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New Therapeutic Targets for the Treatment of High-Risk Neuroblastoma

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ABSTRACT

High-risk neuroblastoma remains a major problem in pediatric oncology, accounting for 15% of childhood cancer deaths. Although incremental improvements in outcome have been achieved with the intensification of conventional chemotherapy agents and the addition of 13-*cis*-retinoic acid, only one-third of children with high-risk disease are expected to be long-term survivors when treated with current regimens. In addition, the cost of cure can be quite high, as surviving children remain at risk for additional health problems related to long-term toxicities of treatment. Further advances in therapy will require the targeting of tumor cells in a more selective and efficient way so that survival can be improved without substantially increasing toxicity. In this review we summarize ongoing clinical trials and highlight new developments in our understanding of the molecular biology of neuroblastoma, emphasizing potential targets or pathways that may be exploitable therapeutically. J. Cell. Biochem. 107: 46–57, 2009.

KEY WORDS: NEUROBLASTOMA; NOVEL THERAPEUTIC TARGETS

Neuroblastoma is the most common extracranial solid tumor of childhood, and accounts for one of every eight pediatric cancer deaths. At least 40% of all children with neuroblastoma are designated as high-risk patients, based on adverse features such as age $\geq$18 months at presentation, the presence of disseminated disease, unfavorable histologic features, and amplification of the *MYCN* oncogene [Park et al., 2008].

Current treatment for high-risk neuroblastoma consists of a coordinated sequence of chemotherapy, surgery, and radiation [Matthay et al., 1999; Pearson et al., 2008]. Resection of the primary tumor is rarely performed at the time of initial diagnosis, since most patients present with extensive metastatic disease. Instead, patients first receive intense multiagent chemotherapy designed to reduce overall disease burden and facilitate later resection of the primary tumor, which is typically performed after several courses of treatment. This initial induction chemotherapy usually consists of varying combinations of alkylating agents, anthracyclines, platinum compounds, and epipodophyllotoxins. Following completion of induction chemotherapy, treatment is consolidated with one or more courses of high-dose chemotherapy with autologous hematopoietic stem cell support. Agents used for high-dose therapy include carboplatin, etoposide, and melphalan (CEM). After recovery from the acute effects of consolidation, patients receive focal radiotherapy to the primary tumor site as well as any residual metastatic sites still identifiable at the completion of induction. Finally, patients receive six courses of the differentiating agent 13-*cis*-retinoic acid (CRA), with the intent of eradicating minimal residual disease that is present in over half of children who have achieved complete remission by imaging criteria. The total length of therapy for most patients is up to 1 year when accounting for the frequent treatment delays and complications.

Even with this aggressive treatment, less than 40% of children are likely to achieve long-term cure [Matthay et al., 1999; Pearson et al., 2008; Zage et al., 2008]. Treatment failures usually arise from the setting of minimal residual disease following high-dose chemotherapy. Although prolonged disease stabilization can be obtained in some children following tumor recurrence, nearly all children who relapse eventually die from disease progression [Lau et al., 2004]. Even patients who appear to achieve a cure with initial therapy remain at risk for developing long-term complications related to treatment, including hearing loss, cardiac dysfunction, infertility, and second malignancies [Laverdière et al., 2005]. These observations underscore the need for more effective and less toxic therapies.

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TABLE I. Selected Ongoing Clinical Trials for Relapsed High-Risk Neuroblastoma

Investigational agents	Class of agents	Patient population	Phase of trial	Sponsor/institution	Comments
Cyclophosphamide + topotecan	Alkylator, Camptothecin	Newly diagnosed	III	COG	Incorporated into induction
Ch14.18	Anti-GD2 antibody	Post-transplant	III	COG	With CRA versus CRA alone
ABT-751	Anti-mitotic	Relapsed	II	COG	Endpoint of PFS
Temsirolimus	mTOR inhibitor	Relapsed	II	Wyeth	Limited-institution study
Temsirolimus	mTOR inhibitor	Newly diagnosed	II	St. Jude Children's Research Hospital	Combined with irinotecan as upfront window
IMC-A12	IGF-1 receptor antibody	Relapsed	I, II	COG	To be paired with temsirolimus in future trials
Beta-glucan	Adjuvant to antibody therapy	Relapsed	I	Memorial Sloan-Kettering Cancer Center	Given together with 3F8 antibody
BSO/melphalan	Chemosensitizer	Relapsed	I	NANT	Requires stem cell support
Fenretinide	Retinoid	Relapsed	I	NANT	Trials for both oral and iv preparations
CEP-701	TrkB inhibitor	Relapsed	I	NANT	Well tolerated oral agent
Nifurtimox	Antiparasitic	Relapsed	I	University of Vermont	Given +/− cyclophosphamide and topotecan
Bortezomib	Proteosome inhibitor	Relapsed	I	University of Michigan Cancer Center	Combined with irinotecan
Bevacizumab	Anti-VEGF antibody	Relapsed	Pilot	Cincinnati Children's Hospital	Given together with vincristine, irinotecan, and temozolomide
Protracted oral etoposide	Anti-angiogenic	Newly diagnosed	Pilot	Texas Children's Hospital	Combined with standard induction chemotherapy
Zactima	Small molecule VEGFR, EGFR inhibitor	Relapsed	I	MD Anderson Cancer Center	Given together with *cis*-retinoic acid
Zoledronic acid	Bisphosphonate	Relapsed	I	NANT	Together with bevacizumab and cyclophosphamide
^{90}Y-DOTA-tyr3-octreotide	Radiopharmaceutical	Relapsed	I	Holden Comprehensive Cancer Center, University of Iowa	Based on adult studies of octreotide-positive tumors
Irinotecan	Camptothecin as radiosensitizer	Relapsed	I	NANT	With ^{131}I-MIBG
MLN8237	Aurora A kinase inhibitor	Relapsed	I	COG	Notable preclinical activity

Each block of sequenced therapy represents an opportunity for improvement, and new therapies should be considered in the context of where they can best fit in to the overall treatment schema. For example, some agents are well suited for induction therapy, while others may be more effective for treating the lower tumor burden that exists after stem cell transplantation. With these concepts in mind, we now review the rationale behind selected clinical trials currently being performed (summarized in Table I). A list of additional therapeutic targets we believe merit consideration for future studies is provided in Table II.

TABLE II. Novel Targets or Approaches With Potential Clinical Utility for Treating High-Risk Neuroblastoma

Target molecule, pathway, function, or potential therapeutic approach	Targeted interaction or mechanism	References
MYCN	Transcriptional regulator of ODC1 expression*	Hogarty et al. [2008]
	Regulation of histone H3 acetylation by MYCN-chromatin interactions*	Cotterman et al. [2008]
	Determinant of NK cell recruitment to tumor foci*	Song et al. [2007]
	Determinant of invasive potential	Tanaka and Fukuzawa [2008]
	Regulation of microRNA expression	Schulte et al. [2008]
Vaccines	Antigens evaluated: MYCB, TH, CD49b, CD59, survivin, GD2, CX3C1	
	Cytokines coexpressed: IL-2, -12, -21	
	GD2-targeted Epstein–Barr virus-specific T-cells*	Pule et al. [2008]
ALK (anaplastic lymphoma kinase)	Inhibition of ALK mutants in a subset of neuroblastoma* (in contrast to inhibition of ALK fusion proteins in lymphoma)	McDermott et al. [2008]
Interferon β or γ	Sensitizes neuroblastoma cells to doxorubicin, temozolomide or cyclophosphamide*	Tong et al. [2008], Sims et al. [2008]
Neuroblastoma-selective therapy	Using tumor-tropic stem-like cells of neural, mesenchymal or lipid origin*	Aboody et al. [2006], Danks et al. [2007]
	Using liposomes targeting GD2 or various membrane proteins	DiPaolo et al. [2008] (review)
	Using liposomes engineered to localize to the extracellular milieu of tumors, to deliver prodrugs	
Invasion/metastasis	ICAM-2 (intercellular adhesion molecule-2) eradicates development of disseminated tumors in a murine model of metastatic neuroblastoma*	Yoon et al. [2008]
	NCAM, ICF-1R, integrin α1, CD44, GPRP	Valentiner et al. [2008]; Qiao et al. [2008]
MDM2	Inhibition of MDM2 sensitizes neuroblastoma cells to DNA-damaging agents, with preferential induction of apoptosis rather than G1 arrest*	van Maerken et al. [2006]
Oncolytic virus-metalloproteinase inhibitor	Inhibition of MMPs enhances the efficiency of oncolytic HSV*	Mahller et al. [2008]

*Molecular targets or approaches discussed in the text.

AGENTS CURRENTLY IN CLINICAL TRIALS

CONVENTIONAL CYTOTOXIC THERAPIES

Since there is a direct relationship between response to induction therapy and event-free survival [Matthay et al., 2003], efforts are ongoing to improve the 50–60% rate of complete + very good partial responses seen with contemporary induction regimens [Matthay et al., 1999; Kreissman et al., 2007]. The incorporation of novel drug combinations with documented activity against relapsed neuroblastoma may be one way of achieving this goal. Approximately one-third of patients with first recurrence of neuroblastoma will achieve a complete or partial response with cyclophosphamide and topotecan [Frantz et al., 2004]. A recent pilot study demonstrated the feasibility of incorporating cyclophosphamide and topotecan into induction chemotherapy in newly diagnosed patients [Park et al., 2006], and the efficacy of this approach is now being studied in a large Phase III trial through the Children's Oncology Group (COG).

In a similar fashion, the combination of another alkylating agent (temozolomide) and camptothecin (irinotecan) is also being evaluated for recurrent disease, with the idea of potential incorporation into induction therapy. In addition to showing activity in relapsed patients [Kushner et al., 2006; Wagner et al., in press], this combination is generally less toxic than other regimens used during induction. This improved tolerance may allow for the addition of targeted agents onto this cytotoxic backbone, which is desirable since many targeted therapies are unlikely to cause regression of bulky tumors when given as monotherapy. Agents which synergize with chemotherapy by enhancing the apoptotic response to treatment, such as the proteosome inhibitor bortezomib [Armstrong et al., 2008] or the anti-insulin-like growth factor type I receptor antibody IMC-A12 [Rowinsky et al., 2007], may be particularly attractive for combination studies.

Another logical strategy is to add anti-angiogenic agents, given the vascular nature of neuroblastoma tumors and the correlation between clinical aggressiveness and extent of angiogenesis [Rössler et al., 2008]. Preclinical studies have suggested that the anti-VEGF antibody bevacizumab normalizes tumor-associated vasculature in neuroblastoma models and improves the intratumoral penetration and efficacy of campthothecins [Dickson et al., 2007]. This study provided the rationale for pilot trials combining bevacizumab with irinotecan-containing regimens, as has been done successfully for colon cancer [Hurwitz et al., 2004] and glioblastoma [Vredenburgh et al., 2007]. Tumor-associated vasculature can also be targeted with low doses of conventional chemotherapeutic agents given frequently over long periods of time. This metronomic strategy may target endothelial cells more than tumor cells, and has been modestly successful in stabilizing disease when using agents such as cyclophosphamide, etoposide, and vinblastine [Kieran et al., 2005; Stempak et al., 2006]. This concept is currently being evaluated in a Texas Children's Hospital trial adding low-dose daily oral etoposide to conventional induction chemotherapy. Of note, endothelial cells had previously been considered attractive therapeutic targets because they were presumed to be of host and not tumor origin. This assumption has recently been called into question by a recent study in which tumor-specific genetic changes (i.e., *MYCN* amplification) were observed in endothelial cells associated with primary tumors, thus raising the question of whether these cells may also be genetically unstable and prone to chemotherapy resistance [Pezzolo et al., 2007].

Modulation of the resistance to chemotherapy by the administration of chemosensitizing agents has also been proposed. One example is the use of buthionine sulfoximine (BSO), a selective inhibitor of glutathione synthesis, together with the alkylating agent melphalan. This combination has shown impressive preclinical synergy [Anderson et al., 2001], and a clinical trial is ongoing which exploits the steep dose-response curve of this combination by using stem cell support following administration of BSO and increasing doses of melphalan. Another potential chemosensitizing approach involves the use of O^6-benzylguanine to circumvent the activity of the DNA repair protein methylguanine-DNA methyltransferase (MGMT), a repair protein frequently expressed in neuroblastoma and likely to confer resistance to the anti-tumor activity of temozolomide [Wagner et al., 2007]. This strategy could potentially improve the 20% response rate seen with single-agent temozolomide in relapsed neuroblastoma [Rubie et al., 2006], although clinical development has been limited by the availability of O^6-benzylguanine and the increased myelosuppression of this combination.

RADIOTHERAPEUTIC TARGETS

Since neuroblastoma tumors are generally radiosensitive, another way to improve treatment outcomes may be to deliver more intensive, tumor-specific radiotherapy. ^{131}I-metaiodobenzylguanidine (MIBG) is selectively taken up by norepinephrine receptors, and intravenous infusion of this radiopharmaceutical represents a method of selectively delivering radiotherapy specifically to tumor cells. One main inclusion criteria for MIBG therapy is demonstration that the tumor is identifiable on a diagnostic ^{123}I- or ^{131}I-MIBG scan, as is the case for approximately 90% of patients. The feasibility and accessibility of this treatment continue to improve, and a growing number of centers worldwide are now capable of delivering this therapy.

^{131}I-MIBG consistently has the highest response rate of any treatment for relapsed neuroblastoma [reviewed in DuBois and Matthay, 2008], and recent trials through the new approaches to neuroblastoma (NANT) consortium have demonstrated that MIBG therapy can be safely combined with high-dose CEM given 2 weeks after ^{131}I-MIBG and using peripheral blood stem cell support to assist hematopoietic recovery [Matthay et al., 2006]. Although MIBG therapy has typically been reserved for patients with relapsed or refractory disease, a European trial has shown this treatment can be given together with induction therapy for newly diagnosed patients [de Kraker et al., 2008]. The COG is planning a pilot trial to test the feasibility of MIBG + high-dose CEM as consolidation for newly diagnosed patients, with the ultimate strategy of using this as a potential treatment arm for patients with "ultra-high-risk" disease that have no response to conventional induction chemotherapy. Other potential strategies include the addition of radiosensitizers such as irinotecan or the histone deacetylase inhibitor SAHA (vorinostat) together with MIBG therapy.

The original method for preparing the clinical formulation of ^{131}I-MIBG was relatively inefficient and contained unlabeled "carrier" *meta*-iodobenzylguanine (MIBG). Unlabeled MIBG was determined to compete with radiolabeled MIBG for cellular uptake by norepinephrine receptors, thereby potentially diminishing the therapeutic effectiveness. Ultratrace™ is a no-carrier added formulation of ^{131}I-MIBG which is now being tested in a Phase I clinical trial for patients with relapsed neuroblastoma, based on the hypothesis that removal of the carrier will result in greater uptake by tumor cells and improved anti-tumor effects.

Additional delivery systems for radiopharmaceuticals include conjugation with the anti-GD2 antibody 3FB. This radiolabeled antibody has been used in newly diagnosed patients in conjunction with the intensive N7 induction regimen at Memorial Sloan-Kettering Cancer Center [Cheung et al., 2001]. Further, since neuroblastoma tumors can also be identified with octreotide scans, the use of radiolabeled octreotide [Forrer et al., 2008] is also being preliminarily explored.

RETINOIDS

The benefit of relatively unconventional "biologic" anti-tumor agents for therapy of neuroblastoma was first demonstrated in a landmark trial by the Children's Cancer Group in which administration of the differentiating agent CRA following recovery from high-dose chemotherapy and stem cell transplantation improved the 3 year event-free survival of patients with high-risk neuroblastoma [Matthay et al., 1999]. Use of CRA in this setting has now become standard therapy. Neuroblastoma is the only common pediatric solid tumor to date in which anything other than conventional cytotoxic chemotherapy is routinely used as part of front-line therapy. The study by Matthay et al. also established the benefit of "maintenance therapy," based on the observation that over half of patients evaluated by conventional criteria to be in complete remission relapsed with progressive disease. This observation inferred that complete cures would require not only elimination of readily identifiable tumor, but also eradication of minimal residual disease that remains after high-dose therapy. CRA exerts growth inhibitory and differentiating effects through its interaction with nuclear retinoid receptors, which regulate expression of multiple target genes. Importantly, the full benefits of this agent likely depend on achieving adequate drug concentrations. This concept is illustrated by a European study of patients with advanced neuroblastoma who had good responses to myeloablative therapy but who showed no benefit from retinoic acid 0.75 mg/kg daily for up to 4 years [Kohler et al., 2000]. These results contrast sharply with the Children's Cancer Group study in which a CRA dosage seven times higher than that used in the European study was administered for 2 weeks every month for 6 months significantly improved event-free survival [Matthay et al., 1999]. In addition to dosage, selection of the appropriate patient population appears important as well. In the first Phase II trial of CRA using a single daily dose of 100 mg/m^2, little activity was seen in patients with bulky relapsed disease [Finklestein et al., 1992], and the agent may have been discarded if attention had not been given to optimizing both the dosing schedule and the patient population to whom it was administered.

Because CRA is fairly well tolerated as a single-agent, it may also serve as a potential backbone on which to build combination therapies. Currently, the COG is performing a Phase III trial comparing single-agent CRA versus the combination of CRA and the chimeric anti-disialoganglioside (GD2) antibody Ch14.18 for the treatment of minimal residual disease. In addition, a pediatric Phase I trial of CRA combined with the histone deacetylase inhibitor SAHA (vorinostat) has been completed [Fouladi et al., 2008], and further study is reasonable based on the preclinical single-agent activity of SAHA and synergy with CRA in neuroblastoma models [De los Santos et al., 2007]. Another combination also being explored is CRA plus ZD6474 (Zactima), a dual small molecule inhibitor of receptors for vascular endothelial growth factor and epidermal growth factor [Natale, 2008].

The success of CRA has also initiated interest in developing retinoids that may be more effective in patients with measurable disease. Fenretinide is a synthetic retinoid analogue that has dose-related cytotoxicity against neuroblastoma cell lines in vitro, in part by increasing intracellular levels of ceramide [Lovat et al., 2004]. Results of a Phase I trial with fenretinide demonstrate that although toxicities are modest, feasibility was a significant issue due to a capsular formulation that is difficult to administer to small children [Villablanca et al., 2006]. This has led to NANT trials of intravenous fenretinide, as well as a new orally administered lipid matrix formulation called Lym-X-Sorb™, designed for a more favorable pharmacokinetic profile [Maurer et al. 2007].

ANTI-GD2 ANTIBODIES

The ganglioside GD2 is a glycolipid that is expressed by almost all neuroblastoma tumors, and as such represents a molecular target that can be exploited both diagnostically and therapeutically. Monoclonal antibodies against GD2 kill tumor cells through both complement and cell-mediated lysis [reviewed in Modak and Cheung, 2007]. Anti-GD2 antibodies have been used in clinical trials for the past decade, and results from hundreds of patients treated with this agent suggest that benefit is greatest when used in patients with minimal residual disease rather than bulky tumors. Early studies with murine antibodies such as 3F8 were complicated by the generation of human anti-mouse antibodies (HAMA) which may limit continued antibody therapy. One of several strategies designed to reduce the development of HAMA is the use of high-dose cyclophosphamide to minimize the immune response [Kushner et al., 2007]. Chimeric mouse-human antibodies such as Ch14.18 also appear to be less immunogenic than 3F8 and potentially improve anti-tumor activity, although up to one-fourth of patients still develop human anti-chimeric antibodies [Ozkaynak et al., 2000].

Other attempts to improve the utility of ant-GD2 antibodies include the use of beta-glucan, a naturally occurring glucose polymer that augments the efficacy of anti-GD2 antibodies in preclinical studies, albeit through poorly understood mechanisms [Cheung and Modak, 2002]. This approach is currently being tested in a clinical trial at Memorial Sloan-Kettering Cancer Center in which oral beta-glucan is given in combination with the murine 3F8 antibody. Cytokines may also be useful to activate effector cells in vivo and improve the benefits of anti-GD2 antibodies [Kushner and

Cheung, 1989; Lode et al., 1998]. A second current trial focuses on administering interleukin-2 and granulocyte–macrophage colony-stimulating factor along with ch14.18 [Gilman et al., in press]. This approach is being studied in a randomized COG Phase III trial in which patients receive CRA alone versus antibody + IL-2 + GMCSF + CRA following high-dose chemotherapy and stem cell transplant. Another strategy is to use a fully humanized antibody linked as a fusion protein to human recombinant IL-2 (hu14.18-IL-2 antibody), theoretically reducing immunogenicity and activating effector cells. As seen with previous anti-GD2 trials, this therapy had encouraging activity for patients with limited disease (i.e., 29% response rate for patients with only bone marrow involvement), but no responses were seen in children with bulky tumors [Shusterman et al., 2008]. A goal of the ongoing COG Phase III study is to better define the benefits of antibody therapy given to a select population of patients presumed to harbor minimal residual disease.

ANTIMITOTICS

The vinca alkaloid vincristine, an inducer of mitotic arrest, has been part of several neuroblastoma induction regimens, although the development of resistance due to expression of MDR1 or to expression of mutated β-tubulin may render this agent less effective. ABT-751 is a novel oral anti-mitotic agent that binds to the colchicine binding site rather than the vincristine binding site on β-tubulin, and inhibits polymerization of microtubules. Importantly, ABT-751 is active against tumor models that are otherwise resistant to vincristine and doxorubicin [Morton et al., 2007]. A COG Phase II study of this compound is currently underway, based on Phase I results showing an encouraging median time-to-progression of 16 weeks in 35 children with relapsed neuroblastoma [Fox et al., 2008]. Of note, the primary objective of the ongoing Phase II trial is to determine the time to progression, rather than response rate, of this regimen. Time to progression represents a new clinical endpoint for neuroblastoma trials, and can be considered for therapies in which long-term administration is feasible and compatible with a good quality of life.

Vinblastine is another vinca alkaloid that may have clinical benefit for neuroblastoma. Preclinical models demonstrate that vinblastine may have cytotoxic effects not just on tumor cells but also on the microvessel density, possibly by downregulating VEGF and VEGFR2 expression [Marimpietri et al., 2007]. These effects are augmented with the addition of the mTOR inhibitor rapamycin, which itself may have downstream effects on angiogenesis [Humar et al., 2002]. Combined treatment with both agents prolonged the lifespan of mice with experimental neuroblastoma compared to either treatment alone [Marimpietri et al., 2007], leading to plans for a NANT trial of vinblastine plus the rapamycin analogue temsirolimus.

Another anti-mitotic agent currently being investigated is MLN8237, an inhibitor of Aurora A kinase. This protein is critical for centrosome maturation and spindle formation during mitosis, and its expression has generally been associated with tumors with gene amplification, poor histologic differentiation, and poor prognosis [Gautschi et al., 2008]. In the pediatric preclinical testing program panel, MLN8237 treatment resulted in maintained complete responses in three of four neuroblastoma models, a relatively impressive result that exceeds the preclinical activity of known active agents such as cisplatin and cyclophosphamide [Houghton et al., 2008]. This agent is currently in Phase I testing through the COG.

BISPHOSPHONATES

Neuroblastoma tumors with unfavorable histologic or genetic features have a remarkable predilection to develop bone metastases, which substantially contribute to the morbidity and mortality of this disease. Neuroblastoma bone metastases recruit osteoclasts to sites of tumor growth, leading to painful destruction of bone. Zoledronic acid (Zometa) is a new-generation bisphosphonate that binds to bone matrix. During pathologic bone resorption the drug is internalized by osteoclasts, leading to induction of apoptosis of these cells possibly by inhibiting farnesyl diphosphate synthase and/or the GTPase proteins Ras and Rho. Zoledronic acid is widely used to delay or treat bone metastases in adult malignancies such as breast and prostate cancer [Lipton, 2008]. Its use is now being studied for neuroblastoma in combination with metronomic oral cyclophosphamide, based on preclinical experiments showing a delay in bone metastases and improved survival of tumor-bearing mice treated with these agents [Peng et al., 2007]. Interestingly, because of its binding to bone matrix, serum levels of zoledronic acid are far less than those achieved in cortical bone; therefore, this agent may be most useful for bone lesions rather than soft tissue or bone marrow disease. A COG pilot trial combining zoledronic acid with conventional chemotherapy for metastatic osteosarcoma is currently underway, and this study should provide some estimation on whether the addition of zoledronic acid may be feasible during neuroblastoma induction therapy.

NIFURTIMOX

Saulnier Sholler et al. [2006] have recently reported the serendipitous response of a patient with progressive neuroblastoma who was being treated for Chagas disease with the anti-parasitic agent nifurtimox. Although the coadministration of cyclophosphamide and topotecan make the attribution of response difficult, nifurtimox had cytotoxic effects against a neuroblastoma cell line in vitro. The mechanism of action was not specifically investigated, but may involve the generation of free radicals as seen in the treatment of parasites. This observation has led to the development of a Phase I trial of nifurtimox alone and in combination with cyclophosphamide and topotecan.

mTOR, IGF-1, and AKT

Signaling through the mammalian target of rapamycin (mTOR) pathway is important for the growth and survival of many pediatric solid tumors, including neuroblastoma. Treatment with mTOR inhibitors causes downregulation of expression of multiple gene products including *VEGF-A* and *MYCN*, and inhibits growth of neuroblastoma xenografts [Johnsen et al., 2008]. Supporting preclinical observations, a recent Phase I trial of the rapamycin analogue temsirolimus demonstrated a complete response in a patient with recurrent neuroblastoma [Spunt et al., 2008]. Phase II testing is ongoing. Temsirolimus is also being studied in combination with irinotecan as an upfront Phase II window during

induction for newly diagnosed patients at St. Jude Children's Research Hospital.

One limitation of mTOR inhibition is the potential for activation of AKT due to circumvention of negative feedback mechanisms [O'Reilly et al., 2006]. This phenomenon occurs in a variety of tumor types, and has been associated with shorter time to progression in a clinical trial [Cloughesy et al., 2008]. Importantly, this escape mechanism can be uniquely abrogated by inhibition of signaling through the insulin-like growth factor type I receptor. This receptor (IGF-1R) is expressed by a majority of neuroblastoma tumors, and its overexpression is associated with anti-apoptotic effects and chemotherapy resistance [Singleton et al., 1996]. Tumor regressions and improved event-free survival have been noted when small molecule inhibitors or antibodies against IGF-1R are used to treat mouse models of neuroblastoma [Liu et al., 1998; Kolb et al., 2008]. As predicted by preclinical investigation with various tumor types, synergy with inhibitors of IFG-1R and inhibitors of mTOR has also been demonstrated against neuroblastoma [Coulter et al., 2008]. The COG is planning Phase I and II trials of the IGF-1R antibody IMC-A12 as a single-agent and in combination with temsirolimus for neuroblastoma patients.

The importance of the AKT pathway in cell survival and proliferation has recently been demonstrated in neuroblastoma, and activation of AKT correlates with worse event-free and overall survival [Opel et al., 2007]. Of note, inhibition of AKT in certain neuroblastoma cell lines destabilizes MYCN protein [Chesler et al., 2006]. Although no specific inhibitors of AKT are clinically available at this time, intense investigation continues in this area, given the role of AKT in multiple pathways associated with tumor growth and response to therapy [Radhakrishnan et al., 2008; Sartelet et al., 2008].

CEP-701

The Trk family of neurotrophin receptors is critical in regulating the biology of neuroblastoma tumors. In particular, the TrkB-mediated signaling pathway functions in an autocrine fashion to promote survival of high-risk tumors [reviewed in Schramm et al., 2005]. This pathway is targeted by the small molecule inhibitor CEP-701 (lestaurtinib). This agent, given orally, is associated with moderate gastrointestinal and hepatic toxicities in an ongoing NANT Phase I trial of relapsed neuroblastoma patients, allowing for its potential combination with conventional chemotherapy regimens such as cyclophosphamide/topotecan or temozolomide/irinotecan. If tolerable, such combinations could then be considered for incorporation into induction therapy.

BORTEZOMIB

Bortezomib is a proteosome inhibitor that is currently approved for use in multiple myeloma. Bortezomib induces apoptosis in part through activation of caspases [Combaret et al., 2008], and inhibits tumor growth and angiogenesis in vivo [Hamner et al., 2007]. This inhibition is associated with improved duration of survival but no complete regressions without regrowth in a mouse model of metastatic disease [Brignole et al., 2006]. Preclinical models also demonstrate synergistic or additive anti-tumor activity with bortezomib and more conventional chemotherapeutic agents [Armstrong et al., 2008]. These preclinical data provided the rationale for a clinical trial at the University of Michigan investigating bortezomib in combination with irinotecan for patients with relapsed neuroblastoma.

STRATEGY FOR INCORPORATION OF NEW AGENTS

Historically, targeted therapies have been first tested in patients with clinical evidence of relapsed or refractory neuroblastoma. However, the experience with CRA and with anti-GD2 antibodies suggests that some agents will show efficacy only in patients with minimal tumor burdens. Therefore, more recent studies in the COG are stratified so that patients with bulk disease are analyzed separately from those whose tumor is only identified by bone marrow evaluation or MIBG imaging, with the hope of identifying agents that have activity provided the tumor burden is sufficiently low. These trials also establish the toxicity profile of the agent, which along with the proposed anti-tumor mechanism of action, provides a basis for determining which agents may be appropriate to combine with specific conventional chemotherapy for use during induction or maintenance therapy or both.

Because the majority of neuroblastoma patients who fail therapy do so after an initial good response, it would be helpful to identify the patients who have increasing minimal residual disease, so that additional treatment could be initiated while the tumor burden is still low. Multiple studies using sensitive methods such as immunohistochemistry or RT-PCR have shown that residual occult neuroblastoma cells remain in the blood or bone marrow following treatment [reviewed in Beiske et al., 2005]. In early trials, serial testing with RT-PCR after completion of therapy has allowed identification of patients destined for relapse before other clinical signs of treatment failure were evident [Burchill et al., 2001; Fukuda et al., 2001; Cheung et al., 2003]. If these results can be validated in a large prospective clinical trial, then intervention with targeted agents for patients identified to have minimal residual disease would be an attractive strategy.

PRECLINICAL INVESTIGATIONS

Of the numerous potential approaches to therapy for neuroblastoma, several have shown compelling results in preclinical models. In the following section, we highlight selected molecules, pathways or strategies that appear promising as points of therapeutic intervention (Table II).

MYCN

Amplification of the *MYCN* gene is an independent prognostic indicator for poor outcome of patients with neuroblastoma, and so this pathway represents an obvious therapeutic target. However, MYCN protein appears to have various functions, and the specific function(s) that contributes to tumor development and progression is unknown. Interestingly, data of Tang, et al. argue that inhibition of MYCN protein function would have anti-tumor effects only in MYCN-amplified, MYCN-overexpressing tumors, consistent with their observation that MYCN-overexpression in

MYCN-non-amplified tumors is associated with favorable clinical outcome [Tang et al., 2006]. Therefore, assuming for discussion that inhibition of MYCN protein function is therapeutically desirable, several MYCN-associated functions merit consideration as molecular targets. At a minimum, MYCN is a transcription factor that positively regulates expression of multiple genes including ornithine decarboxylase (ODC). Interestingly, Hogarty et al. [2008] recently proposed that ODC1 expression is a critical determinant of MYCN-mediated neuroblastoma oncogenesis and that MYCN is a key regulator of ODC1 expression, with the inference being that inhibition of ODC would have anti-tumor efficacy. Of particular interest was the evaluation of combinations of α-difluoromethylornithine (DFMO), an ODC inhibitor, with cyclophosphamide or with cisplatin. DFMO + cyclophosphamide increased the tumor-free survival of 3-month-old TH/MYCN +/+ mice to 80%, compared to 20% with cyclophosphamide alone. While the results with DFMO + cisplatin in this model were not as impressive as DFMO + cyclophosphamide and the toxicity of DFMO may limit its use clinically, the potential utility of ODC1 (or the polyamine synthesis pathway) as a therapeutic target in neuroblastoma merits further investigation.

Another important function of MYCN protein may be its regulation of histone acetylation and methylation through its interactions with thousands of genic and intergenic sites, thereby indirectly influencing the expression and/or function of a myriad of gene products. Based on demonstrated safety in clinical trials of histone deactylase inhibitors, it may be possible to develop tolerated agents that inhibit, in a relatively selective manner, MYCN/chromatin interactions. Whether such inhibitors would nullify the oncogenic functions of MYCN is unknown; but if the hypothesis of Cotterman et al. [2008] is correct then such an inhibitor might impact multiple MYCN-mediated effects simultaneously and circumvent the tumor-promoting functions of this protein.

Song et al. [2007] recently reported that fourfold fewer natural killer T-cells (NKT) were found in the bone marrow of *MYCN*-amplified patients with marrow disease compared to non-amplified controls. Interestingly, NKT localization to tumor sites correlates with favorable outcome in neuroblastoma patients, but no direct evidence supports the hypothesis that NKT cells contribute directly to immune-mediated anti-tumor responses. Further, the significance of the observed fourfold difference in specimens from 25 patients is not known. The suggestion that MYCN initiates or mediates a unique immune escape mechanism in neuroblastoma tumors is intriguing. Unique MYC-mediated protein–protein interactions that enable high-risk neuroblastoma tumors to escape immune recognition might represent useful therapeutic targets.

VACCINES

Alternatively, Himoudi et al. [2008] suggested that, rather than attempt to inhibit MYCN expression or function, a relatively high level of MYCN expression could be exploited by using a vaccine approach to stimulate immune responses to MYCN-expressing cells. This idea is intuitively attractive from the standpoint that normal tissues express low-levels of MYCN and an MYCN-based vaccine may have a commensurately low potential for eliciting an autoimmune response. This idea is also interesting from the standpoint of attempting to exploit a ''negative'' characteristic of aggressive neuroblastoma tumors, which may potentially be more achievable than selectively inhibiting MYCN expression or its multiple functions sufficiently to impact tumor cell viability. However, as has been true for numerous other attempts to design effective vaccines to treat neuroblastoma, the potency of this approach was moderate even in an in vitro assay. These results are similar to the relatively modest anti-tumor responses noted with vaccines to multiple other neuroblastoma tumor antigens including tyrosine hydroxylase [Huebener et al., 2008], CD49b [Yan et al., 2008], CD59 [Donev et al., 2008], survivin [Coughlin et al., 2006], GD2 [Bolesta et al., 2005; Fest et al., 2006], and fractalkine (CX3CL1)[Zeng et al., 2007]. Similarly, equivocal responses have been observed with vaccines engineered to express interleukin-2, -12, and/or -21 [Barker et al., 2007; Croce et al., 2008]. Collectively, these results suggest that ''simple'' immunostimulatory approaches are unlikely to mediate dramatic or sustained responses, due to multiple factors that may include overwhelming tumor burden at time of administration, weakly immunogenic target antigen, tumor-initiated immune evasion, lack of expression of costimulatory molecules by tumor cells, and transient association of T-cells with tumor cells.

Recently, Pule et al. [2008] addressed concerns of tumor-selectivity, duration of survival of targeted T-cells, and strength of immune response by engineering Epstein–Barr virus-specific cytotoxic T-lymphocytes to express a GD2-specific antigen receptor (a sequence derived from the GD2 recognition sequence of the 14G2a antibody). These modified T-cells survived for weeks in vivo and were nontoxic. One patient of 11 who were treated with this vaccine achieved a sustained (>12 months) remission, and 3 others had transient responses. Most enigmatic was the lack of detectable engineered T-cells at necrotic tumor sites in biopsy specimens and the inability to relate responses to tumor size, genetic markers or dose of T-cells, prompting the authors of that study to suggest that ''. . . observed tumor responses may have resulted from indirect mechanisms of cytotoxicty.'' Perhaps, redundancy of function of cellular and molecular components that contribute to immune responses will make it very difficult to develop effective immune-based therapies for neuroblastoma. It is likely that substantial benefits from vaccines may require combination with more conventional approaches.

ALK (ANAPLASTIC LYMPHOMA KINASE)

There is recent interest in the possibility that the gene product of the anaplastic lymphoma kinase (ALK) gene might represent a therapeutic target for neuroblastoma. New studies suggest activating mutations of *ALK* are present in 8–12% of primary high-risk neuroblastoma tumors [George et al., 2008; Mossé et al., 2008]. ALK is a receptor tyrosine kinase that comprises part of a protein fusion that results from a chromosomal translocation present in a majority of large cell lymphomas [Ma et al., 2000]. Several ALK inhibitors have been developed, and tumor cells expressing ALK fusion proteins are exquisitely sensitive to these inhibitors [McDermott et al., 2008]. The situation with neuroblastoma differs somewhat from lymphomas and non-small-cell lung cancers in that the latter

tumors express fusion proteins that include ALK. In contrast, neuroblastomas express mutated forms of ''un-fused'' ALK. Neuroblastoma cell lines that express a specific mutant ALK vary in their sensitivity to ALK inhibitors, suggesting that the anti-tumor responses to ALK inhibitors may also vary. Further development of ALK inhibitors and their combination with conventional chemotherapy are likely forthcoming.

INTERFERONS β AND γ

Developing approaches to increase the efficacy of agents with demonstrated anti-tumor activity for neuroblastoma patients seems an obvious approach toward designing better therapies for this disease. Published data indicate that exposure of neuroblastoma cells to interferon γ prior to doxorubicin [Tong et al., 2008] or exposure to interferon β prior to temozolomide or cyclophosphamide [Rosati et al., 2008; Sims et al., 2008] may enhance the anti-tumor efficacy of these drugs. Because of the systemic toxicity of relatively high-levels of interferon β in plasma, Sims et al. used neural stem/progenitor cells to deliver this cytokine selectively to orthotopic neuroblastoma tumors in murine models, since intravenously administered neural stem/progenitor cells are inherently tumor-tropic. The combination of local expression of interferon β by intravenously administered stem cells and cyclophosphamide significantly enhanced the anti-tumor effect of the cyclophosphamide. Importantly, no increase in plasma concentration of interferon β was detected, suggesting that neural stem cells may comprise effective tumor-selective delivery vehicles in neuroblastoma.

NEUROBLASTOMA-SELECTIVE DELIVERY METHODS

Neural stem cells have also been used to selectively deliver enzymes such as rabbit carboxylesterase (CE), which efficiently activates the prodrug irinotecan. CE-expressing stem cells migrated to tumor sites irrespective of tumor size or anatomic location [Aboody et al., 2006], and in vivo levels of CE expression were sufficient to achieve >1 year tumor-free survival in 90% of mice. Importantly, those studies used clinically relevant doses of irinotecan, and circulating levels of CE and SN-38 (the active form of irinotecan) were not elevated [Danks et al., 2007]. These results demonstrated that stem cell delivery of the therapeutic transgene CE increased the therapeutic efficacy of irinotecan, without added toxicity. The use of stem-like cells harvested from various sources (bone marrow, lipid, or neural tissue) as tumor-selective delivery vehicles has tremendous untapped potential for tumor-selective delivery of well-chosen therapeutic genes and gene products.

While multiple other approaches have been evaluated for their potential to achieve tumor-selective delivery, targeted liposomes have been investigated specifically for neuroblastoma. Liposomes have been designed to bind with high affinity to various membrane proteins or the ganglioside GD2 and used to deliver various chemotherapeutic agents [Pastorino et al., 2006 and reviewed in DiPaolo et al., 2008]. Liposomes have also been designed to localize to the extracellular milieu of neuroblastoma tumors and deliver prodrugs that are activated by enzymes secreted by neuroblastoma cells [Wu et al., 2006]. Cell- or liposome-based delivery of therapeutic moieties has the potential to enhance the therapeutic efficacy and the therapeutic index of multiple approaches to therapy of metastatic and/or residual neuroblastoma.

INVASION/METASTASIS

Specific attempts to limit the metastatic potential of neuroblastoma cells is a worthy therapeutic goal, since patients who die from neuroblastoma usually do so because of uncontrolled metastatic disease. The major obstacle to be overcome for development of this type of approach is the identification of specific molecular targets that act as key control points for the invasive process. Yoon et al. [2008] recently reported the identification of such a protein. These investigators observed that while intercellular adhesion molecule-2 (ICAM-2) had no effect on tumorigenic potential of neuroblastoma cells, expression of this membrane protein eradicated development of disseminated disease in a murine model of metastatic neuroblastoma. ICAM-2 expression limited tumor cell motility, likely through interactions with the actin cytoskeleton, a known primary determinant of cell motility. Further, an association between ICAM-2 expression and favorable outcome was observed by immunohistochemical analysis of primary tumor specimens. Since ICAM-2 is expressed by few normal tissues, ICAM-2-associated molecular interactions may comprise useful targets to limit the invasive potential of neuroblastoma cells.

A primary consideration in evaluating ICAM-2 and other adhesion molecules as potential therapeutic targets is the degree to which each protein controls the invasive process. In the study noted above, 100% of mice injected with neuroblastoma cells expressing relatively high-levels of ICAM-2 survived tumor-free for >1 year, in contrast to 0% survival of mice injected with neuroblastoma cells that expressed little or no ICAM-2. Many other adhesion molecules such as NCAM, IGF-1R, and integrin α1 appear to modulate the invasive potential of neuroblastoma cells, but it is unclear that these proteins regulate essential processes that cannot be performed by other proteins with redundant functions. Therefore, the utility of the latter three proteins as primary regulators of the invasive step of metastasis, and therefore their utility as therapeutic targets, is doubtful. Recent reports suggest that CD44 and gastrin-releasing peptide receptor (GRPR) may also be key regulators of the metastatic potential of neuroblastoma [Qiao et al., 2008; Valentiner et al., 2008], although published data investigating the impact of GRPR on development of metastatic neuroblastoma in a murine model evaluated mice only at relatively short time points. More extensive in vivo experiments will be necessary to adequately evaluate whether GRPR is a key regulator of the metastatic potential of neuroblastoma cells. We propose that pathways in which CD44 and ICAM-2 participate merit further investigation as potential therapeutic targets.

MDM2

Prior to therapy, most neuroblastoma tumor cells express wild-type p53. While p53 has multiple functions, in neuroblastoma cells exposed to DNA-damaging agents the predominant function of p53 is to induce apoptosis [McKenzie et al., 1999]. MDM2 ubiquitinates p53 and targets it for degradation. In theory, inhibition of MDM2

in neuroblastoma cells that express wild-type p53 would increase levels of p53 protein and enhance induction of apoptosis by DNA-damaging agents such as doxorubicin or temozolomide. van Maerken et al. [2006] confirmed that inhibition of MDM2 by nutlin-3 increased the p53 content of neuroblastoma cells expressing wild-type p53. The obvious next experiment is to test the hypothesis that nutlin-mediated inhibition of MDM2 sensitizes neuroblastoma cells that express wild-type p53 to DNA-damaging agents. We could find no reports in the literature that examined this hypothesis. Intuitively, MDM2 inhibitors such as the nutlin class of agents has untapped potential for the treatment of neuroblastoma, and the utility of MDM2 inhibitors needs to be evaluated in combination with chemotherapeutic agents that induce DNA damage.

ONCOLYTIC VIRUS–METALLOPROTEINASE INHIBITOR

While the concept of oncolytic or selectively replicative virus for anti-tumor treatment is not new, few efforts of this type have been reported for treating neuroblastoma. Recently Mahller et al. [2008] evaluated the use of an oncolytic Herpes simplex virus. Particularly noteworthy was the ''arming'' of this virus to express TIMP3, a member of the tissue inhibitor metalloproteinase protein (MMP) family. TIMP3 has been reported to inhibit all known MMPs. Further, relatively low-levels of expression of TIMPs correlate with unfavorable prognosis in neuroblastoma. The hypothesis in the study conducted by Mahller et al. was that inhibition of metalloproteinases would augment the anti-tumor effect of the HSV-derived oncolytic virus. Mice bearing subcutaneous LA-N-5 neuroblastoma xenografts that had been treated with multiple doses of the oncolytic HSV-TIMP3 harbored lower tumor burdens a month after implantation than mice treated with unmodified oncolytic HSV. While a month's follow-up does not provide compelling support for the approach, it is noteworthy that, in contrast to control and HSV only treatment groups, tumors in HSV-TIMP3-treated mice became detectable only after treatment was discontinued. Possibly, optimization of the construct and/or of administration dose and schedule will improve this dual faceted approach. Of particular interest will be the development of strategies that circumvent the need for intratumoral injection, and identification of oncolytic viral approaches which can be combined with conventional chemotherapy.

CONCLUSION

Our understanding of the molecular biology and clinical behavior of neuroblastoma tumors has expanded dramatically in the past two decades. The current challenge is to use this understanding as the basis for translational applications that effectively and selectively target neuroblastoma cells for eradication of minimal residual disease in high-risk patients.

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