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Promising Therapeutic Targets in Neuroblastoma

Katherine K. Matthay[1], Rani E. George[3], and Alice L. Yu[2]

Abstract

Neuroblastoma, the most common extracranial solid tumor in children, is derived from neural crest cells. Nearly half of patients present with metastatic disease and have a 5-year event-free survival of <50%. New approaches with targeted therapy may improve efficacy without increased toxicity. In this review we evaluate 3 promising targeted therapies: (i) ^{131}I-metaiodobenzylguanidine (MIBG), a radiopharmaceutical that is taken up by human norepinephrine transporter (hNET), which is expressed in 90% of neuroblastomas; (ii) immunotherapy with monoclonal antibodies targeting the GD2 ganglioside, which is expressed on 98% of neuroblastoma cells; and (iii) inhibitors of anaplastic lymphoma kinase (ALK), a tyrosine kinase that is mutated or amplified in ∼10% of neuroblastomas and expressed on the surface of most neuroblastoma cells. Early-phase trials have confirmed the activity of ^{131}I-MIBG in relapsed neuroblastoma, with response rates of ∼30%, but the technical aspects of administering large amounts of radioactivity in young children and limited access to this agent have hindered its incorporation into treatment of newly diagnosed patients. Anti-GD2 antibodies have also shown activity in relapsed disease, and a recent phase III randomized trial showed a significant improvement in event-free survival for patients receiving chimeric anti-GD2 (ch14.18) combined with cytokines and isotretinoin after myeloablative consolidation therapy. A recently approved small-molecule inhibitor of ALK has shown promising preclinical activity for neuroblastoma and is currently in phase I and II trials. This is the first agent directed to a specific mutation in neuroblastoma, and marks a new step toward personalized therapy for neuroblastoma. Further clinical development of targeted treatments offers new hope for children with neuroblastoma. *Clin Cancer Res; 18(10); 2740–53. ©2012 AACR.*

Introduction

Neuroblastoma, the most common extracranial solid tumor in children, is derived from primordial neural crest cells that ultimately inhabit the sympathetic ganglia and adrenal medulla. The clinical behavior, which ranges from spontaneous maturation to inexorable progression despite multimodal intensive therapy, is attributable to molecular differences in the tumor. The high-risk clinical prognostic factors of age >18 months and advanced stage are closely associated with unfavorable biologic risk factors, including unfavorable histopathology, tumor amplification of the *MYCN* oncogene, and loss of heterozygosity of 1p and 11q, or other partial chromosome deletions (1). The 5-year event-free survival (EFS) rate for high-risk neuroblastoma is <50%, including patients with metastatic neuroblastoma with ages >18 months, and patients with locoregional or metastatic neuroblastoma with tumor *MYCN* gene ampli-

fication (2). Until recently, the best outcome reported for high-risk neuroblastoma was achieved with intensive combination induction chemotherapy and surgery, followed by myeloablative therapy with hematopoietic stem cell rescue, and then differentiation therapy with isotretinoin (3), the first tumor-targeted therapy with demonstrated activity in neuroblastoma.

Although extensive laboratory studies and some early clinical trials have focused on small-molecule inhibitors and antibodies targeting relevant genetic pathways implicated in neuroblastoma proliferation [e.g., phosphoinositide 3-kinase (PI3K), mTOR, insulin-like growth factor I receptor (IGF-IR), among many others], in this review we will put into perspective 2 of the most successful, extremely tumor-specific agents in current use that may further improve outcome, and evaluate a third, more recent addition to the armamentarium. The first is ^{131}I-metaiodobenzylguandine (MIBG), which targets human norepinephrine transporter (hNET, expressed in 90% of neuroblastoma tumors) for cell-specific uptake and then destroys the cell with the targeted radiation (4). The second is anti-GD2 antibody, which targets GD2 ganglioside (expressed on >98% of neuroblastomas) and mediates immune destruction of the cells. This antibody combined with cytokines improves survival for patients with high-risk disease (5). The third target is the anaplastic lymphoma kinase (*ALK*) gene, which encodes a receptor tyrosine kinase and has now been implicated as an oncogenic driver in neuroblastoma. *ALK* mutations are found in 8% to 12% of neuroblastomas

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at diagnosis (6, 7), and germline *ALK* mutations are responsible for the majority of familial cases (8, 9). Small-molecule inhibitors with proven utility in ALK-rearranged cancers (10) have shown promise in preclinical studies in neuroblastoma, and early-phase clinical trials are under way. Other targeted approaches to treat neuroblastoma and other pediatric malignancies are addressed elsewhere in the Focus section of this issue of *Clinical Cancer Research* (11–15).

Targeting hNET with MIBG

The observation that 90% of tumors are MIBG-avid provides the rationale for using ^{131}I-MIBG as a targeted radiopharmaceutical for high-risk neuroblastoma. MIBG is an aralkylguanidine norepinephrine analog that was originally developed to visualize tissue of sympathetic neuronal origin, and has now become an essential tool for neuroblastoma staging and response (Fig. 1; ref. 16). Early clinical trials (Table 1A) in relapsed neuroblastoma showed that ^{131}I-MIBG was effective at producing significant response rates, and that the only severe acute toxicity was myelosuppression, which could be abrogated by hematopoietic stem cell transplant (17–21). The activity administered has varied: Set total amounts of 50 to 100 mCi were given in earlier European studies regardless of patient size, and one U.K. study based the dose on the amount needed to limit whole-body radiation to 1 to 2 Gy. Studies in the United States used weight-based dosing, which correlated significantly with the whole-body radiation received (22). The maximum practical weight-based dose in a phase I trial was established as 18 mCi/kg. This dose was then tested in a large phase II study of 164 patients, 30% of whom required hematopoietic stem cell support to prevent prolonged myelosuppression. Minor acute toxicities were grade 1 or 2 nausea and vomiting, parotid pain, and (rarely) changes in blood pressure. Late toxicities included a 12% incidence of grade 2 hypothyroidism, despite the routine use of a saturated solution of potassium iodide to block thyroid uptake of free radioactive iodide (23), and the rare occurrence of ovarian failure or myelodysplastic syndrome with acute myeloid leukemia in <5% of patients (24). Of importance, almost all of these studies reported impressive response rates in relapsed disease, and in the largest phase II trial, 37% of patients had a partial response (PR) or complete response [CR (20)].

Increasing tumor radiation dose

Attempts to further improve response rates included increasing the dose and using a different iodine isotope or a higher specific activity form of the molecule (Table 1A). Because of radiation safety limitations, investigators in a recent phase I study increased the dose by administering a rapid-sequence double infusion given 2 weeks apart and supported by hematopoietic stem cell transplant (25). In this fashion, a single patient could receive as much as 42 mCi/kg over a 2-week period; however, the response rate in this small population of patients did not appear to be different from that obtained with the standard 18-mCi/kg dose. Other investigators reported a benefit from repeated MIBG infusions given 6 to 12 weeks apart, with continued improved response in some of the patients with each successive infusion (26, 27). One small phase I trial (28) used ^{125}I-MIBG rather than the usual ^{131}I-MIBG, based on the hypothesis that the different isotope would be more effective for microscopic tumors or single cells, because the Auger electrons travel only a few nanometers, whereas the β particles of ^{131}I are more effective in tumors >1 mm. Myelosuppression was significant despite a lower whole-body radiation dose. Recently, a New Approaches to Neuroblastoma Therapy (NANT) phase I trial of "no-carrier-added" ^{131}I-MIBG was conducted based on preclinical data that nonradioactive carrier MIBG molecules in the standard preparation of MIBG (specific activity: 1.2 MBq/µg) inhibit uptake of ^{131}I-MIBG, resulting in less tumor radiation and increased risk of cardiovascular toxicity (29). In the no-carrier-added preparation of MIBG, virtually every molecule is radioactive (specific activity: 165 MBq/µg). The NANT trial showed toxicity and response profiles similar to those observed with the standard preparation, but this approach had the advantage of being able to be infused over 30 minutes instead of 90 to 120 minutes (30).

Combining MIBG with radiosensitizers or chemotherapy

As another approach to improve efficacy, investigators have designed combination therapies by adding chemotherapy or other radiosensitizers to the MIBG (Table 1A). Exploratory studies showed the feasibility of giving 7 to 15 mCi/kg of ^{131}I-MIBG followed 10 to 14 days later by myeloablative doses of carboplatin, etoposide, and melphalan (31–34). A phase I study of 24 patients with refractory neuroblastoma established the maximum tolerated dose (MTD) at 12 mCi/kg of ^{131}I-MIBG, with carboplatin 1,500 mg/m^2, etoposide 1,200 mg/m^2, and melphalan 210 mg/m^2. The dose-limiting toxicities were mucositis, vascular leak, veno-occlusive disease, and sepsis; however, only 1 toxic death occurred (35). A subsequent NANT phase II study of 50 patients reported the main toxicity as veno-occlusive disease, with 7 out of 50 responses in this highly refractory group of patients (36).

Other studies focused on using ^{131}I-MIBG combined with chemotherapy or radiosensitizers in a nonmyeloablative regimen in refractory patients. Italian investigators treated 16 patients with relapsed or refractory neuroblastoma with 200 mCi ^{131}I-MIBG combined with cisplatin and cyclophosphamide with or without etoposide and vincristine, and obtained 12 PRs (37). Two studies [a European study of topotecan with double infusion of MIBG (38), and a NANT phase I study of irinotecan and vincristine with MIBG (39)] combined MIBG with a camptothecin, with tolerable toxicity and measurable responses. Recent preclinical data showed additive growth inhibition from a combination of a histone deacetylase inhibitor (vorinostat) with radiation in a metastatic murine neuroblastoma model (40). In
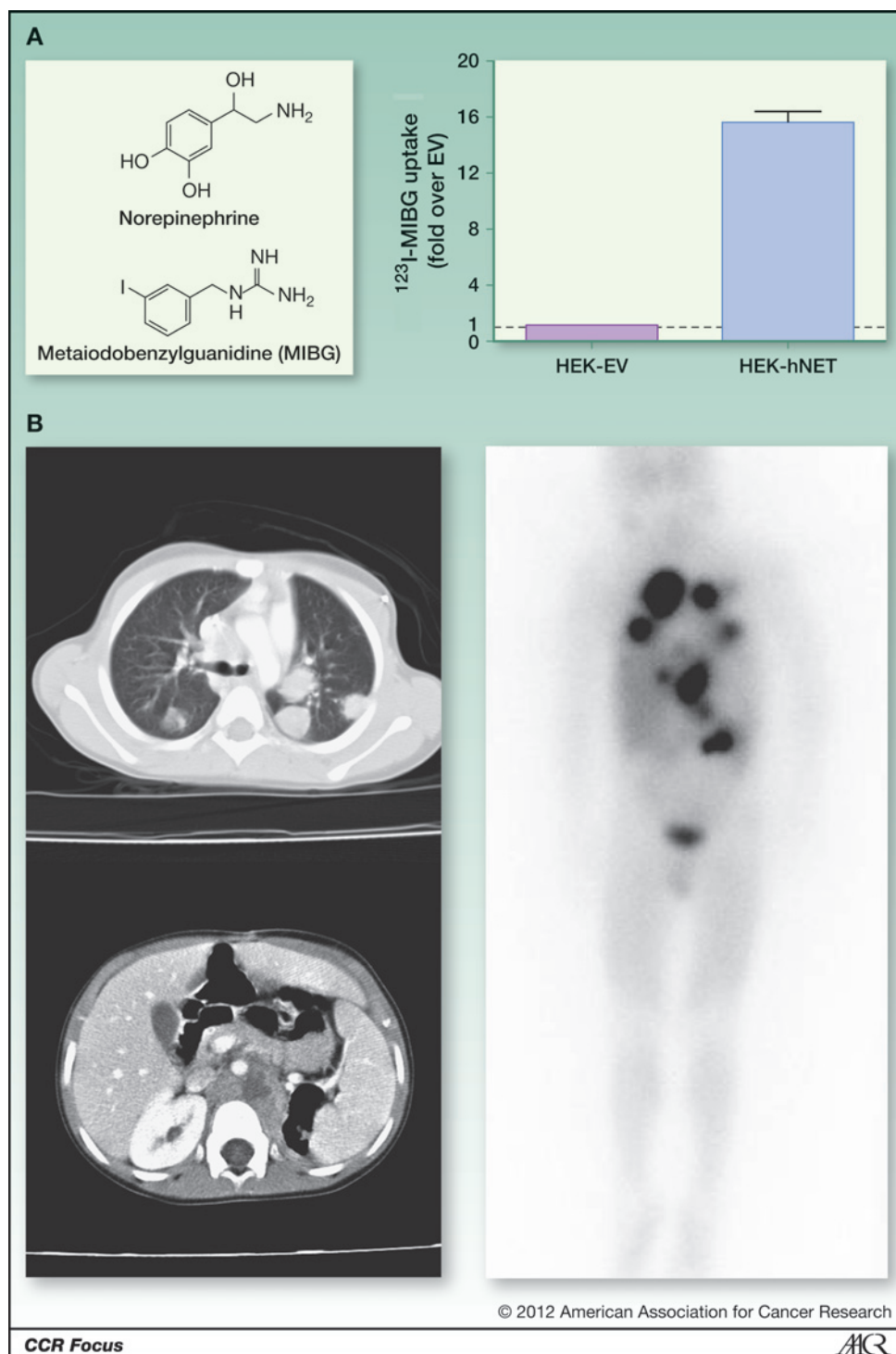
Figure 1. MIBG uptake in neuroblastoma. A, the left panel shows the similar structures of norepinephrine and MIBG, both of which are taken up via hNET. On the right, HEK293 cells transfected with hNET show an ~15-fold higher uptake of ^{123}I-MIBG over the HEK-EV transfected cells. B, the CT scan (left) shows the lung metastases and retroperitoneal mass in a child with recurrent neuroblastoma at the time of ^{131}I-MIBG therapy. The uptake of ^{131}I-MIBG (right) in the tumors is shown at 4 days after infusion of 18 mCi/kg (total activity infused = 340 mCi). EV, empty vector.

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CCR Focus

addition, vorinostat increased uptake of MIBG in neuroblastoma tumors via increased expression of hNET (41). These findings led to a NANT phase I study of vorinostat combined with ^{131}I-MIBG, which is currently enrolling patients at dose level 5 (MIBG, 18 mCi/kg, vorinostat, 230 mg/m^2), and has shown no dose-limiting toxicity to date. Other radiosensitizers are under study in preclinical testing for neuroblastoma and may prove useful in the future (42).

A few recent studies tested the incorporation of MIBG therapy into induction for newly diagnosed patients (Table 1). At a center in Amsterdam, Netherlands, a population of 41 patients who had been treated with 2 cycles of ^{131}I-MIBG before addition of chemotherapy, had a response rate to

Table 1. Key clinical trials of therapies for neuroblastoma targeting hNET with MIBG, GD2 ganglioside with anti-GD2 antibody, and ALK aberrations with small-molecule inhibitors

Trial	Phase	Patients (N)	Agent dose	Results
A. MIBG[a]				
Hutchinson et al. (17)	pilot	14	131I-MIBG (50–220 mCi total)	3 MR; myelosuppression and nausea and transient LFT elevation
Lashford et al. (18)	I/II	25	131I-MIBG 1, 2, 2.5 Gy whole-body dose	Adjusted by whole-body radiation dose with MTD 2.5 Gy; responses 8 PR
Matthay et al. (19)	I	30	131I-MIBG 3–18 mCi/kg	Dose escalation to MTD of 18 mCi/kg; Responses 1 CR, 10 PR, 3 MR
Matthay et al. (20)	II	164	131I-MIBG 18 mCi/kg	Response rate (CR/PR) in refractory or relapsed disease of 37% with 18 mCi/kg; ASCT support given in 30% of patients
Matthay et al. (30)	I	15	no-carrier-added 131I-MIBG 12–18 mCi/kg	MTD with organ and tumor dosimetry of 18 mCi/kg; responses 1 CR, 3 PR, 1 MR
Sisson et al. (28)	I	7	125I-MIBG 0.85–1.35 Gy whole-body dose	Dose limited by thrombocytopenia
Howard et al. (26)	II	28	131I-MIBG 18 mCi/kg	Multiple infusions ($n = $ 2–4) are feasible with 39% response; chronic thrombocytopenia in 46% after final infusion
Matthay et al. (25)	I	21	131I-MIBG × 2 12–21 mCi/kg × 2	Rapid tandem infusion 131I-MIBG with ASCT; MTD 36 mCi/kg; responses 2 PR, 8 MR
Johnson et al. (27)	II	41	131I-MIBG	Two tandem infusions are feasible with 39% response; hematologic toxicity abrogated with PBSC after second infusion
de Kraker et al. (43)	pilot	33	131I-MIBG induction 300 mCi total	131I-MIBG as induction therapy presurgery, 200 mCi followed by 100 mCi in newly diagnosed patients, 57% response
Schmidt et al. (44)	III	40	131I-MIBG end induction	40/111 patients with stage 4 at the end of induction and residual disease got 131I-MIBG, but no impact on EFS in multivariate analysis compared with patients who did not receive MIBG
Gaze et al. (38)	pilot	8	131I-MIBG + topotecan 12 mCi/kg × 2	131I-MIBG (12 mCi/kg × 2) with topotecan tandem therapy; targeted whole-body dose of 4 Gy, showed feasibility
Dubois et al. (39)	I	24	131I-MIBG + VCR/irinotecan 8–18 mCi/kg	131I-MIBG with vincristine and irinotecan dose escalation to MTD 18 mCi/kg; responses 2 CR, 4 PR
Klingebiel et al. (33)	pilot	11	131I-MIBG + CEM 15 mCi/kg	131I-MIBG (15 mCi/kg) with carboplatin, etoposide, melphalan, and ASCT; feasible
Miano et al. (34)	pilot	17	131I-MIBG + Bu/MEL 4–11 mCi/kg	131I-MIBG (4–11 mCi/kg) with busulfan, melphalan, and ASCT; GI toxicity and pneumonitis
Yanik et al. (31)	pilot	12	131I-MIBG + CEM 12 mCi/kg	131I-MIBG (12 mCi/kg) with carboplatin, etoposide, melphalan and ASCT; feasible, good engraftment, mucositis main toxicity

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Table 1. Key clinical trials of therapies for neuroblastoma targeting hNET with MIBG, GD2 ganglioside with anti-GD2 antibody, and ALK aberrations with small-molecule inhibitors (Cont'd)

Trial	Phase	Patients (*N*)	Agent dose	Results
Matthay et al. (35)	I	24	^{131}I-MIBG + CEM 12–18 mCi/kg	^{131}I-MIBG (12–18 mCi/kg) with carboplatin, etoposide and melphalan and ASCT with MTD 12 mCI/kg; response rate 27% in refractory disease
B. Anti-GD2 antibody[b]				
Cheung et al. (49)	I	17	3F8 (murine MAb) 5–100 mg/m^2	Dose escalation; response 7/17(CR/PR/MR); toxicities pain, hypertension, urticaria, and complement depletion
Huang et al. (52)	I	15 (14)[c]	14G2A (murine MAb) 25–500 mg/m^2	Dose escalation; neuroblastoma responses 1 CR, 3 MR, toxicities controllable and reversible, pain, fever, tachycardia, hyponatremia, rash
Murrray et al. (51)	I	18 (5)[d]	14G2A (murine) 50–200 mg/m^2	Dose escalation with MTD of 100 mg/m2; neuroblastoma responses 2 PR; toxicities generalized pain, hyponatremia, fever, rash, paresthesias, weakness, and chronic refractory postural hypotension
Uttenreuther-Fischer et al. (62)	I	15	Ch14.18 (chimeric) 10–200 mg/m^2	Dose escalation with pharmacokinetics showing t1/2 of 66 ± 27.4 hr, which is significantly shorter than 181 ± 73 hr reported in adult patients.
Handgretinger et al. (60)	I	9	Ch14.18 (chimeric) 150–250 mg/m^2	Dose escalation (30–50 mg/m^2/d × 5 days); 5 responses (CR/PR/MR); toxicities pain and urticaria
Yu et al. (61)	I	11 (10)[c]	Ch14.18 (chimeric) 10–200 mg/m^2	Dose escalation; neuroblastoma responses 1 PR, 4 MR. Toxicities of pain, tachycardia, hypertension, fever, urticaria
Yu et al. (64)	pilot	17	Ch14.18 + GM-CSF 200 mg/m^2	Ch14.18 50 mg/m^2/d × 4 and GM-CSF 10 µg/kg/d × 14), 4 CR, 1 PR, 1 MR. Toxicities similar to ch14.18 alone, and transient thrombocytopenia
Yu et al. (65)	II	32	Ch14.18 + GM-CSF 200 mg/m^2	Ch14.18 50 mg/m^2/d × 4 and GM-CSF 10 µg/kg/d × 14), 2 CR, 2 PR, 1 MR; increase in ADCC activity correlated with clinical response.
Frost et al. (56)	I	33 (31)[c]	14G2A + IL-2 10–100 mg/m^2	Dose escalation 14G2A + IL-2; MTD 15 mg/m^2 per day; 1 PR in neuroblastoma and 1 CR in osteosarcoma; toxicities pain and fever
Cheung et al. (57)	II	21/13 R1/R2	3F8 (murine) 100 mg/m^2	Stage 4 NB in first (R1) or second (R2) remission after ASCT treated with 10 mg/m^2 × 5 days; responses by PCR bone marrow (7/12) or scintigraphy ^{131}I-3F8 (6/6)
Cheung et al. (58)	II	136	3F8 + GM-CSF 100 mg/m^2	3F8 in R1 or R2; 5-year EFS for R1 with favorable FCGR2A-131R/R was 52%, vs. 29% in the R/H or H/H group
Ozkaynak et al. (67)	I	19	Ch14.18 + GM-CSF 80–200 mg/m^2	Ch14.18 with GM-CSF post ASCT; MTD 40 mg/m^2/d × 4 days. Toxicities: severe neuropathic pain, fever, nausea/vomiting, urticaria, hypotension, mild to moderate capillary leak syndrome, and neurotoxicity

(Continued on the following page)

Table 1. Key clinical trials of therapies for neuroblastoma targeting hNET with MIBG, GD2 ganglioside with anti-GD2 antibody, and ALK aberrations with small-molecule inhibitors (Cont'd)

Trial	Phase	Patients (N)	Agent dose	Results
Gilman et al. (66)	I	25	Ch14.18 + GM-CSF + IL-2 + isotretinoin 80–160 mg/m^2	MTD of Ch14.18 was 25 mg/m^2/d $\times$ 4 days) given post ASCT; feasible regimen with common toxicities: pain, fever, nausea, emesis, diarrhea, urticaria, mild elevation of hepatic transaminases, capillary leak syndrome, and hypotension
Yu et al. (5)	III	226	Ch14.18 + GM-CSF + IL-2 + isotretinoin 100 mg/m^2	Randomized trial comparing isotretinoin post-ASCT with isotretinoin with immunotherapy (ch14.18 with GM-CSF and IL-2). Immunotherapy was superior to standard therapy by 2-year EFS (66 $\pm$ 5% vs. 46 $\pm$ 5%, $P = 0.01$) and OS (86 $\pm$ 4% vs. 75 $\pm$ 5%, $P = 0.02$)
Osenga et al. (70)	I	28 (27)[e]	hu14.18-IL2 6–42 mg/m^2	MTD of the immunocytokine was 12 mg/m^2/d $\times$ 3 days. Toxicities: hypotension, allergic reaction, blurred vision, neutropenia, thrombocytopenia, and leucopenia; antitumor activity noted in 3 neuroblastoma patients but no measurable responses
Shusterman et al. (71)	II	36	hu14.18-IL2 36 mg/m^2	Responses to 12 mg/m^2/d $\times$ 3 days in 5/23 patients with bone (MIBG) and/or bone marrow disease; responses in 0/13 with soft-tissue disease
Navid et al. (73)	I	32	Hu14.18K332A ongoing escalation	Humanized anti-GD2 mAb with a single point mutation (K322A) reducing complement-dependent lysis); common toxicities: pain, fever, and hyponatremia; well tolerated at higher doses than ch14.18
Batova et al. (75)	Pilot	31	mAb1A7 + QS21	Anti-idiotype anti-GD2 vaccine for patients in R1 or R2; 17/20 in R1 progression-free (median 47 months), 1/11 in R2 progression free
C. ALK inhibitor				
Mosse study chair[f]	I/II	44+	PF-02341066 ongoing escalation	ADVL0912: oral small-molecule inhibitor ALK and C-Met; ongoing phase I trial by COG

Abbreviations: ASCT, autologous stem cell transplant; GI, gastrointestinal; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; LFT, liver function tests; mAb, monoclonal antibody; MR, minor response; OS, overall survival; R1, first remission; R2, second remission.

[a]The trials listed here are only a representative sample of the development of this therapy in phase I and II and combination trials.
[b]Most of the anti-GD2 phase I trials also included a few other GD2-postive malignancies, such as melanoma and osteosarcoma, although the majority of patients entered had a diagnosis of neuroblastoma. Responses are only reported for neuroblastoma.
[c]The number in parentheses indicates the number of neuroblastoma patients enrolled; the remaining patient had osteosarcoma.
[d]The number in parentheses indicates the number of neuroblastoma patients enrolled; the remaining 11 patients had melanoma, and 2 had osteosarcoma.
[e]The number in parentheses indicates the number of patients with neuroblastoma enrolled; the remaining patient had melanoma.
[f]There is no published pediatric trial in neuroblastoma of an ALK inhibitor, but this ongoing study in the COG phase I consortium includes patients with relapsed solid tumors and anaplastic large-cell lymphoma.
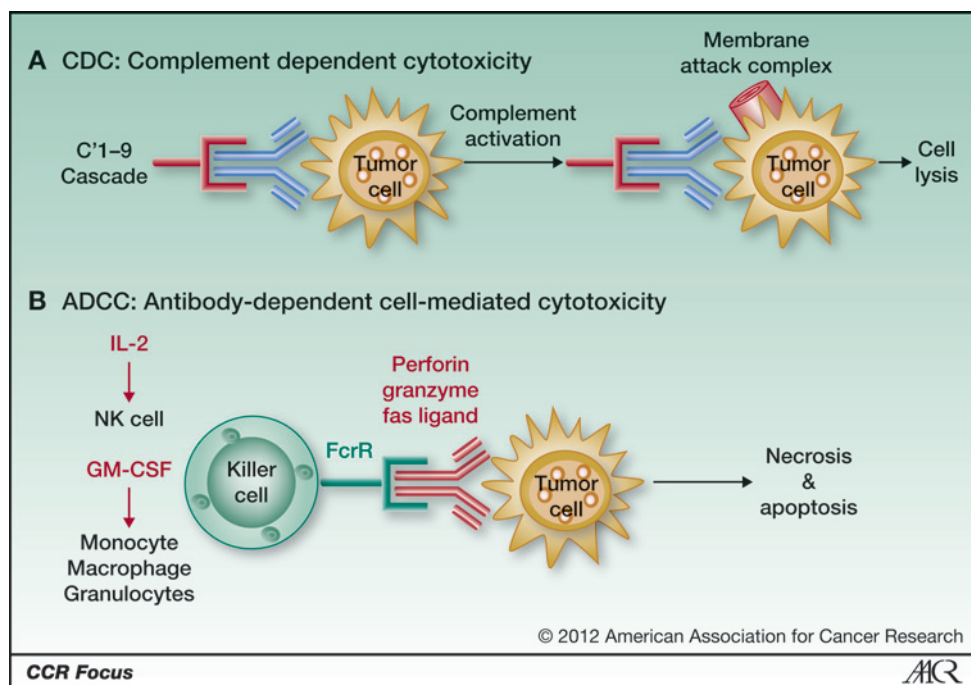
Figure 2. Mechanism of GD2 antibody-targeted destruction of neuroblastoma by CDC and ADCC. A, CDC: The mAb binds to the receptor and initiates the complement cascade, which results in the formation of a membrane attack complex that makes a hole within the cell membrane, causing cell lysis and death. B, ADCC: The Fc fragment of the monoclonal antibody binds the Fc receptors on monocytes, macrophages, granulocytes, and natural killer cells (NK). These cells in turn engulf the bound tumor cell and destroy it. Natural killer cells secrete cytokines that lead to cell death. GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin.

MIBG of 66% (43). German studies evaluated the addition of MIBG therapy at the end of induction and before myeloablative therapy for patients with residual MIBG-positive disease and reported a response rate of 46% but no improvement in overall survival [OS (44)]. A Children's Oncology Group (COG) pilot trial (ANBL09P1) will test the addition of MIBG with vincristine and irinotecan prior to myeloablative therapy for all high-risk patients, regardless of residual disease, and if feasibility and tolerability are shown, a randomized trial will be undertaken.

Future perspectives for MIBG

^{131}I-MIBG therapy is a promising strategy that now requires randomized testing in newly diagnosed high-risk patients. The practicality of this targeted radiopharmaceutical is rapidly increasing, with 9 North American and at least 7 European pediatric centers regularly administering MIBG therapy, and more centers in development. Because of the whole-body radiation that accompanies the tumor dose, it is essential to further test compounds that will increase tumor uptake and sensitivity without increasing normal organ toxicity, and to investigate different radioisotopes, such as the α emitter ^{211}At (45). Better pretherapy tumor dosimetry using single photon emission computed tomography–computed tomography with tracer doses of ^{131}I-MIBG (46), or positron emission tomography–computed tomography with ^{124}I-MIBG (47) may also help to personalize the dose.

GD2-Targeted Immunotherapy of High-Risk Neuroblastoma

Promising results have been observed with immunotherapy targeting the surface glycolipid molecule disialoganglioside (GD2), which is uniformly expressed by neuroblastomas and gliomas, some melanomas, and sarcomas (48, 49). In normal human tissues, GD2 expression is weak and restricted to neurons, melanocytes, and peripheral pain fibers (50). Thus, GD2 is an ideal antigen target for immunotherapy of neuroblastoma. Three first-generation [monoclonal antibodies (mAb) 14G2a, ch14.18, and 3F8] and 3 second-generation (Hu14.18-IL-2, hu14.18K332A, and mAb1A7) GD2-directed antibodies have been investigated for immunotherapy of neuroblastoma (Table 1B, Fig. 2).

First-generation anti-GD2 mAbs

14G2a is an IgG 2a murine anti-GD2 mAb. In 2 phase I trials (51, 52), the dose of mAb 14G2a was escalated from 25 to 500 mg/m^2/course (Table 1B). Toxicities mainly consisted of reversible pain, tachycardia, fever, changes in blood pressure, hyponatremia, and urticaria, and were more severe in adult patients. Pain is thought to occur due to binding of antibody to peripheral nerve fibers expressing GD2 (50). These 2 phase I trials included 19 neuroblastoma, 3 osteosarcoma, and 11 melanoma patients. Therapeutic activity was observed, with 1 CR and 2 PRs in neuroblastoma patients, and 6 minor responses (MR), including 3 in neuroblastoma patients (51, 52). Pharmacokinetic studies revealed a β t 1/2 of 18.3 ± 11.8 hours (53). Because antibody-dependent cellular cytotoxicity (ADCC) is the key antitumor mechanism of therapeutic antibodies, and interleukin 2 (IL-2) was shown to augment lymphocyte-mediated ADCC *in vitro* (54) and antitumor activity of 14G2a *in vivo* (55), a phase I trial of 14G2a in combination with IL-2 was conducted in 31 patients with neuroblastoma and 2 patients with osteosarcoma, with 1 PR in the former and 1 CR in the latter (56).

3F8 is a murine IgG3 anti-GD2 antibody against GD2 that was developed in the 1980s and has shown side effects and indications of antineuroblastoma activity similar to those observed with 14G2A (57). A phase II study of 3F8 showed that 13 of 34 patients with stage 4 neuroblastoma in first or subsequent response remained progression free for 40 to 130 months (57). A follow-up phase II report of 3F8 + granulocyte macrophage colony-stimulating factor (GM-CSF) in 136 patients showed an overall 5-year EFS of 38% for patients without prior relapse, and a better outcome for patients with the *FCGR2A* (R/R) genotype polymorphism, which favors the binding of the IgG3 antibody (58).

MAb Ch14.18 consists of the variable regions of murine IgG3 anti-GD2 mAb 14.18 and the constant regions of human IgG1-κ (59). Phase I clinical trials confirmed activity in relapsed neuroblastoma and a toxicity profile similar to that of mAb14G2a (60, 61). As expected, ch14.18 had a longer than half-life than 14G2a, with a β t 1/2 of 66.6 ± 27.4 hours (60, 62). Because GM-CSF not only raises the number of leukocytes but also enhances their anti-GD2–mediated ADCC (63), a pilot study of ch14.18 + GM-CSF showed responses in patients with recurrent/refractory neuroblastoma (64). This led to a phase II national study, which confirmed the efficacy of ch14.18 + GM-CSF, with 2 CR, 2 PR, 1 MR, and 2 with stable disease in 32 patients with recurrent/refractory neuroblastoma (65). Because most responses occurred in bone marrow or bone, it was hypothesized that this approach would be most effective in the setting of minimal residual disease. Subsequently, the feasibility of giving ch14.18 in combination with GM-CSF, IL-2, and isotretinoin during the early post-transplant period was shown in 2 pilot phase I studies (66, 67).

These clinical trials led to the pivotal randomized COG phase III study (5) to determine whether immunotherapy with ch14.18 combined with GM-CSF and IL-2 on the backbone of isotretinoin would improve survival compared with isotretinoin alone for children with high-risk neuroblastoma in first response after myeloablative therapy and stem cell rescue. Eligible patients were randomized after stem cell transplantation to 6 cycles of isotretinoin (standard) or isotretinoin with 5 intercalated cycles of ch14.18 combined with GM-CSF or IL-2 in alternating cycles (immunotherapy). An analysis of 226 eligible patients showed that EFS was significantly higher for 113 patients randomized to immunotherapy, with 2-year estimated EFS from randomization of 66% ± 5% versus 46% ± 5% (P = 0.0115) for the 113 patients randomized to isotretinoin alone. The immunotherapy group also showed significantly higher OS (86% ± 4% vs. 75% ± 5% at 2 years; P = 0.0223). This approach represents a major advance and is the first effective immunotherapy for high-risk neuroblastoma as well as the first successful immunotherapy to target a nonprotein antigen.

Although ch14.18 in combination with IL-2 and GM-CSF has been shown to improve the outcome of high-risk neuroblastoma, the treatment is associated with significant toxicities, especially in cycles containing IL-2. Furthermore, the exact contribution of cytokines *in vivo* remains unclear.

To address these issues, the Society of Pediatric Oncology European Neuroblastoma Network is conducting a study that randomizes patients with high-risk neuroblastoma to ch14.18 alone or in combination with subcutaneous infusion IL-2 to ameliorate toxicities associated with i.v. administration of IL-2.

Second-generation GD2-targeted immunotherapy and future perspectives

Building on the successful demonstration that ch14.18 + cytokines significantly improved outcome of patients with high-risk neuroblastoma, investigators are conducting a COG phase III trial to collect comprehensive toxicity data for regulatory approval of ch14.18. In addition, immunophenotypes such as FCGR3A and FCGR2A polymorphism (58, 68), and Killer-Immunoglobulin-like Receptors (KIR)-ligand mismatch (69), which may affect ADCC activity and hence clinical response to ch14.18, are under investigation. In addition, second-generation anti-GD2 antibodies and an anti-idiotype antibody vaccine have been developed and are currently in early-phase clinical trials.

Hu14.18-IL2 is a fusion protein of humanized anti-GD2 antibody (hu14.18) and IL-2. The MTD in a phase I trial was 12 mg/m^2/d, ∼50% that of ch14.18. Clinical toxicities were similar to those reported with IL-2 and anti-GD2 mAbs, and antitumor activity was noted in 3 of 27 patients with neuroblastoma, although there were no measurable CR or PRs (70). A phase II study of this immunocytokine showed 5 CRs in 23 patients with neuroblastoma evaluable only by MIBG and/or bone marrow histology, but no responses for patients with measurable disease (71). In this study, patients with KIR-ligand mismatch seemed to be associated with better clinical response to immunotherapy with anti-GD2 (69). A larger phase II study adding GM-CSF is being conducted by the COG.

Hu14.18K332A is a humanized ch14.18 with a mutation to alanine at lysine 322 that limits its ability to fix complement and thereby reduces the pain associated with ch14.18 while retaining its ADCC capabilities. Preclinical studies in rats confirmed that hu14.18K322 elicited substantially less allodynia than ch14.18 (72). Preliminary findings of a phase I clinical trial of hu14.18K322 showed an MTD of 70 mg/m^2/d × 4, with reduced neuropathic pain (73).

Monoclonal antibody 1A7 is an anti-idiotype antibody that is directed against a murine anti-GD2, 14G2a, and in effect mimics the GD2 antigen. Yu and colleagues (74) conducted a clinical trial of mAb 1A7 as a surrogate GD2 vaccine in 31 patients with high-risk neuroblastoma who achieved first or subsequent responses. No systemic toxicities were seen with subcutaneous injections given periodically over 2 years, and only local reactions (transient fever in 4 patients and serum sickness in 1 patient) were observed. All patients generated anti-mAb1A7, and their immune sera displayed complement-dependent cytotoxicity (CDC) and ADCC activities. Sixteen of 21 patients who enrolled during first remission had no evidence of disease progression at a median of 6 years, whereas only 1 of 10 patients in second remission remains progression free.

These findings indicate that mAb1A7 vaccine has little toxicity, is effective at inducing biologically active anti-GD2, and may be useful for controlling minimal residual disease (ref. 75; A.L. Yu and colleagues, unpublished data).

In light of the observed low toxicity profiles of mAb1A7 and hu14.18K332A, it will be desirable to replace ch14.18 with one of these products, or possibly to substitute the Hu14.18-IL2 immunocytokine for the combination of ch14.18 and exogenous IL2. Thus, it will be important to ascertain whether mAb1A7 vaccine or the mutant anti-GD2 mAb will have therapeutic efficacy similar to that of ch14.18 in future clinical trials. Given the documented synergism between chemotherapy and anticancer mAbs such as rituximab (76) and trastuzumab (77), it will be timely to assess the efficacy of anti-GD2 mAbs with chemotherapy for treatment of neuroblastoma. Recently, investigators genetically engineered human T lymphocytes to express GD2-directed chimeric antigen receptors (CAR) by grafting the specificity of 14G2a onto a T-cell receptor (78). These CAR-T cells have been used to mediate tumor regression in patients with neuroblastoma, and offer another testable strategy for GD2-directed immunotherapy (79).

ALK as a Therapeutic Target in Neuroblastoma

The least developed of the targeted therapies for neuroblastoma is that directed to the ALK tyrosine kinase receptor. This lag can be attributed to the relatively recent identification of aberrations in the *ALK* gene (6–9). Nonetheless, these findings have led to clinical testing of ALK targeting in a remarkably short time, largely because of the availability of crizotinib, a dual ALK/MET inhibitor that has shown efficacy in adults with *ALK*-rearranged cancers, such as non–small cell lung cancer (NSCLC), inflammatory myofibroblastic tumor, and anaplastic large-cell lymphoma (10, 80, 81).

ALK meets most criteria to be classed as a valid molecular target (82). Targeting mutated ALK in neuroblastoma is likely to produce a clinical benefit, for the following reasons: (i) point mutations and amplifications, which occur in ∼8% to 10% and 2%, respectively, of primary neuroblastoma tumors, are oncogenic both *in vitro* and *in vivo*, leading to constitutive phosphorylation of ALK and of downstream signaling molecules that are critical for cell proliferation and survival (6–9). Of ∼12 mutations reported in neuroblastoma, the most common affect the R1275 and F1174 residues (83). (ii) Cells expressing mutated *ALK* exhibit oncogene addiction with ALK inhibition leading to cell death (6). (iii) Most importantly, these aberrations are amenable to inhibition by small molecules, unlike the most unequivocal genetic marker of aggressive neuroblastoma, amplification of the *MYCN* oncogene (6).

Crizotinib and other ALK inhibitors

The first drug to be approved by the U.S. Food and Drug Administration (FDA) for treatment of *ALK*-rearranged cancers was crizotinib, an orally bioavailable ATP-compet-

itive 2,4-pyrimidinediamine derivative [PF2341066; Pfizer Inc. (84)]. Crizotinib binds to the inactive conformation of *ALK* and has shown striking efficacy against ALK-rearranged tumors such as NSCLC and inflammatory myofibroblastic tumor. In early-phase clinical testing, the overall response rate was 57% in a population of 82 patients with *EML4-ALK*–positive NSCLC (10, 80, 81). Currently, crizotinib is being tested in an ongoing phase I/II trial for children with neuroblastoma and other solid tumors bearing *ALK* mutations and rearrangements (NCT00939770, ClinicalTrials.gov). Preclinical testing showed the sensitivity of neuroblastoma cell lines with *ALK* amplification and the R1275Q mutation to crizotinib, marked by complete and sustained regression of xenografts (85), By contrast, the F1174L mutation, which possesses a greater transforming capacity than other *ALK* mutations (83) and is associated with a poor response to standard therapy (83), showed only minimal response in cell lines and none at all in neuroblastoma xenografts bearing this mutation (85). ALK^{F1174L} also mediates acquired resistance to crizotinib in adults with *ALK* translocation-positive cancers (86). The structural basis of this resistance is not yet understood, but it could reflect the observation that this mutation is not in direct contact with the crizotinib-binding site within the ATP-binding pocket (86), or that it has a greater affinity for ATP than crizotinib (85). In view of the lack of sensitivity of ALK^{F1174L} to crizotinib, the pediatric phase I study has been amended to increase the recommended phase II dose based on preclinical data that suggest that the resistance can be overcome in a dose-dependent manner (85).

Aggressive efforts to develop new ALK inhibitors are under way. An example is CH5424802 [Chugai Pharmaceuticals Ltd. (87)], an orally available benzo[b]carbazole derivative that was shown to potently inhibit the growth of neuroblastoma cells expressing amplified *ALK*. This agent also displayed *in vitro* inhibitory activity against neuroblastoma cells expressing ALK^{F1174L}, which was shown to be comparable to that achieved in cells with wild-type (WT) ALK. Whether CH5424802 will show efficacy for *in vivo* models of neuroblastoma with mutated *ALK* remains to be seen. Additionally, LDK378 (Novartis Pharmaceuticals), an orally active inhibitor of the ALK kinase, is currently being tested in phase I trials in adult patients (NCT01283516, ClinicalTrials.gov), and may hold promise for the treatment of neuroblastoma patients with mutated ALK.

As observed with other kinase inhibitors, eventual resistance to crizotinib and other ALK inhibitors will likely develop, either through mutations within the ALK kinase domain or by upregulation of an alternative signaling pathway (86, 88). This phenomenon has already been described in adult patients and will undoubtedly emerge in children who initially respond to crizotinib. Preclinical models of resistance are needed to enable prediction of resistance mechanisms and aid in the design of drug combinations to delay or circumvent resistance. Effective targeting of resistance mutations

would be aided by resolution of the crystal structure of ALK in its active confirmation, and of the F1174L mutant in particular.

## Future prospects for clinical application of ALK targeting

Although the number of patients whose tumors have *ALK* aberrations is approximately half that associated with *MYCN* oncogene amplification, there are several reasons why inhibition of ALK would be a feasible therapeutic option in neuroblastoma. First, ALK is expressed on the surface of most neuroblastoma tumor cells (89) and is restricted to the brain following development. Thus, ALK, like GD2, would be an ideal tumor-associated antigen to target with immune-based therapies (Fig. 3). Anti-ALK antibodies may be beneficial not only for patients with aberrant ALK in their tumors, alone or in combination with small-molecule inhibitors (90), but also for the larger number of patients whose tumors express WT ALK.

Second, a proportion of neuroblastoma cells that over-express phosphorylated ALK that is neither mutated nor amplified respond to ALK depletion by undergoing apoptosis. Such tumor cells are also responsive to ALK inhibition by small molecules (91) and may even be amenable to RNA interference-based therapeutic strategies (92). Thus, ALK is apparently activated in such cells by alternative mechanisms, suggesting that ALK inhibition may be a useful therapeutic strategy for a larger subset of patients than previously projected.

Third, ALK inhibition may provide an effective targeting strategy against *MYCN*-amplified tumors, which account for nearly half of all high-risk neuroblastomas. ALK^{F1174L} cosegregates with *MYCN* amplification in patients, and this combination is associated with a particularly poor prognosis, as shown by the fatal outcome of 9 of 10 children with ALK^{F1174L}/*MYCN* amplified tumors (83). ALK^{F1174L} also has been shown to potentiate the oncogenic activity of MYCN *in vivo*, although by itself, ALK^{F1174L} appears to be insufficient to cause the malignant transformation of neural crest–derived stem cells (93, 94). The latter observation may in part explain the occurrence of *ALK* mutations in low-stage neuroblastomas. Both WT and F1174L ALK upregulate MYCN expression *in vitro* (95), and *in vivo* (R. George, unpublished data). Moreover, these 2 genes signal through a common signaling pathway, PI3K/AKT/mTOR, and targeting ALK with short hairpin RNAs has been shown to inhibit cell growth in tumor lines with concomitant *MYCN* amplification (6). Together, these observations suggest that inhibition of ALK, whether WT or mutant, could be a means of inducing the death of *MYCN*-amplified tumor cells, either alone or in combination with emerging strategies that target MYC, such as BET bromodomain inhibition (96).

Neuroblastoma has been at the forefront of pediatric cancers in terms of stratification of patients based on molecular aberrations, the prototypical example of which is *MYCN* amplification. More recently, the discovery of *ALK* mutations has bolstered the prospects of effective

Figure 3. Current and future prospects for targeting ALK in neuroblastoma. Mutated ALK could be targeted by small-molecule inhibitors alone or in combination with inhibitors of downstream signaling pathways. ALK-directed immunotherapy such as anti-ALK antibodies could induce cytotoxicity by directly inhibiting the mutated receptor or by provoking a cytotoxic immune response. Nanoparticles carrying ALK siRNA could result in inhibition of ALK activity, leading to cell death. ERK, extracellular signal-regulated kinase; JAK, Janus-activated kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription.
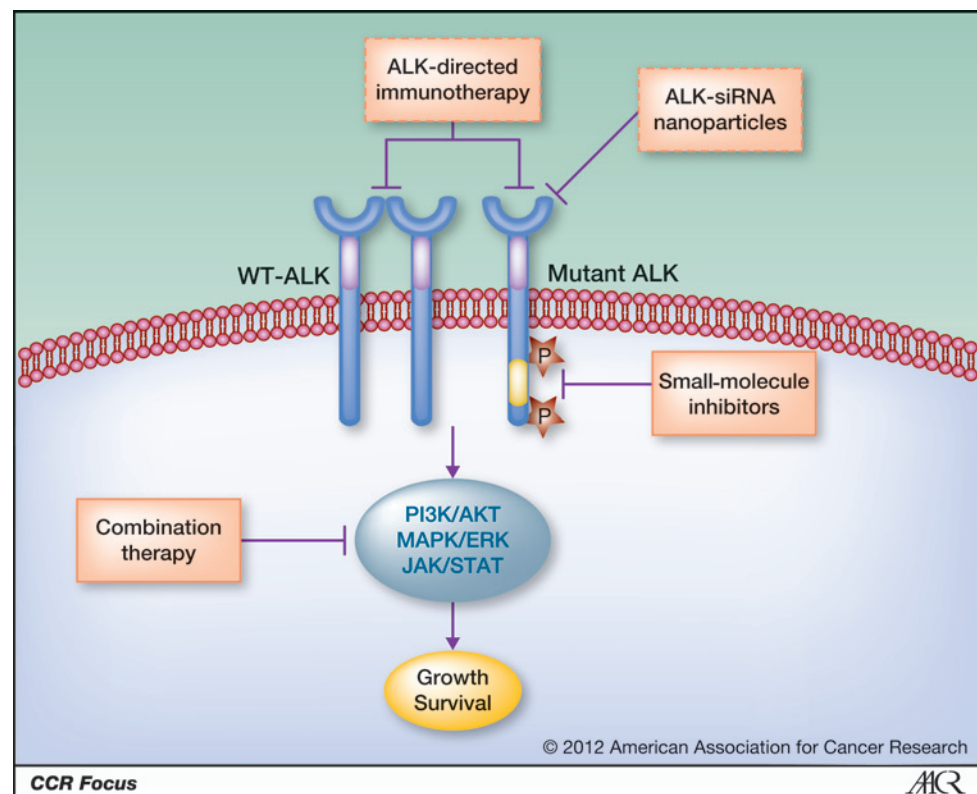
molecularly targeted therapy for high-risk tumors, although considerable work needs to be done before ALK inhibition can occupy the same high-priority ground as hNET-MIBG and anti-GD2 antibody therapy.

Conclusions

The targeted approaches to high-risk neuroblastoma discussed above will provide new ammunition to fight both measurable and microscopic disease in patients. The remaining challenges will be to determine how to combine these agents with standard cytotoxic agents and when to introduce them during therapy, whether in the neoadjuvant setting, during consolidation, or to treat minimal residual disease. Perhaps the greatest challenge for investigators of this orphan disease is to achieve worldwide access to and commercialization of these therapies. MIBG and anti-GD2 antibodies have both been under investigational testing with proven activity in neuroblastoma since the 1980s, yet because of the very small market, neither has been developed commercially. It is hoped that the impressive activity of MIBG in phase II studies, and the recent success in a randomized trial of the ch14.18 mAb, will prompt the acceptance of new drug applications by the FDA. Paradoxically, an ALK small-molecule inhibitor has already been approved based on activity in the more-common adult cancers, although it is still in early clinical trials for neuroblastoma. Anti-GD2

antibodies and MIBG both provide targeting for most neuroblastomas, but the acute toxicity of the former and the radiation isolation and possible late effects of the latter limit their utility to pediatric centers with expertise. Although oral ALK inhibitors are currently limited to a subset of tumors, they offer a truly personalized approach to treatment.

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