RESEARCH ARTICLE

Influence of Glycosylation on the Development and Treatment of Neuroblastoma

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ABSTRACT

Neuroblastoma is a solid malignancy observed in pediatric patients developing when neuroblasts are unable to mature, leading to unregulated proliferation and tumor formation. Neuroblastoma is heterogeneous and aggressive in nature, leading to high treatment failure, morbidity, and mortality rates. Lewis family glycans, as part of the Core 2 O-glycans, play a key role in neuroblastoma malignant cell behavior in MYCN-amplified cell lines. Current treatment approaches for neuroblastoma include chemotherapy, surgery, and radiation. These approaches are faced with physiological and cellular barriers, including the less understood role of glycosylation in development and treatment. Studies have confirmed that the inhibition of mucin glycosylation has improved effectiveness of cytotoxic drug agents employed against solid malignancies such as with pancreatic cancer, yet little research is available regarding the influence of glycosylated proteins for other diseases. This article explores genetic defects associated with neuroblastoma such MYCN gene amplification at the time of diagnosis, as well as clinical approaches and therapeutic challenges encountered during treatment. Additionally, the article reviews experimental and clinical evidence in support of the influence of glycosylation in neuroblastoma development, and possible unfavorable impact of glycosylation on drug therapy.

Keywords: Neuroblastoma, MYCN, Glycosylation, Mucin, pediatric cancer
Background

Neuroblastoma is the most common malignancy found in children under one year of age, representing approximately 6% of all childhood cancers.\(^1\)\(^,\)\(^4\) It is the third most common childhood cancer and most common extracranial tumor in children where 15% of the cases lead to death.\(^3\) More than 700 cases are diagnosed every year in the United States.\(^1\) Around 90% of cases are diagnosed in children by the age of 5, and the disease is rarely observed in children over the age of 10.\(^1\)\(^,\)\(^3\)\(^,\)\(^4\)

According to the United States SEER (Surveillance Epidemiology and End Results) program, neuroblastoma and hepatoblastoma had the highest incidence rates between 1975-1995.\(^3\) Historically, early diagnosis before age of 5 improved the overall 5-year survival rate. For high-risk patients there is a 50% survival rate while low-risk patients estimated around 95%.\(^2\) In a retrospective study from 1973-2013, the incidence of neuroblastoma decreased overtime due to the advancement of diagnostic techniques which helped differentiate neuroblastoma from other tumors. There was no significant difference in disease occurrence between genders or between Caucasians and African Americans. However, African American and Native American patients were likely to have higher risk of disease and a lower chance of survival compared with Caucasians. Asians had a relatively lower incidence compared to other races. The study concluded that the combination of early diagnosis and clinical interventions contributed significantly to improved patient survival rates, and better clinical outcomes overall.

Neuroblastoma is a result of malignant cell formation involving immature nerve cells, also known as neuroblasts.\(^2\) Typically, neuroblasts mature or have the ability to undergo apoptosis to prevent malignant formations.\(^1\) However, on occasion, neuroblasts do not fully mature and neuroblastoma develops. These immature nerve cells continue to proliferate forming a palpable mass in the individual.\(^1\) The malignant masses generally arise in the adrenal gland but may also form in the neck, chest, or spinal cord.\(^2\) Common initial signs and symptoms include a palpable mass, proptosis, periorbital ecchymosis, bowel dysfunction, abdominal discomfort associated with low appetite, hepatomegaly, fever, hypertension, and leg weakness.\(^4\) It is not uncommon for metastasis to occur at the time of diagnosis in children.\(^1\) In fact, at the time of diagnosis two-thirds of patients have clinical evidence of metastasis.\(^5\) Common sites of metastasis include the lymph nodes, liver, lungs, bone marrow, and central nervous system.\(^5\) With only 1 to 2% of neuroblastoma cases being familial, the majority of cases are sporadic, or not inherited.\(^1\) Little is known about the cause of neuroblastoma, and no known preventative measures can be taken.

At diagnosis, neuroblastoma is categorized by its risk level as either low, intermediate, or high risk.\(^6\) Some determinants of risk level include the child’s age, DNA ploidy, chromosomal aberrations, and amplification of the MYCN gene.\(^7\) These determinants of risk stratification establish the prognosis of the child. The worst prognosis has been reported for children >18 months of age having diploid or near tetraploid cells and showing amplification of the MYCN genes.\(^7\) MYCN is a proto-oncogene responsible for cell growth and division.\(^6\) When the gene is upregulated, the cells are less likely to respond to treatment.\(^6\) MYCN-amplified cell lines also have higher Lewis glycan expression profile.\(^8\) These sugar moieties have been shown to enhance cell-cell interaction, assisting with the invasive nature and metastasis of neuroblastoma cancer cells.\(^9\) The enzyme responsible for the Core 2 Structure of O-glycans, C2GNT1, was found to be highly expressed in MYCN-amplified cell lines.\(^8\) When C2GNT1 siRNA was introduced to neuroblastoma cell lines, there was a significant decrease in cell adhesion, migration, and proliferation.\(^8\) Therefore, Lewis family glycans, as part of the Core 2 O-glycans, play a key role in neuroblastoma malignant cell behavior in MYCN-amplified cell lines.

MYCN amplification downregulates three endothelial cell proliferation inhibitors, SI-1, SI-2, and SI-3.\(^10\) This downregulation offsets the delicate balance between endothelial stimulators and inhibitors, leading to angiogenesis and progression of the neuroblastoma tumor as well as the poor prognosis associated with MYCN amplification.\(^10\) Another mechanism led by MYCN amplification that creates the shift toward endothelial stimulators is the downregulation of LIF, leukemia inhibitory factor, which is responsible for the inhibition of endothelial cell proliferation.\(^11\) By mechanistically reversing the effects of the inhibitor, the balance shifts towards endothelial growth, resulting in tumor progression and metastasis.\(^11\) Another genetic feature of neuroblastoma that results in poor prognosis are mutations in the ATRX tumor suppressor gene.\(^12\) The mechanism by which mutations in this gene confer worse outcomes is not completely understood, but there is evidence that mutations in the ATRX gene increase with the age of diagnosis. Despite both ATRX mutation and MYCN amplification resulting in a worse prognosis, the two appear not to occur simultaneously.\(^12\)

Another common gene mutation found in neuroblastoma is that of the anaplastic lymphoma
kinase (ALK) gene. ALK is highly expressed in the developing fetus and neonatal brains. ALK is a gene that codes for ALK tyrosine kinase receptors that participate in signal transduction and are important in cell growth and differentiation. Similarly to MYCN, ALK overexpression is responsible for the proliferation, migration, and invasion of tumor cells causing the cell to grow and divide uncontrollably. This mutation is found more in patients with high-risk neuroblastoma. About 75% of familial neuroblastoma and 10% of sporadic cases contain mutations in the ALK gene. There is also potential for ALK gene amplification. When MYCN is amplified there is often co-amplification due to their proximity to each other on chromosome 2p. Hasan et al. observed that the promoter region of the ALK gene was a target of MYC proteins. A genetic knockdown of MYCN resulted in a decrease in the expression of ALK. MYCN amplification also resulted in overexpression of ALK, signifying that MYCN and MYC proteins are regulators for ALK gene transcription and expression.

The GALNT14 gene is yet another noteworthy player in the general context of genetic predispositions to neuroblastoma. This gene is located on chromosome 2p and encodes N-acetylgalactosaminyltransferase 14 (GALNT14). The GALNT family enzymes play a critical role in the initiation of O-glycosylation. The GALNT14 transition mutation resulting in thymine substitution for cytosine at position 802 (c.802C>T) predisposes an individual to neuroblastoma. In addition, overexpression of GALNT14 contributes to the invasiveness of neuroblastoma resulting in poorer overall survival. De Mariano et al. identify a statistically significant relationship between MYCN amplification and GALNT14 overexpression. It is proposed that it is likely due to its proximity to MYCN and ALK genes on chromosome 2p. As clearly shown, several genes predispose an individual to neuroblastoma and therefore can serve as potential targets for treatment.

A predictive factor for prognosis and progression in neuroblastoma is the presence of the CD44 glycoprotein on the surface of the cell. CD44 is responsible for cell-to-cell interactions, leukocyte homing and activation, tumor migration and metastasis, and growth. A functional CD44 receptor is expressed only in the absence of MYCN amplification. In MYCN-amplified cell lines, the CD44 receptor is either not present or not functional, potentially due to the overexpression of N- and O-glycosylation. Those in the high-risk category of neuroblastoma tend to lack a functional CD44 receptor and contain MYCN amplification, ultimately leading to high metastasis and poor prognosis.

Rather than traditional TNM staging, neuroblastoma is classified through a pre-surgical staging system and a post-surgical staging system. The International Neuroblastoma Risk Group Staging System (INRGSS) is the pre-surgical staging system used to determine metastasis of the primary tumor using image-defined risk factors (IDRF). The presence of IDRFs indicates challenges and barriers for the removal of the primary tumor. The INGRSS has 4 stages including L1, L2, M, and MS. The L1 and L2 stages are defined as the primary tumor having no metastasis but differ in the absence or presence of IDRFs. The M and MS stages categorize the tumor as metastatic to secondary sites. MS is more clearly defined as any child under 18 months of age having tumor metastasis to the skin, liver, or bone marrow. The International Neuroblastoma Staging System (INSS) is the post-surgical stratification system that classifies the disease based on localization of tumor, lymph node involvement, residual cancer cells, metastasis, and resectability. Stages of the INSS include Stage 1, 2A, 2B, 3, 4, and 4S, with Stage 4 having the most extensive metastasis. Unlike the INRGSS, INSS cannot be used to determine the best treatment option prior to surgery. Therefore, the INGRSS is more commonly used to determine the risk group and tumor staging to develop treatment plans for patients.

Risk categories are used to determine the appropriate treatment regimen. As the total number of risk categories and factors increase, the treatment plans become more aggressive. Low-risk patients are treated by surgery alone followed by observation. Newborns with adrenal tumors are typically observed to prevent any complications that may arise during surgery. Intermediate-risk patients undergo chemotherapy. Common chemotherapy agents include etoposide, doxorubicin, and cyclophosphamide. Surgery may be done prior to chemotherapy to improve the effectiveness. If cancer progresses after chemotherapy, radiation is used. Treatment for high-risk patients can be categorized into three phases: induction, consolidation, and post-consolidation. During the induction phase, the child undergoes chemotherapy and surgery. Consolidation includes the use of myeloablative treatment, which introduces extreme doses of chemotherapy, followed by stem cell therapy to assist with reestablishing cells in the bone marrow. Post-consolidation is used to prevent the recurrence or relapse of neuroblastoma using the following treatments: radiation, immunotherapy, or retinoid therapy. The three-phase therapy used in patients
with high-risk neuroblastoma does not come without significant acute and long-term adverse effects. In a retrospective review of high-risk neuroblastoma patients who received high-dose chemotherapy followed by stem cell recovery, 86.9% of patients experienced late effects, including hearing, dental, endocrine system, and growth effects. Despite the adverse effects of the three-phase therapy, one can argue that the benefit of therapy outweighs the risk of neuroblastoma progression and further metastasis. The five-year survival rate in patients with high-risk neuroblastoma is only between 40 to 50%. Due to the low survival rate and late effects of current treatment, new developments are needed to enhance therapeutic treatment effects and better the overall survival rate.

Issues with current therapies

Various mechanisms for malignancy and resistance to current therapies are present in neuroblastoma. These mechanisms render current treatment less efficacious toward the malignant tumor and more toxic to the body’s natural systems and cellular function. Neuroblastoma is heterogeneous and aggressive in nature, leading to high treatment failure, morbidity, and mortality rates. High-risk neuroblastoma patients, who have an increased risk for recurrence and treatment failure, have a 5-year survival rate of about 40% due to these treatment barriers and the aggressive potential of cancer itself. An estimated 50 to 60% of high-risk and 5 to 15% of low to intermediate-risk patients will face a relapse at one point in their life, most likely within 5 years of treatment. Approximately two-thirds of patients diagnosed with neuroblastoma will have a secondary tumor metastasis at some point throughout their diagnosis. Neuroblastoma’s tendencies to be metastatic, malignant, and aggressive in nature present barriers to the effective remission of pediatric patients.

The structure and function of the tissue vasculature and the presence of high interstitial pressure are both barriers that hinder treatment in most cancer tumors which is seen in neuroblastoma cell lines. Neuroblastoma secretion of proangiogenic elements leads to an intricate network of new tumor vasculature. This vasculature is noted with varying degrees of diameter, irregular pericyte coverage, inconsistent formations with loops and trifurcations, and varying permeability and blood perfusion. Low blood perfusion through the vasculature leads to low drug delivery, as well as a hypoxic and acidic tissue environment. This microenvironment has been shown to favor neuroblastoma immunosuppression, drug resistance, metastasis, and poor prognosis. High interstitial pressure within the tumor creates a negative pressure that impedes drug uptake, leading to decreased therapeutic levels within the cell. Low drug uptake, drug resistance, and immunosuppression created by abnormal tumor vasculature and interstitial pressure serve as a barrier to neuroblastoma treatment.

Glycosylation also presents a barrier to drug delivery within the malignant tumor interstitial tissue. Type O-, S-, and N-glycosylation are aberrantly expressed on MYCN-amplified neuroblastoma cell lines. Increased rates of glycosylation, particularly of Lewis glycans, lead to an increase in proliferation, adhesion, and migration, ultimately leading to higher rates of malignancy and metastasis. Overexpression of the MUC1 gene, an oncogene responsible for the production of mucin-1, has been shown to lead to extensive O-glycosylation of glycoproteins located on the cell surface of a variety of cancer cell types. Overexpression leads to a reduction in integrin-mediated cellular adhesion properties with the extracellular matrix, ultimately leading to increased metastasis and invasiveness of the tumor. MUC1 has also been shown to suppress T-cell responses, leading to immunosuppressive properties and loss of antitumor immunity. The role of MUC1 in neuroblastoma has not been thoroughly explored but is thought to contain similar characteristics when compared to other cancers. Glycosylation also serves as a physical barrier against the uptake of therapeutic agents. Although glycosylation inhibition has been shown to be a useful tool for therapy in other pediatric cancers, as seen in Table 2, little research has been done to view its potential role in improving the uptake of chemotherapeutic agents in neuroblastoma. As seen in Table 1, there have been several agents used to target glycosylation in neuroblastoma to reduce cell survival and metastatic potential. Increased glycosylation has been suggested to represent a barrier impeding chemotherapeutic drug uptake and treatment, as seen in Table 2.
Another feature of neuroblastoma cells resulting in low treatment efficacy is mutations in the ATRX tumor suppressor gene which increases with the age of diagnosis.\textsuperscript{12} Neuroblastoma cells with mutations in the ATRX tumor suppressor gene have been found to serve as a predictor for poor prognosis, similarly to MYCN amplification, yet unrelated as described previously.\textsuperscript{22} The ATRX gene is believed to play roles in telomere maintenance and regulation of gene expression, but the mechanism by which ATRX mutations confer worse outcomes in neuroblastoma patients is not completely clear.\textsuperscript{12} Although the mechanisms are unclear, ATRX mutations have been shown to result in poor overall survival, which contributes to the decrease in survival rates as age at the time of diagnosis increases.\textsuperscript{12} Having knowledge of the presence of ATRX mutations could potentially help identify patients who may require alternative therapy to increase their longevity and current therapy lacks in this area.\textsuperscript{12} While ATRX mutations have not been shown to occur in cells that were also MYCN amplified, Zeineldin et al. performed an in-vivo mouse study that induced the inactivation of the ATRX gene in cell lines that were MYCN amplified.\textsuperscript{22} While mutation of the ATRX gene and MYCN amplification showed worse prognosis individually, their collective presence led to both tumor reduction and better mouse survival.\textsuperscript{22} Both MYCN amplification and the mutated ATRX gene resulted in the cancer cells being overwhelmed, ultimately leading to their demise. Thus, dampening the tumor suppressor gene in human MYCN-amplified cell lines could potentially lead to a better prognosis, but this has yet to be shown.

Studies support the notion that MYCN dictates the cellular expression of TrkA, in non MYCN-amplified cell lines, or TrkB, in MYCN-amplified cell lines.

Table 2. Influence of glycosylation on general cancer development and treatment. Summary of select clinical trials on the effects of glycosylation inhibition in cancer treatment.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Cancer type</th>
<th>In vitro/ In vivo</th>
<th>Results</th>
<th>REF.</th>
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<tr>
<td>Pan-sialyltransferase inhibitor 3Fax-Neu5Ac</td>
<td>Multiple myeloma (MM1S &amp; MM1S\textsuperscript{He} &amp; MM1S\textsuperscript{H})</td>
<td>In vitro, In vivo</td>
<td>Exposure of mice to 3Fax-Neu5Ac ↑ survival by ↑ sensitivity to the agent bortezomib. In vitro, 3Fax-Neu5Ac did not reverse resistance to bortezomib, but did ↓ the interactions between the myeloma cells, E-selectin, MADCAM1, and VCAM1, showing sialyltransferase inhibition resulted in ↓ extravasation and retention in bone marrow.</td>
<td>[49]</td>
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| Benzyl-α-GalNAc (O-glycosylation inhibitor) and neuraminidase | Pancreatic cancer (Capan I, HPAF-II, U-87) | In vitro          | - Exposure of cells to benzyl-α-GalNAc resulted in ↑ antiproliferative activity of 5-fluorouracil against two pancreatic cancer cell types, Capan I and HPAF-II compared to control (U-87 MG glioblastoma).  
- Neuraminidase did not show ↑ efficacy of 5-fluorouracil. | [50] |
| Tunicamycin             | Gastric cancer (SGC7901, SGC7901/ADR, SGC7901/VCR, BGC823, MKN45, AGS, GES) | In vitro          | - Pre-exposure of multidrug resistant gastric cancer cells to tunicamycin enhanced sensitivity to cytotoxic drug adriamycin.  
- The results observed were proven to be due to tunicamycin’s role as an N-glycosylation inhibitor, rather than its role as an endoplasmic reticulum stress-inducer. | [51] |
| C1GALT1 siRNA, itraconazole | Head and neck squamous cell carcinoma (SAS, OEC-M1, FaDu) | In vitro, In vivo | - Exposure of HNSCC cells to siRNA to knockdown C1GALT1 or with itraconazole ↓ tumor growth. | [52] |
The presence of multidrug resistance in neuroblastoma due to the overproduction of P-glycoproteins has become prevalent in recent years. Overexpression of the MDR1 gene within the tumor cells leads to increased activity of the P-glycoproteins on the cell membrane. These proteins act as pumps within the membrane, actively using energy to pump out chemotherapeutic agents from the tumor, leading to diminished concentrations of therapeutic agents and treatment failure. Common oncology agents such as vinca alkaloids, anthracyclines, and epipodophyllotoxins are substrates for the P-glycoproteins on the tumor cell membrane. The more differentiated the neuroblastoma cell line, the higher the rates of P-glycoprotein expression. Studies have shown increased mortality in patients who have increased P-glycoprotein expression neuroblastoma. It has been found that MDR1 and MYCN genes are coactivated during the metastatic process, leading to increased expression of both and therefore more chemotherapeutic challenges. Using a diagnostic tool to identify the presence of P-glycoproteins guides therapy and patient outcomes. Agents to inhibit the P-glycoprotein transporter have been identified as possible treatment enhancers to increase cytotoxic agent concentrations within the tumor, therefore increasing cell death and therapeutic efficacy. Overexpression of P-glycoproteins results in multidrug-resistant tumors and metastases. Despite the potential to achieve remission after consolidation therapy in high-risk individuals, drug-resistant tumors result in the presence of minimal residual disease (MRD). The mechanism behind MRD involves epithelial-mesenchymal transition and the ability of cells to migrate throughout the body. The heterogeneity of neuroblastoma and MRD put the individual at increased risk for relapse, thus utilizing tests to detect MRD in neuroblastoma patients is crucial.

As stated previously, at the time of diagnosis neuroblastoma has mostly metastasized throughout the body. If the metastasis has occurred in the central nervous system, then additional barriers to treatment now exist. To target tumor metastases in the brain, the blood-brain barrier (BBB) must be exploited. While the BBB exists to prevent the passage of foreign and unwanted substances into the CNS, it serves as a major obstacle when trying to treat tumors in the brain. The BBB consists of several cells with tight junctions and adhesion proteins to separate the blood from the CNS. In mice models, brain tumors have resulted in some alterations in the BBB making the barrier more permeable. Despite increased permeability, the accumulation of therapy remained minimal. One method of drug delivery involves the use of a hyperosmotic solution such as mannitol followed by chemotherapy. This has shown promising reversible alteration of the BBB with little to no added toxicities. Hyperosmotic solutions work by widening the tight junctions of the BBB due to the shrinkage of endothelial cells caused by the differences in osmolarity, allowing the chemotherapeutic agent to accumulate in brain metastases. In addition, studies have shown the use of nanoparticles as a method of drug delivery across the BBB. A glucose-coated gold nanoparticle has shown significant effects on tumors in the brain. Due to the sugar coating, the endothelial cells in the brain willingly take up the gold nanoparticle, allowing for it to take effect. Also, the utilization of a gold nanoparticle compared to other nanoparticles allowed for increased selectivity of brain endothelium.

Efflux pumps like p-glycoproteins, prevent the accumulation of chemotherapy in the brain. However, as previously stated, efflux pump inhibitors can be utilized to enhance the efficacy of therapeutic agents. Yanagisawa et al. studied the effects of the chemotherapeutic-sensitizing agent biricodar (VX-710) in addition to traditional chemotherapeutic drugs to circumvent the effects of efflux pumps. The study found little to no toxicity and adverse effects associated with its use, in addition to increased modulator activity with biricodar when compared to the drug alone or in combination with the other chemotherapeutic sensitizing agents, cyclosporine A and PSC 833. While six different cell lines were studied, the modulator response of biricodar differed between cell lines due to p-glycoprotein expression variability. Another potential drug targeting neuroblastoma metastasis beyond the BBB is lignan honokiol. Exposure of neuroblastoma to honokiol resulted in cell shrinkage, decreased cell viability, DNA fragmentation, and apoptosis through the cytochrome c-caspase pathway. Honokiol was found to pass through cerebral endothelial cell tight
juncti
ons and mice BBB, leading to activation of caspases -9, -3, and -6 and cellular apoptosis.31 Despite the many studies that have been performed demonstrating mechanisms of BBB exploitation, the blood-brain barrier, nonetheless, remains a limitation to treatment and current therapies need further research to better target metastasis to the brain.

Lastly, inflammation and malnutrition generated by the neuroblastoma cells have been linked to mortality and low treatment efficacy.32 Neuroblastoma has also been shown to decrease adipose and muscle tissue composition in pediatric patients.32 In addition, it is known to decrease Lactobacillus and other synergistic gut microbiota bacteria, leading to nutritional absorption depletion.32 Some chemotherapeutic agents for neuroblastoma, such as cyclophosphamide, require an adequate gut microbiome to be an effective treatment mechanism against malignancy.32 Malnutrition and inadequate microbiotic environment for chemotherapeutic agents cause high rates of mortality and low therapeutic efficacy in neuroblastoma patients. Neuroblastoma has been shown to increase pro-inflammatory cytokines, TNF-α and IL-6, which are linked to an increase in patient catabolism due to a cachexic state.32 Inflammation near the tumor produces a physical barrier and prevents the uptake of chemotherapeutic agents within the tumor itself.32 Therefore, systemic inflammation and malnutrition lead to poor patient outcomes and increased treatment toxicity.

Effective treatment approach

Current treatment for neuroblastoma depends on the risk group classification of the patient.6 Determining risk group classification is important to be able to provide the most effective treatment with the least complications. Low and intermediate-risk groups have far better outcomes after treatment than the high-risk group. Moving up risk levels coincides with more intense and invasive treatment options. While high-risk patients have the most complex treatment procedures, they also have the lowest survival rates.

Low and intermediate-risk groups have 95% and 90% survival rates, respectively.6 Low-risk patients are the simplest cases of neuroblastoma. These are patients in stages 1 and 2, whose tumors have not metastasized and are located on one side of the body.6 The tumors also lack MYCN gene amplification, indicating a better prognosis.6 Due to these characteristics, surgery and observation are appropriate for these patients. Usually, surgery alone is enough to remove the tumor and cancerous cells. In infants, neuroblastomas have been shown to spontaneously regress so it is best to observe patients before putting them at risk for complications due to surgery.33 At this level, chemotherapeutic agents are only used if the patient is experiencing symptoms, such as spinal cord compression.1 Intermediate patients require chemotherapy as a first-line treatment approach.5 These patients are in stage 3.6 Tumors at this stage are mostly in the area they originated in but have begun to move to the other side of the body and are not easy to remove with surgery. Chemotherapy can be used alone or in conjunction with surgery. In the latter cases, chemotherapy will be done to reduce the size of the tumor before surgery is performed. The only exception to this treatment regimen is once again the case of infants. The goal is to avoid the use of chemotherapeutic agents in such young patients.1 As such, surgery and observation will be done first. Radiation will only be done at this risk level if the patient has not responded to chemotherapy.5 Overall, these patients require a more intense treatment regimen than low-risk patients, but have very favorable survival rates.

The issues with treatment arise when treating high-risk patients.6 High-risk patients can be at any stage of the disease; however, they also have MYCN gene amplification and unfavorable histology.5 Patients at this risk level undergo a three phase, intense treatment cycle.6 During the induction phase, a combination of different chemotherapeutic agents is used to try and get cancer into remission. Common combinations of therapy include cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide; this combination is known as COJEC and is given as a rapid treatment.34 Other standard treatments include vincristine, cisplatin, etoposide, and cyclophosphamide, known as OJEC.34 The difference between standard and rapid treatment is simply the timing between cycles.34 These common agents can be combined in a variety of ways and adjusted according to patient response to induction therapy. Following induction therapy, a consolidation phase follows. This phase includes myeloablative therapy and stem cell transplant.33 Myeloablative therapy is the use of high-dose chemotherapy to destroy bone marrow cells.33 Stem cell transplant is used to repopulate the bone marrow with healthy cells. Myeloablative therapy followed by stem cell transplant has been shown to increase survival rates in patients.35 The final phase is the post-consolidation phase. The goal of this phase is to prevent the disease from coming back and includes
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Despite the intense treatment regimens currently being employed, survival rates for these patients are still low. Recent studies have begun to look at targeting the glyco-biology of tumor cells to develop more effective treatments. N- and O-glycosylation of proteins is necessary for normal cell function. Changes in glycosylation have been associated with malignant tumor cells of a variety of different cancers. In neuroblastoma, O-glycosylation has especially shown to be associated with more aggressive forms. Irregular expression of glycoproteins, as seen in Figure 1, leads to cancer cell proliferation, immune evasion, and extravasation. Overexpression of complex N-glycans contributes to malignant phenotypes. The conversion of hybrid N-glycans to complex N-glycans is controlled by an enzyme encoded by the MGAT2 gene. Complex N-glycans are highly branched and contribute to cell growth and invasiveness of neuroblastoma cell lines. Hall et al. looked at the effects of changing the ratio of hybrid to complex N-glycans that were expressed on neuroblastoma cell lines by silencing the MGAT2 gene. They found that decreasing complex N-glycans also decreased cell growth and invasiveness. In a later study, Hall et al. found a relationship between N-glycans and matrix-metalloproteinases (MMPs). When the complex N-glycans were altered and reduced, MMP-2 activity was significantly decreased, slowing the invasiveness of neuroblastoma. In 2011, Del Gross et al. showed that by inhibiting N-glycosylation in neuroblastoma cell lines expression of a mutant ALK receptor was decreased, which resulted in a decrease in cell survival. Inhibition of N-glycosylation via tunicamycin administration has also been shown to reduce the number of functional sodium channels on the cellular surface. A decrease in N-glycosylation makes cells more sensitive to proteolytic degradation which leads to a decrease in protein synthesis, folding, and insertion of sodium channels and other proteins on the cell surface. The reduction in active sodium channels is yet another potential target to improve cellular death and patient survival in neuroblastoma.

Figure 1. Glycosylation Inhibition. Schematic diagram of the effects of an O-glycosylation inhibitor on the presence of O-linked glycans.
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Figure 2. Glycosylation Inhibition and Application of Chemotherapeutic Drugs. Schematic diagram of the effects of benzyl-alpha-GalNAc, an O-glycosylation inhibitor and its potential to enhance the accumulation of chemotherapeutic agents. Adapted from European Journal of Cancer.

In neuroblastoma, the type-O glycosylation variety has been linked to the most aggressive form of disease. Berois et al. observed that increased GALNT13 expression, which encodes for a glycosyltransferase involved in mucin type-O glycosylation, correlates with poor clinical outcomes and bone marrow metastasis in human neuroblastoma cells. While an earlier study showed that high expression of B4GALT3 correlates with better prognosis and survival in neuroblastoma patients, a more recent study done by Chang et al. revealed that in intermediate and high-risk patients, increased B4GALT3 expression independently predicts poorer outcomes. Chang et al. focused on the knockdown of a glycosyltransferase, B4GALT3, which is responsible for modifying β1-integrins. The knockdown resulted in decreased tumor growth, migration, and invasion. It is becoming apparent that inhibition of glycosylation might play a beneficial role in the treatment of neuroblastoma. Kalra and Campbell showed that the inhibition of type O glycosylation increased the cytotoxic effects of 5-FU in pancreatic cells. Such findings support the notion that the glycosylation of neuroblastoma cells could well impede drug uptake and effects. Additional studies designed to probe the mucin glycosylation effect, confirmed that a ‘zone of exclusion’ formed around the cellular environment of mucin-expressing cells limited the uptake of nanoparticles. The observation was specific to the pancreatic cell line employed.

Concluding Remarks

Currently, there are effective treatments for neuroblastoma, especially when it comes to low and intermediate-risk patients, but there are also many limitations to treatment with all risk groups. Barriers to treatment include those that come with nearly all solid mass tumors, such as increased interstitial pressure impeding drug entry into the tumor and irregular tumor vasculature leading to unsuccessful drug transport. Successful treatments are often impeded due to neuroblastoma characteristics, such as the induction of malnutrition...
in diagnosed patients as well as the expression of O-linked, S-linked, and N-linked glycans on the surface of some neuroblastoma cells increasing malignancy and metastasis while decreasing the likelihood of positive health outcomes.\textsuperscript{8,32} There is some evidence that inhibition of glycosylation may be a potentially beneficial treatment option for neuroblastoma, as illustrated by the clinical studies outlined in Table 1 and those on other cancers in Table 2, but there is an apparent need for further research on the topic with regards to neuroblastoma specifically. Neuroblastoma is a common pediatric malignancy and the high failure rates associated with treatment, especially in high-risk patients, requires our attention. The development of a more targeted treatment to increase drug uptake and enhance cytotoxicity represents a next step in neuroblastoma therapy.

**Conflict of Interest**

No conflicts of interest exist in this work, financial or otherwise.

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### Table 1. Influence of glycosylation on pediatric neuroblastoma development and treatment. Summary of select clinical trials on the effects of glycosylation inhibition in neuroblastoma cell lines.

<table>
<thead>
<tr>
<th>Agent</th>
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<th>In vitro/In vivo/clinical</th>
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<tbody>
<tr>
<td>Tunicamycin</td>
<td>Neuroblastoma (SH-SY5Y, KELLY, UKF-NB3 &amp; NB1)</td>
<td>In vitro</td>
<td>• In ALK mutated or amplified neuroblastoma cell lines, inhibition of N-glycosylation ↓ phosphorylation of ALK and its downstream effectors (AKT, ERK ½, STAT) and ↓ survival of cancer cells.</td>
<td>[38]</td>
</tr>
</tbody>
</table>
| C2GNT1 siRNA           | Neuroblastoma (CHP-212 & SK-N-AS) | In vitro                  | • C2GNT1 siRNA inhibited C2GNT1 which ↓ adhesion, proliferation, and migration in CHP-212 and SK-N-AS.  
• C2GNT1 siRNA resulted in ↓ Sialyl Lewis X and Lewis Y expression and ↓ E-selectin and P-selectin binding in CHP-212                                                                                                                                   | [8]  |
| Okadaic Acid           | Neuroblastoma (KELLY)         | In vitro                  | • Treatment with okadaic acid, a phosphatase inhibitor, ↓ dephosphorylation of proteins, and the addition of N-acetylglucosamine to the proteins.  
• Okadaic acid resulted in an accumulation of phosphorylated proteins in the nucleus and the cytosol, while O-linked N-acetylglucosamine levels ↓ more in the nucleus than cytosol (53% vs 15%).  
• The change in O-linked N-acetylglucosamine levels in neuroblastoma cells was not due to ↓ in UDP-GlcNAc precursor levels.                                                                                                                                                                                                                         | [47] |
| B4GALT3 siRNA          | Neuroblastoma (SK-N-DZ, SK-N-BE, SK-N-AS, & SH-SY5Y) | In vitro, in vivo, Clinical | • Expression of B4GALT3 correlated with advanced stages of neuroblastoma, greater in high-risk patients, associated with unfavorable histology, and connected to a poor survival rate.  
• In SK-N-DZ cells transfected with siB4GALT3, which knocked down B4GALT3, overall tumor growth was suppressed in vivo.  
• B4GALT3 knockdown also resulted in ↓ blood perfusion and diameter of blood vessels in the SK-N-DZ tumors.  
• B4GALT3 ↑ migration & invasion of SH-SY5Y & SK-N-AS.                                                                                                                                                                                                                                                       | [43] |
| Statins                | Neuroblastoma (SH-SY5Y & STA-NB10) | In vitro                  | Statins inhibited efflux by p-glycoprotein.  
• Atorvastatin elicited similar effects to tunicamycin on glycosylation of the P-glycoprotein, ↓ glycosylation and the transporters function.  
• Administration of statins with doxorubicin resulted in ↑ doxorubicin accumulation in the cancer cells.                                                                                                                                                                                                                                                                  | [48] |
| Sh-C1GALT1/ pLKO.1-puro| Neuroblastoma (Human NB cells, SK-N-BE, SK-N-SH, GI-LI-N) | In vitro, In vivo          | • Knockdown of C1GALT1 by sh-C1GALT1/pLKO.1-puro increased the proliferation, colonization, invasion, and migration of NB cells.  
• Down-modulating C1GALT1 expression increased O-glycosylation of surface protein TrkA which decreased its function and downstream effects. This may contribute to the malignant type of NB cells.                                                                                                                                                                      | [53] |
Influence of Glycosylation on the Development and Treatment of Neuroblastoma

References

48. Sieczkowski E, Lehner C, Ambros PF, Hohenegger M. Double impact on p-


