Oncolytic Viroradiotherapy for Neuroblastoma

Honors Research Thesis
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Abstract

Neuroblastoma is the most common extra-cranial solid tumor in childhood and the leading cause of childhood cancer mortality. MIBG (meta-iodobenzylguanidine), an analogue of noradrenaline, is a form of targeted radiation therapy for high-risk neuroblastoma when bound to ^{131}Iodine. MIBG enters cells through the norepinephrine transporter (NET), a protein expressed on the surface of neuroendocrine cells, including most neuroblastoma cells. ^{131}I-MIBG then radiates the cells it enters and induces cytotoxicity in surrounding cells. However, ^{131}I-MIBG is not always effective, likely in part due to low NET expression in high-risk neuroblastomas. Oncolytic virotherapy is a promising therapeutic approach currently in clinical trials. Previously, our lab has shown that preclinical models of neuroblastoma are sensitive to oncolytic herpes simplex virus (oHSV) therapy. oHSV can also be used to deliver the NET transgene to tumor cells to increase susceptibility to ^{131}I-MIBG. In the present study, we are investigating the efficacy of HSV1716/NET in increasing NET expression and thereby increasing the efficacy of ^{131}I-MIBG. We will also evaluate if ^{131}I-MIBG enhances viral replication. The results to date indicate that neuroblastoma cell lines are susceptible to HSV1716/NET, and upon viral infection, there is effective transfer of the NET gene resulting in an increase in ^{131}I-MIBG uptake.

Introduction

Each year, roughly 13,500 parents will hear the words "your child has cancer." However, there is hope for these families in the fact that survival rates have increased from 10 percent to 80 percent in the last 40 years (Howlader, N., Noone, A. M., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S. F., ... & Cronin, K. A., 2011). Despite these major advances, the survival rates remain much lower for high-risk cancers and for patients with metastases.

Neuroblastoma is the most common extra-cranial solid tumor in childhood. Although neuroblastoma accounts for less than 10% of childhood cancers, it accounts for 15% of pediatric cancer deaths (Park, J.R., Eggert, A., and Caron, H., 2010). While the survival rates for low- or intermediate-risk patients remains fairly high, the event free survival rate for high-risk patients is less than 50% (Cohn, S.L., Pearson, A., London, W.B., Monclair, T., Ambros, P.F., Broderu, G.M., Faldum, A., Hero, B., Iehara, T., Machin, D., Mosseri, V., Simon, T., Garaventa, A., Castel, V., and Matthay, K.K., 2009). Despite mulit-modal therapy including surgery, chemotherapy, radiation, immunotherapy, and stem cell transplant, outcomes remain poor. Therefore, alternative therapies are urgently needed for the treatment of high-risk patients, especially children with relapse or refractory disease.

Neuroblastoma is derived from neural crest cells, and thus uniquely expresses the norepinephrine transporter (NET) on its cellular membrane, making it a unique cancer for the use of targeted therapies and tumor-selective imaging. One novel therapeutic agent is meta-iodobenzylguanidine (MIBG), an analog of norepinephrine, which enters cells through the NET. MIBG can be tagged with radioisopes ^{123}Iodione or ^{131}Iodine, which are used for imaging or therapy, respectively. While 90% patients with neuroblastoma have a positive ^{123}I-MIBG scan, only 30% of patients have a clinical response to ^{131}I-MIBG (Matthay, K., Yanik, G., Messina, J.,

Quach, A., Huberty, J., Cheng, S., Veatch, J., Goldsby, R., Brophy, P., Kersun, L.S., Hawkins, R.A., and Maris, J.M., 2007). Targeted radiotherapy is a promising approach for neuroblastoma, but escalating dosing has only lead to increased toxicity rather than increased ^{131}I-MIBG uptake into the tumors (Matthay, K.K., Reynolds, P., Seeger, R.C., Shimada, H., Adkins, E.S., Haas-Kogan, D., Gerbing, R.B., London, W.B., and Villablanca, J.G., 2009). A study by the Children's Oncology Group found that higher risk neuroblastoma patients have less ^{123}I-MIBG uptake, which could be due to a lower level of NET expression. ^{131}I-MIBG uptake and therapeutic efficacy could be improved in neuroblastoma patients by increasing the level of NET expression.

Oncolytic virotherapy, the use of a genetically modified virus to selectively target and kill cancer cells, is a promising therapeutic approach. This therapy uses an attenuated virus, called an oncolytic virus, which has been mutated to not cause an infection in normal cells, but can specifically target cancer cells. Oncolytic viruses, specifically oncolytic Herpes Simplex Virus-1 (oHSV) with its large genome, can be used as viral vectors to aid in cancer gene therapy. Previously, our lab has shown that preclinical models of neuroblastoma are sensitive to oHSV therapy (Parikh, N.S., Currier, M.A., Mahller, Y.Y., Adams, L.C., Pasquale, B.D., Collins, M.H., and Cripe, T.P., 2005**)**. Therefore, oHSV therapy can be used to deliver the NET transgene to neuroblastoma to increase susceptibility to ^{131}I-MIBG.

Focus of the research

The purpose of the research is to gather preclinical data to determine whether oHSV therapy coupled to NET gene therapy can effectively enhance ^{131}I-MIBG uptake in

neuroblastoma cells, resulting in greater cytotoxicity of the combined therapies than either therapy alone.

Significance of the project

Finding a way to enhance ^{131}I-MIBG uptake in neuroblastoma is critical to the success of this therapy in clinic. Currently, patients receive this treatment as a Phase 1 or 2 medications and are eligible only if they had a relapse or refractory disease that was unresponsive to conventional therapies. These patients tend to have minimal clinical response that is likely due to developed resistance (Matthay et al., 2007). Additionally, this project research may provide rationale for using ^{131}I-MIBG earlier in the treatment process.

Our translational research can be used to provide better outcomes for children with relapsed or refractory neuroblastoma. HSV1716 is already in clinical trials at Nationwide Children's Hospital (NCT00931931) and the hospital now has an operating ^{131}I-MIBG room. The preclinical data gathered in this project will be the first step towards getting this combination therapy in a clinical trial. In addition to improving neuroblastoma susceptibility to targeted radiotherapy, the ultimate goal of this project is to provide rationale for using oncolytic viroradiotherapy in other cancer types.

Definitions of Key Terms

Oncolytic virotherapy- A treatment using a virus that infects and kills cancer cells but not normal cells.

Attenuated virus- A viable virus that is no longer harmful made possible by deletion of virus genes.

Radiation Therapy- The use of high-energy rays to kill cancer cells

Targeted Radiation- The use of tumor-seeking molecules to deliver radiation directly to the cancer

Metastatic disease- When cancer has spread from its original location to other locations in the body

Refractory disease- When cancer resists treatment and is never improved

Relapse disease- The return of the disease after a period of improvement

Neural crest- A temporary structure in embryonic development that gives rise to structures associated with the nervous system

Norepinephrine- A hormone and neurotransmitter in the body

Transgene- A gene that has been transferred from one organism to another

Endogenous- Originating from within an organism or cell

Exogenous- Originating from outside an organism or cell

Background

The human body is made up of many types of cells that are highly regulated. However, cells can sometimes being to replicate and divide with little or no control. When this happens, the uncontrolled cell growth can destroy nearby cells and start to invade other parts of the body, becoming what is known as cancer. Unfortunately, this unregulated replication can occur in children. Although pediatric cancer only accounts for 1% of all cancer cases, it is the second leading cause of death in children, exceeded only by accidents (Howlader et al., 2011). Childhood cancer spans all racial, ethnic, and socio-economic groups, ending the life of one in every five children affected.

Pediatric cancers can be separated into 3 main categories: leukemias, lymphomas, and solid tumors. A solid tumor arises from cells that usually make an organ. Neuroblastoma is the most common extra-cranial solid tumor, counting for roughly 10% of all childhood cancer cases (Park et al., 2010). The average age of neuroblastoma diagnosis is 17 months (Maris, 2010). Neuroblastoma is derived from neural crest cells, or progenitor cells of the sympathetic nervous system. The mechanism of cancer formation is unknown, but it is speculated to be due to defects in embryonic genes controlling the neural crest development. Neuroblastoma can be located anywhere along the sympathetic nervous system, but it occurs most frequently in the abdominal cavity, and more specifically the adrenal gland. At time of diagnosis, 50% of patients present with metastases, or tumor spread (Park et al., 2010). Neuroblastoma is often diagnosed through presenting symptoms, pathological confirmation of tissue sample, or through tumor-selective imaging.

Upon diagnosis, patients with neuroblastoma are classified by a staging system called the International Neuroblastoma Staging System. Patients are placed into pretreatment risk groups based on a combination of factors: age, tumor histology, MYCN amplification status, and the presence of other genetic aberrations (Park et al., 2010). For example, patients older than 18 months are associated with poorer outcomes. Poorer outcomes are also associated with patients that have amplified MYCN, a known neuroblastoma oncogene. The risk groups determine which treatment a patient will receive. Event free survival rates correlate with each risk group and determines how intense of a therapy regimen is needed to achieve the best outcomes while minimizing side effects.

Due to advances in therapy, the survival rates of neuroblastoma patients with low- or intermediate-risk tumors are above 50%, with the survival rate above 85% for very low-risk

patients (Cohn et al., 2009). Standard therapies for these children with neuroblastoma include a combination of observation, surgery, chemotherapy, or radiation therapy with varying intensities. Surgical resection is typically the first step in patients who have localized tumors, and it is often the only treatment necessary. However, if the cancer is not all removed with surgery or there are metastases at the time of diagnosis, the patient will usually receive chemotherapy or radiation therapy.

Although many pediatric patients with neuroblastoma benefit from standard therapies, the survival rate of high-risk patients remains low at less than 50% (Cohn et al., 2009). The high risk is dependent on the age of the patient, whether the tumor is MYCN amplified, and how much of the tumor can be removed by surgery. Compared to the lower risk groups, patients in this group receive intensive multi-modal therapies that include chemotherapy, radiation therapy, and stem cell transplant. Despite using the most intensive therapies for these patients, the outcomes remain poor. 50% of high-risk neuroblastoma patients will develop relapsed or refractory disease (Park et al., 2010).

As mentioned, neuroblastoma tumors can be diagnosed with tumor-selective imaging using meta-iodobenzylguanidine (MIBG), a tumor-seeking molecule that can be tagged with radioactive iodine. MIBG is an analog of the catecholamine norepinephrine and enters cells through the norepinephrine transporter (NET), a transmembrane protein expressed on the surface of neuroblastoma cells. MIBG is shuttled across the membrane by NET in a process that is energy dependent. The uptake is proven to be NET specific shown by decreased uptake in the presence of competitive inhibitors, such as DMI (Smets, L.A., Loesberg, C., Janssen, M., Metwally, E.A., and Huiskamp, R. 1989). ^{123}I-MIBG is used for imaging and ^{131}I-MIBG is used for treatment, due to differences in radiation emissions. Once inside the cells, ^{131}I-MIBG exhibits

cytotoxic effects by emitting β and γ radiation, which damages the DNA of the cells. ^{131}I-MIBG also induces cytotoxicity through the radiation induced bystander effect (RIBE). RIBE are effects that occur in cells that have not been directly irradiated, but experience cytotoxic effects due to the signals released from neighboring irradiated cells (Mothersill & Seymour, 2004). ^{131}I-MIBG is currently in use as a therapy for relapsed or refractory neuroblastoma patients.

Even though 90% of patients have a positive ^{123}I-MIBG scan, a recent study found that only about 30% of children who receive ^{131}I-MIBG therapy respond to the treatment, and it is usually a partial response (Matthay et al., 2009). A report by the Children's Oncology Group found that high-risk patients had lower NET protein expression and usually had ^{123}I-MIBG non-avid scans, indicating that the level of NET is important in mediating uptake of MIBG (Dubois, S.G., Geier, E., Batra, V., Yee, S.W., Neuhaus, J., Segal, M., Martinez, D., Pawel, B., Yanik, G., Naranjo, A., London, W.B., Kreissman, S., Baker, D., Attiyeh, E., Hogarty, M.D., Maris, J.M., Giacomini, K., and Matthay, K.K., 2012). ^{131}I-MIBG is currently used at maximum dose in treatment, therefore finding a way to increase NET in high-risk patients will improve the efficacy of the current doses used in ^{131}I-MIBG therapy.

One possible way to increase the expression of NET on the surface of neuroblastoma cells is through gene transfer. Oncolytic virotherapy may be the needed therapeutic combination to targeted radiotherapy because a virus can be used as a vector for gene transfer and the virus will also aid in cell killing. Herpes simplex virus, type-1 (HSV-1) can be employed as an oncolytic virotherapy by attenuation through genetic engineering. HSV-1 is an enveloped double-stranded DNA virus that belongs to the virus family *herpesvidae* (Shen & Nemunaitis, 2006). HSV-1 can enter a cell through interaction with proteins on the cell membrane. HSV-1 is also an ideal virus for therapy because of its ability to vastly propagate itself once inside a cell.

Once the virus enters the cell, it is transported to the cell nucleus. There it uses the host cell's machinery to make more virus particles from virus DNA, making 80 new viruses from just the one virus that originally entered the cell (Shen & Nemunaitis, 2006).

HSV1716 is an attenuated version of the wild type, or naturally occurring, HSV-1. This clinically safe virus was created through the deletion of both copies of a critical gene RL1, or ICP34.5 (MacLean, A.R., Ul-Fareed, M., Robertson, L., Harland, J., and Brown, S.M.,1991). ICP34.5 is a necessary gene for HSV-1 infection of normal cells. This gene counteracts the effects of PKR signaling, which is a defense mechanism activated by the host cell upon virus infection. PKR signaling normally leads to inhibition of viral replication in a host cell, and therefore HSV-1 contains a gene to overcome the replication inhibition. Without ICP34.5, HSV1716 is unable to cause infection in normal cells because it cannot overcome the cell's defense mechanism. Cancer cells are constantly dividing and replicating and have no need for an internal virus defense mechanism. Because most cancer cells have defective PKR signaling, ICPd34.5 is not necessary for HSV-1 infection of cancer cells (Hammill, A.M., Conner, J., and Cripe, T.P., 2010; Friedman, G.K., Pressey, J.G., Reddy, A.T., Markert, J.M., and Gillespie, G.Y., 2009). Therefore, HSV1716 will be able to cause infection in cancerous cells inside the body while not affecting normal cells.

HSV1716 is known for its broad tropism, and other oncolytic HSV's have been shown to be an effective oncolytic virus for preclinical models of neuroblastoma (Parikh et al., 2005). Additionally, HSV1716 has 150 bp (basepairs) of non-coding genes, rendering the virus an ideal candidate for cancer gene therapy (Shen & Nemunaitis, 2006). Therefore, HSV1716 can be used as a delivery agent of the NET transgene for use in combination with ^{131}I-MIBG. Quigg et al. first engineered HSV1716 with the bovine NET transgene, resulting in HSV1716/NET. The

bovine NET transgene cassette was added in place of both deleted copies of the RL1 gene in HSV1716. HSV1716/NET was proven as a suitable vector for transgene delivery, and its combination with ^{131}I-MIBG increased cell killing in a non-NET expressing tumor (Quigg, M., Mairs, R.J., Brown, S.M., Harland, J., Dunn, P., Rampling, R., Livingstone, A., Wilson, L., and Boyd, M., 2005). HSV1716/NET followed by exposure to ^{131}I-MIBG exhibited anti-tumor efficacy in a human xenograft model of non-NET expressing cancers (Sorenson, A., Mairs, R.J., Braidwood, L., Joyce, C., 2012). The previous HSV1716/NET studies serve as rationale to investigate the translational application of the combination therapy in neuroblastoma as a way to enhance NET expression.

Research Aims and Related Hypotheses

Aim 1: Evaluate HSV1716/NET cytotoxicity and replication in neuroblastoma cell lines. A panel of neuroblastoma cell lines will be tested with the oncolytic virus HSV1716/NET and HSV1716 to verify viral replication and cell killing. It is important to determine that HSV1716/NET is a viable virus in neuroblastoma cell lines. The toxicity and viral production will be measured using cell survival (MTS) assays to determine how well the cancer cells are killed and how well the virus is propagated. The hypothesis for Aim 1 is neuroblastoma cell lines are susceptible to HSV1716/NET infection.

Aim 2: Characterize endogenous NET expression and exogenous NET expression upon HSV1716/NET infection in neuroblastoma cell lines. The panel of neuroblastoma cell lines will be evaluated for baseline NET expression, which will be confirmed through detection of RNA expression using quantitative RT-PCR (qRT-PCR). After establishing the baseline NET

expression, the same panel of neuroblastoma cell lines will be used to determine NET gene transfer. The cell lines will be infected with HSV1716/NET and post viral infection, the increase in NET transgene expression will be measured. It is important to investigate whether any increase in the NET transgene is from the virus-derived NET transcript. HSV1716/NET contains the bovine NET transcript; thus, post viral infection, the bovine NET and human NET transgenes will be compared. The hypothesis for Aim 2 is infection with HSV1716/NET will deliver the NET transgene and increase the exogenous NET expression in NB cell lines.

Aim 3: Determine ^{131}I-MIBG uptake and cytotoxicity in neuroblastoma cell lines in combination with oHSV therapy. Three neuroblastoma cell lines will be chosen for MIBG studies based upon low, intermediate, and high levels of NET expression and viral production, determined by the results of Aim 1 and Aim 2. ^{131}I-MIBG uptake will be measured post viral infection at varying time points to evaluate optimal timing of ^{131}I-MIBG exposure after infection. Half of the cells will also be treated with DMI (a competitive inhibitor of NET) to evaluate whether ^{131}I-MIBG uptake is NET specific. ^{131}I-MIBG uptake will also be measured after infection with both HSV1716/NET and HSV1716 to determine if the viral NET gene mediates uptake. Additionally, the cytotoxicity of ^{131}I-MIBG will be measured in combination with HSV1716/NET to evaluate whether there is a combined effect. The hypothesis for Aim 3 is ^{131}I-MIBG uptake is NET specific and is enhanced upon HSV176/NET viral infection in neuroblastoma cells. The second hypothesis is the cytotoxicity of radiation and virotherapy will be greater in combination than either therapy alone.

Methodology

This research study will be completed in the research laboratory of Timothy Cripe, MD PhD in the Center of Childhood Cancer and Blood Diseases in the Research Institute at Nationwide Children's Hospital. The Center of Childhood Cancer and Blood Diseases is affiliated with the James Comprehensive Cancer Center at The Ohio State University Wexner Medical Center. This research is being completed under the supervision of Pin-Yi Wang and Keri Streby. The technical methodology of each Aim is explained below.

Aim 1: Cell survival/MTS assay and virus production. A panel of neuroblastoma cancer cell lines was used for the experiments in this study (CHLA-20, CHLA-90, CHLA-119, CHLA-136, CHP-134, IMR-32, NB-1643, NB-EBc1, SK-N-AS, SK-N-BE(2), SK-N-SH, and SH-SY5Y). The cell lines were obtained from the Pediatric Preclinical Testing Program (PPTP) database (directed by Dr. Peter Houghton, NCH) and from ATCC. The cell lines were confirmed with STR and mycoplasma negative. Vero, African Green Monkey kindey, cell line was purchased from ATCC and used in plaque assay. All cell lines were maintained in the media recipies recommended by ATCC. The oncolytic viruses, HSV1716 and HSV1716/NET, were provided by Virttu Biologics (Glasgow, UK). NB cell survival was measured by MTS assay. All 12 cell lines were seeded into a 96-well plate at a density of 3000 cells per well and cultured overnight. The cultures were then infected with HSV1716/NET or HSV1716 at MOI's (molecules of infection) of 0.001, 0.01, 0.1, and 1. The cell survival was determined after 2, 4, and 6 days compared to uninfected controls. For the viral production assay, cells were seeded in a 12-well plate at a density of 1×10^5 neuroblastoma cells per well and cultured over night. The cells were then infected with low concentrations of HSV1716/NET (MOI= 0.01). At 2, 24, 48, and 72 hours

post infection, the cell lysates were collected and titered by standard plaque assay on vero cells (Dulbecco & Vogt, 1953).

Aim 2: mRNA expression and gene transfer. Total RNA was isolated from ~1 x 10^6 cells of each neuroblastoma cell line in culture using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). cDNA was generated from RNA through reverse transcription using SuperScriptII Reverse Transcriptase (Life Technologies). The cDNA of each cell line will be used to perform quantitative polymerase chain reaction (qPCR) with Power SYBR Green PCR Master Mix (Life Technologies) and human NET primer. The reaction was performed using Appiled Biosystems 7900 Real-Time PCR system (Life Technologies). The samples were run at 50°C for 2 minutes, 95°C for 5 minutes, 40 cycles of 94°C for 15 seconds, 58°C for 35 seconds, and 72°C for 35 seconds followed by a standard dissociation stage to determine the melting temperature of each amplification product. The resulting mRNA detection was presented as expression fold relative to an internal standard, the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For the gene transfer study, select neuroblastoma cell lines (CHLA-20, CHLA-90, CHLA-119, CHLA-136, NB-1643, SK-N-AS, and SK-N-BE(2)) were seeded in a 6-well plate at a density of 1 x 10^5 NB cells per well and cultured overnight. Cells were either uninfected (as control) or infected with HSV1716/NET (MOI= 0.1) in duplicate. 24 h post virus infection. RT-qPCR was performed on the 7 samples from the gene transfer as described above, except the cells were also tested with the bovine NET primer in addition to the human NET primer.

Aim 3: ^{131}I-MIBG uptake and cytotoxicity. Neuroblastoma cell lines CHLA-20, SK-N-BE (2), and SK-N-AS were chosen for the ^{131}I-MIBG studies based on levels of NET expression and viral production. For the uptake studies, cells were either uninfected (as control) or infected with HSV1716/NET (MOI=0.5). 24, 48, or 72 hours post infection, the cells were exposed to 1 MBq

of ^{131}I-MIBG for 2 hours. Half of the cells were treated with 2mM DMI (desmethyimipramine), a competitive inhibitor of NET. ^{131}I-MIBG cellular uptake was measured on a gamma counter (counts per million per cell). To prove viral gene mediated uptake, cells were either uninfected (as control), infected with HSV1716/NET (MOI= 0.3), or infected with HSV1716 (MOI= 0.3). All cells were exposed to 1 MBq of ^{131}I-MIBG at the same time point of 24 hours. ^{131}I-MIBG and oHSV cytotoxicity was measured either in combination or separately. Cells were seeded in 96-well plates at an intial density of 3000 cells per well and were cultured over night. Cells were infected with HSV1716/NET (MOI = 0, 0.01, 0.1, or 1). 24 h post infection, cells were exposed to 0, 0.1, 0.5, or 1 MBq of ^{131}I-MIBG for 2 hours. MTS assay at day 2, 4, and 6 was performed to determine cell survival as described in Aim 1. The percent survival was compared to uninfected controls.

Results

HSV1716/NET exhibits a wide range of virus replication and exhibits cytotoxic effects in neuroblastoma cell lines. It has been previously shown that HSV1716 effectively replicates in preclinical models of neuroblastoma (Parikh et al., 2005). The oncolytic virus HSV1716 with the added bovine NET transgene, HSV1716/NET, showed similar replication in neuroblastoma cells as the parent virus (viral constructs shown in fig.1). The replication assays performed on the panel of 12 neuroblastoma cell lines (6 cell lines shown) revealed varied viral production between all samples (fig. 2). At 72 hours post virus infection, the neuroblastoma cells showed between 0 and 4 logs increase of viral production across all cell lines. SK-N-SH had a 4-fold increase in viral production after 72 hours, whereas NB-1643 had no viral production.

HSV1716/NET is effective in neuroblastoma cell line killing (6 cell lines shown). Some cell lines were more susceptible, like SK-N-SH, and some were more resistant, like NB-1643 (fig. 3). The HSV1716/NET cell survival results followed a similar trend to that of HSV1716 (data not shown).

Endogenous NET mRNA varies across neuroblastoma cell lines. Most neuroblastoma endogenously expresses NET. We evaluated the baseline NET mRNA expression of all 12 cell lines (fig. 4). The results show a range of baseline NET expression; the fold expression varies from 0.0001 to 0.05 relative to GAPDH.

HSV1716/NET effectively delivers the NET transgene. In the gene transfer assay, we compared the mRNA expression of human NET transgene and the bovine NET transgene post infection (fig. 5). The results confirm that the NET transgene is transferred to the neuroblastoma cells by the virus. The uninfected controls express no bovine NET, indicating the enhanced expression is due to the virus.

HSV1716/NET enhances ^{131}I-MIBG by NET gene transfer. Three neuroblastoma cell lines (CHLA-20, SK-N-BE(2), and SK-N-AS) were chosen for the ^{131}I-MIBG *in vitro* studies based on varying endogenous NET expression, HSV1716/NET cytoxicity, and viral production. In both CHLA-20 and SK-N-BE(2) cells, ^{131}I-MIBG uptake was enhanced at 24 and 48 hours post viral infection compared to the uninfected controls (fig. 6). ^{131}I-MIBG uptake begins to diminish at 72 hours in both cell lines. NET specific uptake is also demonstrated by minimal ^{131}I-MIBG uptake in cells treated with DMI.

To further examine that the viral NET transgene mediates the enhanced uptake, neuroblastoma cells (SK-N-AS, SK-N-BE(2), and CHLA-20) were exposed to ^{131}I-MIBG post viral infection with HSV1716/NET and HSV1716 (fig 7.). One group was uninfected to serve as a control. At 24 hours post infection, cells infected with HSV1716/NET displayed a 2-3 fold increase in ^{131}I-MIBG uptake compared to uninfected cells or cells infected with the virus without the NET transgene. The uninfected control and HSV1716 had very similar ^{131}I-MIBG uptake.

^{131}I-MIBG and HSV1716/NET exhibit varying cytotoxicity in neuroblastoma cells. To determine the additive effect of targeted radiation and an oncolytic virus, two neuroblastoma cell lines (CHLA-20 and SK-N-AS) were exposed to varying doses of ^{131}I-MIBG post HSV1716/NET infection at varying MOIs (fig. 8). CHLA-20 demonstrated an additive cytotoxic effect when exposed to both virus and radiation, but it was not synergistic. CHLA-20 was very susceptible to radiation as nearly all cells were killed at 0.5 and 1 MBq exposure, regardless of the HSV1716/NET infection. The additive effects seen in CHLA-20 were not present in SK-N-AS. SK-N-AS cells were resistant to viral infection, except at an MOI of 1. Resistance to radiation was evident in SK-N-AS because there was minimal cell death when the cells were only exposed to ^{131}I-MIBG and not HSV1716/NET.

Figures

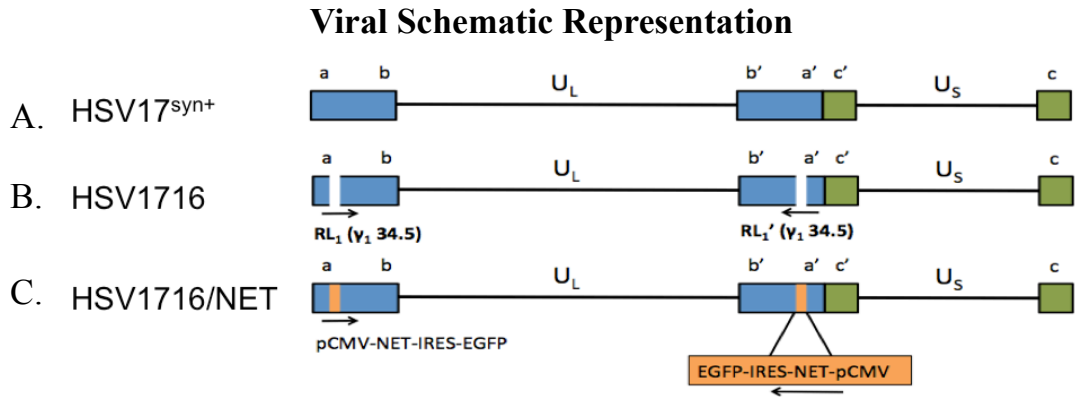

Figure 1. Schematic representation of the (a) HSV17^{syn+} genome, (b) the HSV1716 genome, and (c) the HSV1716/NET genome.

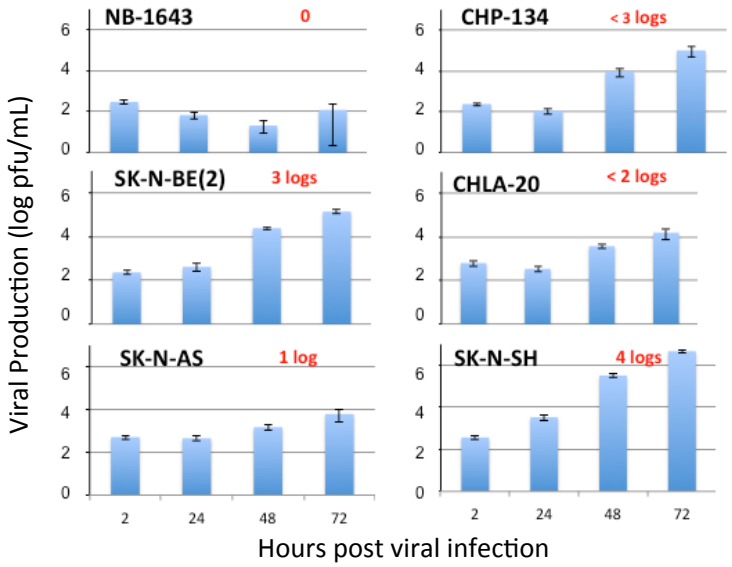

Figure 2. HSV1716/NET infected neuroblastoma cell were plated on Vero cells for 2, 24, 48 and 72 hours and amount of virus produced was determined by plaque assay.

HSV1716/NET exhibits cytotoxic effects in neuroblastoma cell lines

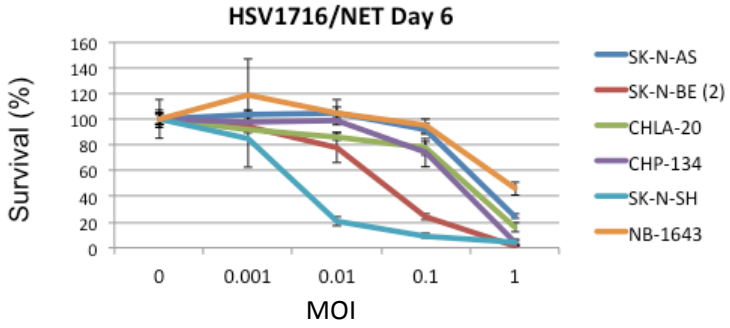

Figure 3. Cells were infected with HSV1716/NET at varying MOIs. MTS assays day 6 is shown. The percent survival was compared to uninfected controls.

Endogenous NET mRNA varies across neuroblastoma cell lines

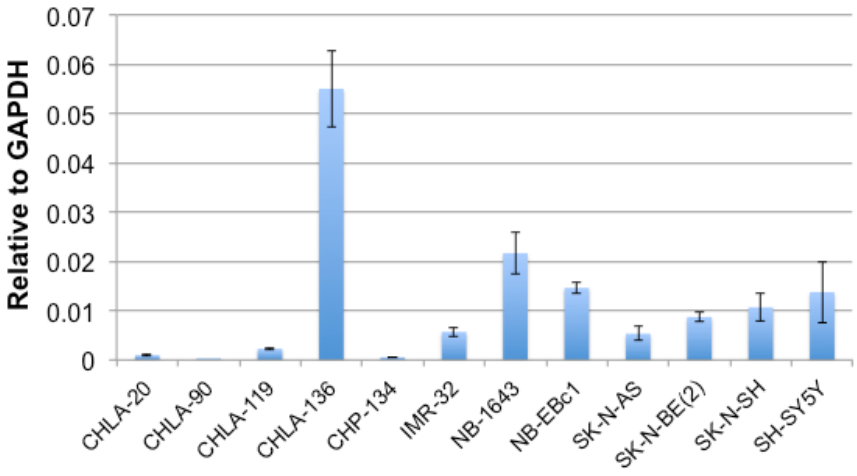

Figure 4. RT-qPCR analysis for mRNA expression of NET. Data are presented relative to GAPDH.

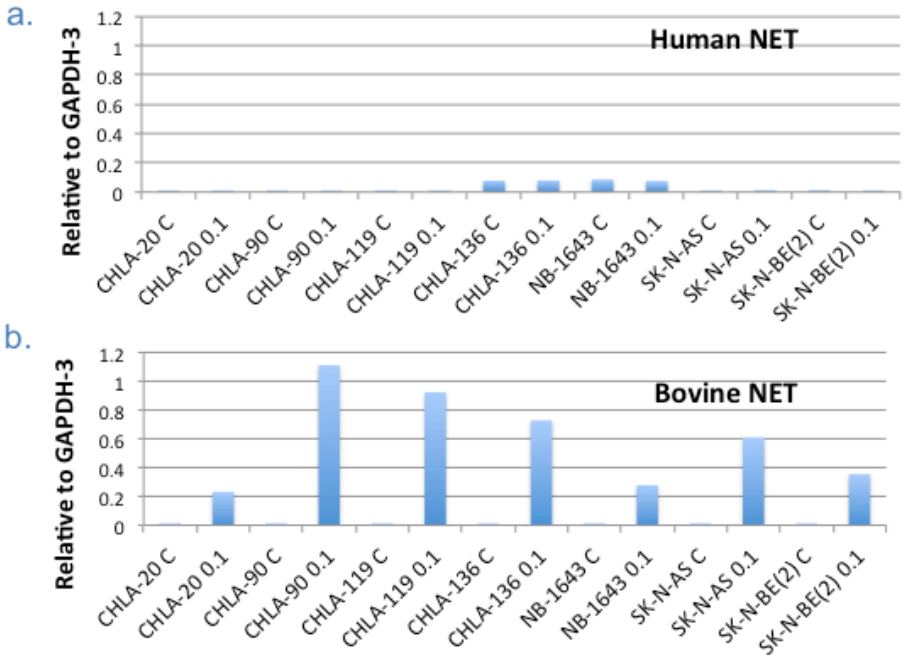

Figure 5. (a) RT-qPCR analysis for RNA expression of human NET and **(b)** virus-derived (bovine) NET post virus infection. Data are presented relative to GAPDH.

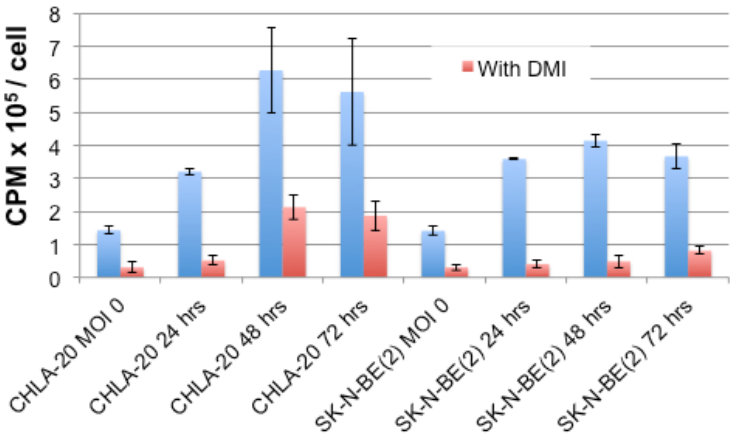

Figure 6. Cells were either uninfected (as control) or infected with HSV1716/NET (MOI=0.5). 24, 48, or 72 hours post virus infection, the cells were exposed to 1 MBq of ^{131}I-MIBG for 2 hours. Half of the cells were treated with 2mM DMI (DMI competitively inhibits NET).

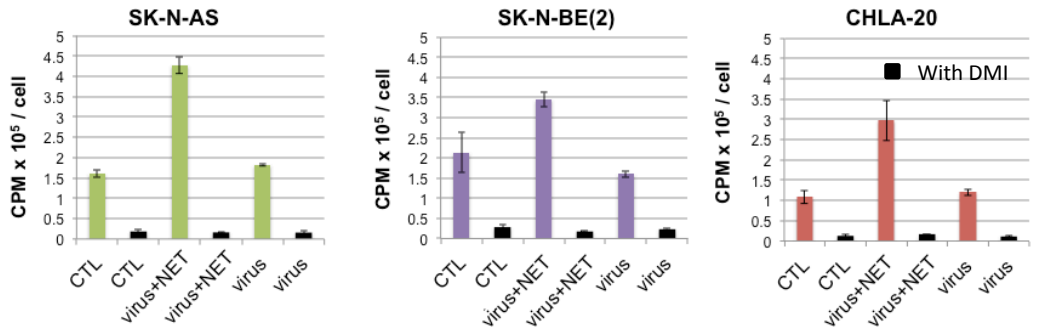

Figure 7. Cells were either uninfected (control), infected with HSV1716/NET (MOI=0.3), or HSV1716 (MOI=0.3). 24 hours post virus infection, the cells were exposed to 1 MBq of ^{131}I-MIBG for 2 hours. Half of the cells were treated with 2mM DMI (DMI competitively inhibits NET).

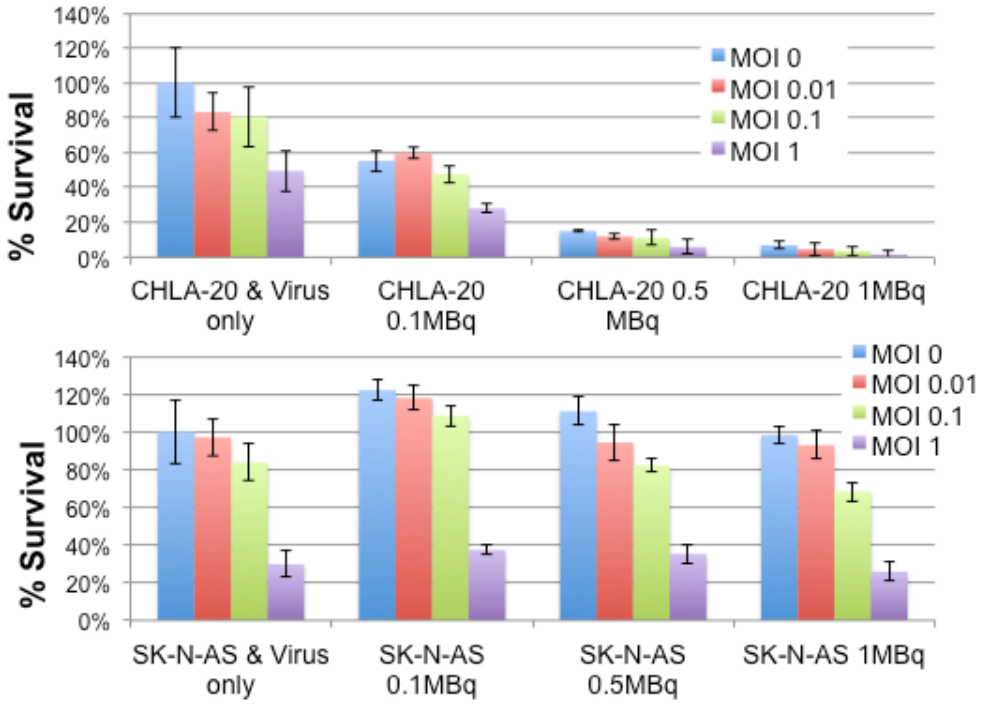

Figure 8. Cells were infected with HSV1716/NET at varying MOIs. 24 hours post virus infection, cells were exposed to ^{131}I-MIBG for 2 hours. MTS Assays day 4 is shown. The percent survival was compared to uninfected controls.

Discussion

Targeted radiation is a promising approach to neuroblastoma because it can greatly increase the therapeutic ratio and limit the normal-tissue toxicity typically associated with radiation. However, radionuclide therapy is only available for high-risk patients as a last option and is likely limited by radiation resistance developed from previous therapies.

This research evaluated the use of oncolytic virotherapy (HSV1716/NET) as a cancer gene therapy to deliver the NET transgene to preclinical models of neuroblastoma. The *in vitro* data confirmed the heterogeneity of neuroblastoma across several cell lines. HSV1716/NET replication and cytotoxicity assays were similar to the parent virus, thus indicating the transgene does not inhibit viral efficacy in neuroblastoma cells. We confirmed our initial hypothesis that HSV1716/NET can induce neuroblastoma cytotoxicity by viral infection. However, the cell lines varied in sensitivity to the virus, even though the cell lines were all the same tumor type. We also show variation in HSV1716/NET viral production/replication among the different neuroblastoma cell lines. Further research could investigate why such variation exists among cell lines of the same cancer in an *in vitro* setting. The diverse panel of neuroblastoma cells gives a representative sample that may resemble what is often seen in patients in the clinic.

We also determined each cell line had a different amount of endogenous NET. As neuroblastoma cell lines are serially passaged in culture, NET expression declines; thus, the NET expression we found may not accurately reflect the endogenous NET expression of the primary tumor sample at the time it was removed from a patient. The establishment of a varied NET expression prior to viral infection is important so we can determine if HSV1716/NET can increase NET expression in neuroblastomas with low NET expression as well as neuroblastomas

with high NET expression. If we can increase NET across all levels of NET expression, this will further enhance the selectivity of ^{131}I-MIBG uptake in tumor cells as compared to normal cells.

HSV1716/NET not only induces cytotoxicity, but increases the exogenous NET expression in the neuroblastoma cells. As HSV1716/NET contains the bovine NET transgene, we compared human and bovine NET transcripts post viral infection to determine whether any observed change was due to increased endogenous NET or production of exogenous NET. We confirmed the increase in NET transcripts is due to the successful gene transfer of NET by HSV1716/NET, and not due to a viral infection-induced increase of the endogenous NET. This confirms our hypothesis that HSV1716/NET effectively delivers the NET transgene and increases exogenous, not endogenous, NET expression

We next proved our hypothesis that HSV1716/NET enhances ^{131}I-MIBG uptake in neuroblastoma cell lines. The enhanced uptake peaked at 48 hours post-viral infection and then declined. The increase in uptake from 24 to 48 hours suggests the virus was able to increase the NET transgene with more replication cycles. However, the increase is not consistent with the decreased uptake seen at 72 hours. The decrease was possibly due to neuroblastoma cell death before exposure to ^{131}I-MIBG, either caused by viral cell killing or over-crowding of the cells in the plates due to insufficient cell-killing/cell overgrowth. Further studies are necessary to investigate the optimal timing of the combined therapy. We then demonstrated that the observed increase in ^{131}I-MIBG uptake was mediated by the viral transgene in HSV1716/NET. Neuroblastoma cells had similar ^{131}I-MIBG uptake when uninfected or infected with the virus without the NET transgene (HSV1716), but had enhanced uptake when infected with HSV1716/NET. This confirms that the enhanced ^{131}I-MIBG uptake seen in cells infected with HSV1716/NET is due to an increase in exogenous NET.

When the cytotoxicity of the combined therapies was examined, an additive effect was observed in the CHLA-20 cell line. This cell line was very sensitive to both the virus and radiation, but the observed additive effect was not synergistic. Alternatively, the SK-N-AS cell line was only slightly sensitive to the virus at an MOI of 1 and not sensitive to radiation at all. The results indicate that SK-N-AS is resistant to radiation, but because no synergy was observed in either cell line, another model might be necessary to further investigate cytotoxicity and synergy. The majority of cytotoxicity from ^{131}I-MIBG is thought to be due to the radiation induced bystander effect (RIBE), which is diminished in the 2D (2 dimensional) model used in our study. Previous findings show that 3D models have 80% cell kill after exposure to ^{131}I-MIBG, likely due to RIBE (Mothersill & Seymour, 2004). The initial cytotoxicity results are promising with one cell line, but in order to determine the true synergistic effect of the combined therapies, we will investigate a 3D model, either *in vitro* or *in vivo.*

There are various ways to increase NET expression in neuroblastoma cells through gene transfer, but the use of oncolytic virotherapy is unique in that the virus itself can contribute an additional cytotoxic effect to the cells. The oncolytic virus is thought to enhance the efficacy of ^{131}I-MIBG radiotherapy, but ^{131}I-MIBG might also enhance the cytotoxic effect of the virus. Advani et al. found that ionizing radiation enhances the replication of an oncolytic HSV-1 (Advani, S.J., Sibley, G.S., Song, P.Y., Hallahan, D.E., Kataoka, Y., Roizman, B., and Weichselbaum, R.R. 1998). We are currently investigating the effects of ionizing radiation from ^{131}I-MIBG on the viral replication of HSV1716/NET.

The current research found HSV1716/NET to effectively deliver the NET transgene and enhance ^{131}I-MIBG uptake. Additional work is needed to further investigate additive and synergistic effects of the combined therapies as well as the effect of ionizing radiation on viral

reproduction. The current findings will also be investigated *in vivo* to continue gathering preclinical data with the goal of bringing oncolytic viroradiotherapy into clinical trials to improve the outcomes of children with neuroblastoma. In the future, HSV1716/NET can be used to treat tumors that do not have NET and bring targeted radiotherapy to more cancer types.

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