumor xenografts but demonstrated at best modest activity [32].

2.3.5. MAP kinase pathway inhibitors

The Ras/Raf/MEK/ERK axis is abnormally activated in pediatric cancers by mutations in *BRAF* and *KRAS* genes. Multiple inhibitors of this pathway, including selumetinib and tipifarnib, have been tested in the context of pediatric CNS tumors since the *BRAF V600E* mutation was recently identified as a driving factor in subsets of pediatric low grade gliomas [181]. Although selumetinib showed limited *in vitro* and *in vivo* activity as a single agent in a panel of 44 pediatric xenograft models [182], it was highly active against a juvenile pilocytic astrocytoma xenograft (BT-40) harboring the *BRAF V600E* mutation, causing complete regressions [182]. Selumetinib is now in phase I clinical trial for recurrent or refractory low-grade gliomas (NCT01089101). Tipifarnib, a Ras farnesyltransferase inhibitor, was evaluated in a phase I trial for refractory sarcomas and brain tumors or neurofibromatosis, where it was well tolerated in children but showed limited activity [183]. In a more recent trial of newly diagnosed DIPG patients, tipifarnib administered with irradiation offered no clinical advantage above irradiation alone [184], although Ras status (presence of mutations, or aberrant activity in tumors) was not reported.

2.3.6. Monoclonal antibody therapy

Cetuximab is a mAb directed against EGFR, and approved for treatment of *KRAS* wild-type metastatic colorectal cancer, and squamous cell carcinoma of the head and neck. The combination of cetuximab and irinotecan proved safe in children and active against CNS tumors [185], leading to ongoing phase II evaluation in high-grade astrocytomas and DIPG, in combination with radiotherapy (NCT01012609). Recently, a case report described intra-arterial cerebral infusion of cetuximab and bevacizumab (a monoclonal VEGF antibody) for multiply recurrent pediatric ependymoma, producing stable residual disease [186]. Another EGFR mAb used to treat pediatric brain tumors is nimotuzumab, which could be safely administered over long periods, and was associated with improved survival in 23 high-grade glioma patients [187]. The potential use of nimotuzumab in radioimmunotherapy (when conjugated to (177)Lu) was also recently tested in A431 epithelial carcinoma xenografts, where locoregional application demonstrated antitumor activity [188]. Less encouragingly, discordant responses in adult *versus* pediatric high-grade glioma patients have been reported for bevacizumab. Adults with recurrent high-grade glioma treated with bevacizumab and irinotecan achieved radiographic responses in 20/32 (63%) cases [189], whereas only 2/12 (16.7%) pediatric patients achieved a partial response, and 5/12 (45.5%) experienced diffuse invasive recurrence [190]. Similarly, a phase II study of the combination of

bevacizumab and irinotecan in pediatric cases of recurrent ependymoma showed disease stabilization in 2/13 (15.4%) patients [191]. On the other hand, the same combined therapy produced objective responses with clinical improvements in 7/9 pediatric low-grade gliomas [192].

2.3.7. Tyrosine kinase inhibitors

A number of small molecule EGFR inhibitors have been tested in children with CNS tumors. Erlotinib in combination with rapamycin produced prolonged disease stabilization in 2/19 (10.5%) low-grade glioma patients [178]. Erlotinib was also tested in combination with radiotherapy in two independent phase I trials for high-grade gliomas and astrocytomas. The first study reported no objective radiologic responses to therapy, but prolonged disease stabilization in 16/23 (70%) patients [193], while the second reported that 4/18 (22%) patients achieved partial responses [194]. A phase I trial of erlotinib in combination with temozolomide produced stable disease or partial response in 2/13 (15%) patients with CNS tumors [110]. Gefitinib is an EGFR/ERBB2 inhibitor that has been proposed for the treatment of malignant gliomas, brain stem tumors and medulloblastoma following preclinical results using mouse xenografts [195]. Phase I trials have indicated that gefitinib is well tolerated in children as a single agent, and in combination with irinotecan or radiotherapy [196]. A more recent phase II clinical trial of gefitinib and radiotherapy in brainstem gliomas indicated partial responses in 6/43 (14%) patients, and long-term progression-free survival in this usually fatal disease [197]. Another dual EGFR/ERBB2 inhibitor lapatinib proved safe and induced prolonged stable disease in 13/59 (22%) CNS tumor patients [198]. Through a cytotoxicity screening approach, lapatinib also demonstrated antitumor activity against AT/RT cell lines *in vitro* and xenografts *in vivo* [199]. Another EGFR/ERBB2 inhibitor, AEE788, produced prolonged survival and tumor growth suppression in xenograft models of ependymoma [200] and medulloblastoma [201], respectively.

Other tyrosine kinases such as VEGFR, PDGFR and MET are potential therapeutic targets for pediatric CNS malignancies, due to their aberrant activation in pediatric brain tumors [202,203]. However, the VEGFR inhibitors sunitinib and vandetanib gave disappointing results in childhood brain cancer clinical trials. In particular, sunitinib showed cardiotoxicity in children with previous exposure to anthracyclines or cardiac radiation [204], whereas vandetanib in combination with radiotherapy induced longer progression-free survival in only 3/35 (8.5%) DIPG patients [205]. Recently, a combination of VEGFR and PDGFR inhibitors (vandetanib and dasatinib) did not improve the poor prognosis of newly diagnosed DIPG patients, who showed 2-year survival rates of less than 10% [206]. The VEGFR inhibitor cediranib (AZD2171) produced tumor growth delay in 1/3 medulloblastoma xenografts when administered as a monotherapy [120], but showed additive effects with rapamycin and vincristine towards D645 xenografts [121]. A phase I clinical trial in children with recurrent or progressive CNS tumors is nonetheless underway (NCT00326664). Foretinib, a multi-kinase inhibitor of MET and PDGFRA, reduced tumor growth and metastasis in xenograft models of disseminated medulloblastoma [207], whereas the polo-like kinase (PLK1) inhibitor BI2536 suppressed cell growth and enhanced radiation sensitivity in 5 medulloblastoma cell lines (ONS-76, Daoy, D283, D425 and D458) [208].

2.3.8. DNA topoisomerase inhibitors

A number of DNA topoisomerase inhibitors have shown activity in the treatment of childhood CNS malignancies. Topotecan in particular produced complete or partial responses in 10/36 (28%) newly diagnosed high-risk medulloblastoma patients [209], and consolidated remission of poor prognosis pediatric CNS tumors when combined with carboplatin [210]. Two phase II trials of topotecan as part of combination therapy in high-risk pediatric brain tumors are currently underway (NCT01342237, NCT01756989). Similarly, irinotecan as a monotherapy proved active in children with relapsed CNS tumors, producing partial

or complete responses in subsets of glioma [211] and medulloblastoma patients [212], and objective responses in children with recurrent medulloblastoma when combined with bevacizumab [213]. Etoposide in combination therapy has also successfully completed phase I [214] and II trials [215] for pediatric CNS tumors, with 22/28 (79%) patients showing complete or partial responses to carboplatin, etoposide, and high-dose methotrexate [215]. As described for neuroblastoma, investigational topoisomerase poisons such as namitecan and Genz-644282 have produced some encouraging results in the preclinical setting. In the PFSK primitive neuroectodermal tumor xenograft model, namitecan treatment induced complete responses and prevented tumor relapse, but only slowed tumor growth in medulloblastoma Daoy xenografts [147], whereas Genz-644282 produced partial regression of D645 glioblastoma xenografts, but progressive disease in mice bearing BT-45 medulloblastoma xenografts [216].

2.3.9. HDAC and Aurora kinase inhibitors

As in leukemia and neuroblastoma, HDAC inhibitors have also demonstrated activity against pediatric CNS tumors. The anticonvulsant valproic acid was tested in a phase I study in patients with refractory solid or CNS tumors, and gave partial or minor responses in single patients with glioblastoma multiforme and brainstem glioma, respectively [217]. This led to further phase II studies in combination with radiotherapy and bevacizumab (NCT00879437). Entinostat decreased proliferation and potentiated the effects of ionizing radiation in AT/RT tumor cell lines [218], while both panobinostat and vorinostat showed tumor growth inhibition and radio-sensitization in the HD-MB03 medulloblastoma mouse model [219]. Phase I trials of vorinostat as either a single agent [38], or in combination with temozolomide [220] or bortezomib [135] produced cases of disease stabilization, but no objective responses. Vorinostat was also tested in combination with the AURKA inhibitor alisertib, showing additive cytotoxicity in Daoy medulloblastoma cells [134]. The importance of Aurora kinases as potential therapeutic targets for childhood brain malignancies is highlighted by *AURKB* being highly and consistently overexpressed in the majority of high-grade glioma (8/11) and DIPG (6/9) patients [221]. Alisertib alone also produced objective responses in medulloblastoma and glioblastoma xenografts, including a maintained complete response (BT-46 cells) and a partial response (D456 cells) [35].

2.3.10. Hsp90 inhibitors

Hsp90 inhibitors represent candidate agents for the treatment of pediatric brain tumors. In the preclinical setting, geldanamycin and its analog 17AEP-GA showed potent inhibition of adult LN18, LN229 and T98G glioblastoma cell proliferation, survival, migration and invasion *in vitro* [222]. NVP-AUY922, a synthetic Hsp90 inhibitor, also strongly inhibited the proliferation of pediatric SF188 and KNS42 glioblastoma cell lines [223]. Recently, another Hsp90 inhibitor ganetespib was tested on the *BRAF*-mutant BD-40 astrocytoma xenograft model, but showed no antitumor activity [40]. In a phase I trial for 17AAG in patients with relapsed/refractory solid tumors, the best response was stable disease in 5/17 (29%) patients, including 1/4 ependymoma patients [224].

2.3.11. CNS tumor summary

In addition to poor survival rates, CNS tumors are associated with significant long-term neurologic effects in survivors that result from radio- and chemotherapy. Targeted agents with an improved therapeutic window could spare normal cells in the developing brain, and even agents delaying radiotherapy treatment could significantly reduce adverse long-term effects [225]. Although anti-tumor activity in preclinical models has not always been confirmed in early phase clinical trials, these results may not reflect those that could be obtained at initial diagnosis. In summary, the significant recent advances in our understanding of the molecular changes driving pediatric CNS tumors require more time to fulfill their promise in the clinic.

2.4. Sarcomas

Pediatric sarcomas are a heterogeneous group of neoplasms characterized by specific chromosomal translocations (Ewing sarcoma, synovial sarcoma and ARMS) [226], or complex karyotypes and gains of whole chromosomes (osteosarcoma and ERMS) [227,228].

2.4.1. Ewing sarcoma

Ewing sarcoma is a rare tumor of bone or soft tissues, and is characterized by the fusion of the *EWS* gene to those encoding the transcription factors FLI1 or ERG in 85% and 10% of cases, respectively [229]. Multiple targets have been demonstrated for EWS/FLI1, including IGF binding protein 3 (IGFBP-3) [230], which increases IGF-1 bioavailability, and the Aurora kinases [231].

2.4.1.1. Insulin-like growth factor type 1 receptor (IGF-1R) inhibitors. As EWS translocations activate the IGF pathway, inhibition of insulin-like growth factor type 1 receptor (IGF-1R) function may target Ewing sarcoma [232]. However, despite encouraging pre-clinical data [233,234], clinical trial results for IGF-1R targeted agents have been disappointing [232]. A phase I trial of AMG 479, a mAb targeting IGF-1R induced partial or complete responses in Ewing sarcoma patients [235], but more recent phase II trials using similar agents such as R1507 [236], figitumumab [237] and cixutumumab [238] reported only partial responses in patients with recurrent or refractory disease. A limitation of these trials however was that patients were not selected on the basis of IGF-1R expression. Several trials are currently evaluating the activity of novel IGF-1R antibodies such as teprotumumab as a monotherapy (NCT00642941), or in combination with mTOR inhibitors (NCT00880282, NCT00927966).

2.4.1.2. Aurora kinase inhibitors. Although the AURKA inhibitor alisertib induced complete responses in 1/5 Ewing sarcoma xenograft models (SK-NEP-1) [35], single-agent alisertib only stabilized disease in a phase I trial that included Ewing sarcoma patients [133]. A phase II trial has recently been completed, although results are not yet available (NCT01154816). The pan-Aurora kinase inhibitor tozasertib was shown to selectively and potently reduce the viability of Ewing sarcoma cell lines, and demonstrated synergies with etoposide and doxorubicin [239]. Dual inhibition of AURKA and AURKB in Ewing sarcoma may therefore be more potent than AURKA inhibition alone [239].

2.4.1.3. Other tyrosine kinase inhibitors. Surface expression of c-KIT and PDGFRB in Ewing sarcoma cell lines predicted that Ewing sarcoma may respond to imatinib, which produced concentration-dependent inhibition of cell growth *in vitro* in multiple Ewing sarcoma cell lines, and of tumor growth *in vivo* [240]. However, single-agent imatinib produced disappointing results in phase II clinical trials, with only 1/24 (4%) [241] or 1/5 [242] Ewing sarcoma/PNET patients achieving partial responses. This occurred despite the immunohistochemical detection of KIT or PDGFRA being a requirement for patient selection in one trial [242]. The multi-targeted kinase inhibitor pazopanib induced tumor growth delay as the best response in 5 Ewing sarcoma xenografts [243], while cediranib showed modest anti-tumor activity as a single agent, but additive effects against EW5 cells in combination with rapamycin [121]. Dasatinib inhibited proliferation without inducing apoptosis in 3 Ewing sarcoma cell lines [124], whereas gefitinib and vandetanib inhibited cell line proliferation at only micromolar concentrations [244], indicating that Ewing sarcomas may not depend on EGFR and VEGFR pathways for survival [244]. More recently, *MET* and *ALK* mutations were demonstrated in Ewing sarcomas, and *in vitro* treatment of five patient-derived cell lines with MET and/or ALK inhibitors produced IC50 values within the low micromolar range [245]. Further research will be required to determine whether MET/ALK

inhibitors could be used to treat a subset of Ewing sarcoma patients [245].

2.4.1.4. HDAC and Hsp90 inhibitors. The HDAC inhibitor entinostat (MS-27-275) showed *in vitro* cytotoxicity in Ewing sarcoma cell lines, and marked regression of established TC71 Ewing sarcoma xenografts [137]. More recently, vorinostat induced significant tumor growth delay in 3/5 Ewing xenograft models, but no objective responses [246], and no objective responses in combination with bortezomib in a subsequent phase I clinical trial [135]. Similarly, both the Hsp90 inhibitors ganetespib [40] and AT13387 [247] produced little anti-tumor activity and no objective responses in 2 and 5 Ewing sarcoma xenograft models, respectively [40,247].

2.4.1.5. DNA topoisomerase inhibitors. Encouraging results have come from the testing of TOP1 and TOP2 inhibitors, where irinotecan and etoposide were both well-tolerated and active in high-risk or refractory/relapsed Ewing sarcoma patients in combination regimes [248,249]. Topotecan in combination with cyclophosphamide induced stable disease or partial responses in 4/13 (31%) and 3/13 (23%) relapsed Ewing sarcoma patients, respectively [250], while objective responses were attained in 7/14 (50%) patients with recurrent or progressive disease when vincristine was added to the protocol [251]. *In vivo* studies also revealed that Genz-644283 [216] and namitecan [252] produced maintained complete responses in SK-NEP-1 [216] and TC-71 [252] Ewing sarcoma xenografts at well-tolerated doses.

2.4.1.6. Ewing sarcoma summary. A number of challenges emerge from a consideration of targeted therapy use in Ewing sarcoma. While targets and pathways downstream of oncogenic fusion proteins provide a wealth of therapeutic targets in Ewing sarcoma [226], the clinical benefits from targeted therapy have been limited, and not always been explained by target expression [242,253]. Limitations to drug diffusion and penetration in bone may reduce the activity of high molecular weight agents such as mAbs [253], and the relevance of preclinical results obtained in subcutaneous xenograft models. Intra-tumor heterogeneity also makes a strong case for the use of multiple targeted therapies in combination regimes [253].

2.4.2. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a group of malignancies that resemble developing skeletal muscle, and is the most common soft-tissue sarcoma in children and adolescents [228]. RMS is divided into two major histologic subgroups, ERMS and ARMS, which account for 60% and 30% of cases, respectively [228]. ERMS is characterized by chromosome gains (chromosomes 2, 8, 12, 13) and loss of heterozygosity for chromosome 11p15.5 [228]. In contrast, ARMS is characterized by fusions of the transcription factors PAX3 and PAX7 with FOXO1 in 70% and 10% ARMS patients, respectively [254]. Great efforts have been made to directly alter the expression or function of these fusion proteins, through the use of antisense oligonucleotides [255], transcriptional repressors [256] and vaccines against the fusion region [257]. As for neuroblastoma (Section 2.2.2), differentiation therapy has been attempted in RMS cell lines *in vitro*. Treatment of RD and Rh30 cells with retinoic acid induced stereo-specific growth inhibition, but not differentiation [258], whereas 12-O-tetradecanoylphorbol-13-acetate treatment of RD cells induced myogenic differentiation [259]. More recently, fenretinide was shown to reduce PAX3–FOXO1 fusion protein levels in ARMS cell lines, and to significantly delay *in vivo* tumor growth in ARMS Rh4 xenografts, compared to untreated controls [260].

2.4.2.1. Tyrosine kinase and PI3K/mTOR inhibitors. PAX3/7-FOXO1 downstream targets include MET, PDGFR and IGF/IGFR, so therapies targeting these molecules or pathways may be useful for RMS treatment. Transgenic expression of the MET ligand hepatocyte growth factor in *Ink4a/Arf*−/−mice induced RMS with extremely high penetrance and short

latency [261], while shRNA silencing of MET inhibited RD18 proliferation and invasiveness *in vitro*, and induced regression of RD18 xenografts [262]. High levels of phosphorylated MET were detected in 14/24 (58%) RMS patient samples and CW9019 and Rh30 ARMS cell lines, where treatment with the MET inhibitor SU11274 significantly inhibited cell proliferation and migration *in vitro* [263].

Following the demonstration that *PDGFRA* is a transcriptional target of PAX3–FOXO1 [264], PDGFRA inhibition using RNA interference or imatinib reduced mouse ARMS cell line proliferation *in vitro*, and imatinib or PDGFRA neutralizing antibody treatment led to disease regression or stabilization *in vivo* [265]. The selective PDGFR inhibitor CP-673,451 also reduced tumor volume, Ki67 staining and PDGFR phosphorylation of RUCH2 xenografts *in vivo* [266]. The multi-target tyrosine kinase inhibitor pazopanib delayed growth of Rh30 xenografts and enhanced survival, both as a single agent and in combination with topotecan [127]. However when used at a lower dose, single-agent pazopanib significantly improved event-free survival in only 2/5 ARMS/ERMS xenograft models, and produced no objective responses [243].

Targeting IGF/IGFR as downstream effectors of PAX3/7–FOXO1 has been tested using neutralizing antibodies and small molecule inhibitors. IGF-1R antibodies such as alpha IR-3 [267], IMC-A12 [268], and the IGF-1R inhibitor BMS-754807 in combination with cetuximab [233] commonly inhibited growth of RMS xenografts. In RMS, IGFR survival signaling is primarily maintained through the AKT pathway, and activation of PI3K/mTOR signaling [269]. Concomitant use of IGFR antibodies and rapamycin resulted in additive inhibition of Rh30 and RD cell growth and survival [270], and the PI3K inhibitor XL147 induced tumor growth delay as a single agent, and significantly improved event-free survival in 6/6 RMS xenografts [32]. However a phase II trial of single-agent temsirolimus induced prolonged stable disease in 1/16 (6%) RMS patients [99]. IGF-1R can also signal through the MEK/ERK cascade, and the specific MEK inhibitor U0126 reduced anchorage-dependent and -independent RD cell proliferation [271]. U0126 also showed synergistic interactions with radiotherapy in 3 RMS cell lines *in vitro*, and significantly improved progression free survival of mice bearing TE671 RMS xenografts [272].

Over 80% of ARMS patients carry altered *ALK* gene copy number [273], identifying ALK as a potential therapeutic target in ARMS patients. ALK and IGF-1R were subsequently shown to be co-expressed in the majority of ARMS patient samples examined, and simultaneous targeting of ALK and IGF-1R (using NVP-TAE684 and the R1507 antibody, respectively) produced synergistic effects in the ARMS cell line Rh41 and Rh30 [274]. A phase I trial (NCT01742286) is now open to evaluate the safety profile of the ALK inhibitor LDK378 in pediatric cancer patients with known *ALK* alterations. Similarly, a phase II trial (NCT01524926) is underway to investigate the efficacy of crizotinib in locally advanced or metastatic tumors, including ARMS patients harboring specific alterations leading to ALK and/or MET activation.

Finally, a number of other kinase inhibitors have been tested in RMS cell lines and xenografts, such as erlotinib, alisertib and AZD1480, which are EGFR, Aurora kinase and JAK/STAT inhibitors, respectively. While primary tumors from a transgenic ARMS model expressed EGFR, these were unaffected by erlotinib treatment [275]. In contrast, alisertib induced maintained complete responses in Rh65 ARMS xenografts [35,36], and significantly improved event-free survival in 4/5 other RMS xenografts, including Rh18 [35]. Similarly, AZD1480 reduced Rh18 ERMS cell viability *in vitro*, significantly inhibited tumor growth *in vivo*, and significantly improved the survival of mice bearing Rh18 xenografts [132].

2.4.2.2. Hsp90 inhibitors. It is worth discussing the role of Hsp90 inhibitors as potential therapies for RMS, as despite demonstrating little *in vivo* antitumor activity against other pediatric tumor types, alvespimycin induced objective responses in Rh30 ARMS xenografts [276]. Hsp90 was highly expressed in RMS (SMS-CTR and Rh30) but not in control cell lines, and geldanamycin and its analogs tanespimycin, 17AEP-GA and 17DMAP-GA reduced RMS cell proliferation, migration and invasion, and induced apoptosis [277]. In contrast, ganetespib and AT13387 demonstrated only low activity against a number of RMS xenografts, and produced no objective responses [40,247].

2.4.2.3. DNA topoisomerase inhibitors. As previously described (Sections 2.1.4 and 2.2.5), topoisomerase inhibitors such as etoposide have been studied in clinical trials as reinduction or maintenance therapy for pediatric malignancies [278,279]. In relapsed or refractory RMS patients, irinotecan showed anti-tumor activity and a good tolerance profile as a single agent [280], as well as additive effects when administered with vincristine [281]. Similarly, the combination of topotecan with vincristine and doxorubicin induced objective responses in a small cohort of 6 patients with refractory or recurrent RMS, and was well tolerated [282]. More recent *in vivo* studies also indicated that namitecan, Genz-644282 and gimatecan were highly active in a number of RMS xenograft models [148,216,252]. As described for Ewing sarcoma (Section 2.4.1.5), namitecan produced RD/TE-671 RMS xenograft regression at low doses and additive effects with the antiangiogenic agents bevacizumab and sunitinib [252], while Genz-644282 induced complete responses in Rh18 and Rh28 ARMS and Rh30 ERMS xenografts [216]. Prolonged daily treatment with low doses of gimatecan also produced significant tumor regression in RD/TE-671 RMS tumor xenografts [148].

2.4.2.4. RMS summary. Outcomes in children with RMS vary greatly, from 95% to 15% 5 year event-free survival for patients with low- or high-risk disease, respectively [283]. Future clinical trials using targeted therapy must therefore focus upon improving outcomes for intermediate- and high-risk patients [283]. While many agents have been tested in the preclinical RMS setting, patient numbers limit those that can progress to clinical trial, particularly in the case of low frequency targets. However there is optimism that agents such as IGF-1R mAbs and ALK inhibitors may shortly deliver long-awaited improvements in outcomes for RMS patients.

2.4.3. Synovial sarcoma

Synovial sarcoma is the most common non-RMS soft tissue sarcoma in adolescents, and is characterized by translocations that fuse *SS18* (*SYT*) to *SSX1*, *SSX2*, and, more rarely, to *SSX4* [226]. As such, SS18 fusion proteins represent a major therapeutic target in synovial sarcoma, and siRNA down-regulation of *SS18–SSX* transcripts reduced anchorage-independent growth of synovial sarcoma cell lines *in vitro*, and significantly delayed tumor formation *in vivo* [284]. Expression microarray profiling of synovial sarcomas bearing SS18–SSX translocations identified *IGFBP2*, *ERBB2* and *BCL-2* up-regulation [285], with overexpression of these and other proteins such as EGFR being confirmed by immunohistochemistry [285–287]. Whereas treatment of synovial sarcoma cell lines with the IGF-1R inhibitor NVP-AEW541 impaired cell growth and significantly increased apoptosis [288], a phase II trial of gefitinib in patients with advanced EGFR-positive synovial sarcoma produced only stable disease as the best response [289]. Following frequent BCL-2 detection in synovial sarcoma [285–287], the BCL-2 antisense oligonucleotide (G3139) was tested against FU-SY-1 synovial sarcoma cells *in vitro*, resulting in significantly increased sensitivity to doxorubicin treatment [290]. In a phase I trial, G3139 induced prolonged stable disease in 2/5 synovial sarcoma patients in combination with doxorubicin and cyclophosphamide [291]. In summary, these results suggest that continued attempts to target either SS18–SSX fusion proteins and/or their downstream targets are likely to improve outcomes in patients with this rare tumor type [226].

2.4.4. Osteosarcoma

Osteosarcoma is a primary bone malignancy characterized by heterogeneous genetic complexity [227], but as with other childhood

sarcomas, may also be driven by altered IGFR signaling, as well as EGFR/ERBB2 and Wnt pathways [292].

2.4.4.1. Tyrosine kinase inhibitors. Several studies have evaluated the effects of IGF-1R inhibition on childhood osteosarcoma cell lines and xenografts. The IGF-1R small molecule inhibitor picropodophyllin dramatically reduced *in vitro* colony formation by osteosarcoma cell lines [293], while NVP-AEW541 [234] and the mAb SCH-717454 (Robatumumab) delayed osteosarcoma xenograft growth either as monotherapy [294,295], or in combination with cyclophosphamide [295]. The IGF-1 receptor antagonist RG1507 subsequently produced stable disease in 2/3 osteosarcoma patients during a larger phase I trial in children with refractory or relapsed solid tumors [296].

EGFR inhibitors have also been tested in osteosarcoma cell lines, where the EGFR inhibitor 324674 (a 4,6-disubstituted pyrimidine) reduced migration and colony formation of 5/5 and 3/3 osteosarcoma cell lines, respectively, and the invasiveness of 143B cells [293]. However, the EGFR inhibitors gefitinib and afatinib did not reduce the viability of 4 osteosarcoma cell lines in a dose-responsive manner [297]. Although ERBB2 expression has been variably reported in pediatric osteosarcoma [298], ERBB2 positivity has been associated with reduced event-free and overall survival in osteosarcoma patients [299]. However, trastuzumab in combination with cytotoxic chemotherapy did not improve outcomes in metastatic osteosarcoma patients with ERBB2-positive disease, relative to those of ERBB2-negative patients not receiving trastuzumab [300].

Osteosarcoma primary cultures [301] and cell lines [302] have been reported to express PDGFRA and/or PDGFRB and to respond to PDGF stimulation *in vitro* [301,301]. Nonetheless, imatinib inhibited cell proliferation *in vitro* at only micromolar concentrations in PDGFR-expressing patient-derived osteosarcoma cells [301] and MG-63 cells [302], and a phase I trial of single-agent imatinib produced no responses in 10 patients with refractory/relapsed osteosarcoma [241]. Other PDGFR inhibitors have been tested in osteosarcoma, with pazopanib in combination with topotecan inhibiting the growth of KHOS xenografts [127], whereas in a phase I study, sunitinib induced stable disease in one of 2 osteosarcoma patients, but no objective responses [204]. Cediranib (a VEGFR inhibitor) produced complete responses in 1/5 osteosarcoma xenografts [120] and showed additive effects with cisplatin and rapamycin [121].

Other kinases may have particular significance in osteosarcoma, and further therapeutic application. For example, MET overexpression converted primary human osteoblasts into osteosarcoma cells that displayed the transformed phenotype *in vitro*, and distinguishing features of human osteosarcomas *in vivo* [303]. The MET inhibitors K252a [304] and SU11274 [293] reduced HGF-induced cell proliferation and migration of Saos-2 and U2OS osteosarcoma cell lines [304], and reduced MNNG cell migration, and colony formation by TE85, 143B and MNNG cells [293], respectively. siRNA screening demonstrated the sensitivity of osteosarcoma cell lines towards PLK1 depletion [305], and the PLK1 inhibitor GW843682X inhibited proliferation of MNNG–HOS and OST osteosarcoma cell lines that were described as highly resistant to standard anticancer drugs such as doxorubicin and cisplatin [123].

2.4.4.2. Notch and Wnt pathway inhibitors. Notch signaling is upregulated in primary osteosarcoma samples and cell lines, and genetic or chemical down-regulation of Notch signaling both significantly reduced the growth of SJSA-1 xenografts *in vivo* [306]. The γ-secretase and Notch pathway inhibitors RO4929097 and PF-03084014 delayed *in vivo* tumor growth in 4/6 [307] and 5/5 [308] osteosarcoma xenografts, but produced no objective responses. Wnt signaling controls bone development and regeneration, and overexpression of numerous Wnt pathway components have been reported in osteosarcoma [309]. For example, Wnt10b ligand was detected in 33/44 (75%) osteosarcoma cases and showed a trend towards association with reduced overall survival [310]. Inhibition of Wnt/β-catenin signaling with CCT036477 enhanced

Saos2 cell line sensitivity to methotrexate, and showed synergistic effects with Notch inhibition [311]. Targeting the Wnt pathway using recombinant proteins, small molecules, antibodies, or *via* downstream transcriptional targets are all in preclinical development [309], although no agents are yet in clinical trial for osteosarcoma.

2.4.4.3. PI3K/mTOR inhibitors. Inhibition of the PI3K/AKT/mTOR axis has also been proposed for osteosarcoma patients, and PI3K/AKT inhibitors such as GSK690693 and MK-2206 have been tested against osteosarcoma xenograft panels. Both agents significantly improved event-free survival in 6/6 osteosarcoma xenografts *in vivo* as single agent therapy, but produced no objective responses [312,313]. More encouragingly, rapamycin induced maintained complete responses in OS-33 osteosarcoma xenografts *in vivo* [314]. Its derivative everolimus, in combination with sorafenib, also showed antiproliferative and proapoptotic effects, producing tumor growth inhibition, antiangiogenic and antimetastatic effects in the MNNG–HOS xenograft model [315]. Phase II studies of everolimus in children and adolescents with refractory or relapsed osteosarcoma (NCT01216826) are ongoing, and of sorafenib and everolimus in relapsed and non-resectable osteosarcoma (NCT01804374).

2.4.4.4. Hsp90, HDAC and DNA topoisomerase inhibitors. As for neuroblastoma [154], Hsp90 inhibitors have shown interesting results towards osteosarcoma cells *in vitro*. Geldanamycin inhibited osteosarcoma U-2OS and Saos-2 cell proliferation at micromolar concentrations [154], although U-2OS cells were markedly more sensitive to the analog SNX-2112 [155]. Both agents demonstrated synergism with cisplatin [154,155]. HDAC inhibitors such as FR901228 and trichostatin A demonstrated activity towards osteosarcoma cells *in vitro* and *in vivo*, particularly when used in combination therapy [316,317]. The HDAC inhibitor MS-27-275 also increased apoptosis and promoted regression of lung metastases in osteosarcoma xenografted mice, with no evidence of significant toxicity [318]. Vorinostat produced prolonged stable disease in one of 2 osteosarcoma patients in a phase I trial, without major toxicities [38], and is under further evaluation in pediatric oncology patients (NCT01422499). Investigational TOP1 and TOP2 inhibitors recently demonstrated high activity in osteosarcoma xenografts, with objective responses to Genz-644284 in 4/5 osteosarcoma xenografts that included 2 maintained complete responses [216], and complete responses to namitecan in U2-OS xenografts [252].

2.4.4.5. Osteosarcoma summary. Osteosarcoma patient outcomes have failed to improve substantially over the past decades, due to low disease prevalence combined with high degrees of tumor heterogeneity [214, 319]. Most targets will therefore be present at low frequency, and primary and secondary resistance to targeted agents will be common. Disease heterogeneity also presents difficulties in extrapolating preclinical results obtained in small numbers of cell lines or patient xenografts, although limited drug penetration may also explain why encouraging preclinical results from subcutaneous osteosarcoma xenografts have not been recapitulated in subsequent phase I trials. More rigorous preclinical testing prior to phase I trial initiation is therefore required [319]. Targeting metastatic progression as opposed to existing lesions may also improve outcomes in osteosarcoma patients [320]. Such targeted agents might show no activity against established lesions, and would require the use of different experimental and clinical trial end-points, but could potentially be applied to a number of solid tumors [320].

3. Conclusions

The use of tailored treatments has already proven successful in the management of adult cancer patients. Von Hoff and colleagues demonstrated the achievement of longer progression free survival in 27% of patients with refractory solid tumors treated with drugs suggested by molecular profiling [321]. More recently, Kim et al. described the

importance of tumor profiling in "real-time" biopsies that predicted tumor responsiveness in heavily pre-treated advanced NSCLC patients [322].

Results from early clinical trials demonstrate that the use of molecularly targeted therapies is generally safe in the pediatric population [30, 38,72,114,135,164,185,187,198], and that particular drugs have shown promising results. Among them, imatinib, dasatinib and sorafenib were found active in Ph + ALL, CML and ALL pediatric patients, respectively [11,20,22]. A large number of tyrosine kinase inhibitors including crizotinib, erlotinib, gefitinib and lestaurtinib have been used to treat children with refractory/relapsed neuroblastoma [108,110,111,114]. Erlotinib and gefitinib have been successfully employed to treat high-grade gliomas [193,194,197], while IGFR mAbs show promising results in pediatric sarcomas [235]. These encouraging results indicate that molecularly targeted therapies have great further potential for the treatment of childhood cancers.

Despite these promising results, the literature published to date also highlights problems that need to be overcome, to fulfill the promise of molecularly targeted therapies for childhood cancer. A large number of biomarker-matched drugs are tested in clinical trials, and then dismissed. In general, 90% of novel targeted therapies do not transition to routine use, despite good results in the preclinical setting [323]. This can be due to unexpectedly high toxicities, lack of objective efficacy, and/or because agents present no measurable advantages over those already in use [323]. In terms of preclinical studies, the assumption that childhood cancer *in vivo* models recapitulate the actual disease has not always proved correct [225,240–242]. Efficacy of targeted therapies will be greatly influenced by intra-tumoral heterogeneity, meaning that more homogenous models such as cell line xenografts may overestimate the efficacy subsequently demonstrated in patients [319]. Other findings may reflect the fact that the efficacy of targeted therapies cannot always be evaluated using the standard methods used for conventional chemotherapy, due to off-target effects, requirements for prolonged dosing at lower levels, and general cytostatic rather than cytotoxic effects [324]. In other words, tumor growth inhibition could represent a more adequate measurement of activity rather than tumor regression in the assessment of single agent efficacy, highlighting the importance of combination trials. This is particularly important in the pediatric population where the goal must be to eradicate disease in the long term.

Another challenge is the low frequency at which predictive markers are often detected in tumors (Table 2), meaning that only a minority of patients may benefit from a specific targeted therapy [325,326]. This problem can become particularly acute in rare diseases such as childhood cancers. Efficacy of targeted therapies may be underestimated if preclinical models do not represent the full range of clinical disease, or if patients are not appropriately selected in clinical trials [323]. Another consideration is the presence or development of drug resistance, due to either activation of alternative pathways, or the presence or acquisition of further mutations that confer a selection advantage to a subgroup of cancer cells [327]. In this context, it is important to consider the combination of multiple agents and/or the use of less selective drugs, in order to inhibit multiple pathways at the same time, or the same pathways at multiple levels [328].

In summary, our understanding of altered signaling pathways responsible for childhood malignancies is changing treatment approaches and improving the outcomes of pediatric patients. A huge effort has been made to prove the safety of molecular targeted therapies in the pediatric population, but patient results have sometimes been considered disappointing. It is clear that conventional methods to evaluate clinical efficacy of novel therapies do not always apply to targeted agents, due to the genetic complexity and heterogeneity of tumors, and the modes of action of these novel drugs. Therefore, the testing of targeted agents in combination with traditional chemotherapeutics needs further investment, in order to produce long-lasting clinical benefits. At the same time, preclinical models need to be improved for the assessment of optimal dosing to obtain additive effects in combination therapy, while clinical trials with patient selection based on molecular profiling will avoid administering target-specific agents to patients who are unlikely to benefit. Finally, of the 12 known pathways that are aberrantly activated in cancer, at least 140 different genes have been described to carry driver mutations [329], but only a small percentage of these are currently "actionable" in the context of targeted therapy. Identifying new actionable targets will be essential to expand the benefits of targeted therapies to a broader range of patients.

Conflict of interest

The authors declare no conflicts of interest.

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