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Evaluation of Daily Oral Kinase Inhibitors in Pediatric SPNET Tumor Xenografts

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Honors Thesis

Defense: April 19th, 2012
Introduction

Throughout the human body cells grow and die at different rates. Each tissue has specific cellular requirements that define the growth and death of its cells. For example red blood cells are replaced frequency whereas brain cells slowly die with age and are not replaced. Cancer comes from a fundamental break down in the regulation of growth. These new growing cells reach a point when they are too abundant. When they have surpassed the body’s need for new cells, they form large masses called tumors. Tumors are deadly when they become malignant. Malignant cells have the ability to invade and spread throughout other tissues of the body. They can also establish secondary growth areas in a process known as metastasis. Recruiting the blood supply and leaching nutrients from other areas of the body, tumors become a burden that ultimately weakens the host. A tumor’s ability to grow and spread is rarely impeded by the immune system. This is because tumors have a unique ability to evade the immune system, making cancer a silent killer (Darnell, 1990).

Medulloblastoma

Currently over 20,000 children under the age of 15 in the United States are undergoing treatment for a Brain or Central Nervous System (CNS) tumor. Brain tumors represent the most common solid tumor of all pediatric cancers. Unfortunately barely 50% of these patients will survive the upcoming year, with a quarter surviving a decade (CBTRUST, 2011). The majority of these incidents are in children between 3-7 years of age (Gottardo & Gajjar, 2008). Medulloblastomas represent the most common malignant pediatric brain tumor with approximately 400 cases annually diagnosed in the United States (CBTRUST, 2011). The causation of medulloblastoma is unknown in most
patients. Although medulloblastomas occur frequently in patients under 18 years of age, they comprise less than 1% of all adult primary CNS tumors (Packer et al., 1999). The World Health Organization, or WHO, classifies medulloblastoma as a primitive neuroectodermal tumor (PNET) but distinguishes five subtypes of medulloblastoma: classic, anaplastic, large cell, nodular desmoplastic, and medulloblastoma with extensive nodularity (Louis et al., 2007).

Children diagnosed with medulloblastoma five decades ago faced almost certain death. Clinical presentation varies with age, usually with signs of irritability, vomiting, hydrocephalus, truncal ataxia, lethargy, and headache. Medulloblastomas appear on CT or MRI scans as solid, heterogeneous, contrast-enhancing masses (Khatua et al., 2012). The advancements in medulloblastoma outcomes for children represent a successful achievement for pediatric neuro-oncology. Today’s treatment includes surgical resection, craniospinal irradiation, and chemotherapy curing 80-85% of children diagnosed with average or standard risk medulloblastoma and 70% of those classified with high risk disease (Gottardo & Gajjar, 2008). Standard risk patients are over the age of 3 years with localized disease and without anaplastic subtype; all other patients are considered high risk. Risk stratification in medulloblastoma is currently based on age, metastatic status, extent of surgical resection, and histological presence or absence of diffuse anaplasia (Leary & Olson 2012).

Unfortunately these impressive improvements in survival are achieved at a high cost of quality of life. For this reason patients under 3 years of age are not treated with craniospinal radiation, thus contributing to the poor prognosis of these patients. Many survivors experience significant long-term neurocognitive and neuroendocrine effects.
which are most commonly the cause of the resulting deterioration in patient quality of life (Gottardo & Gajjar, 2008). This is due to the standard dose of 23.4 units of absorbed radiation, or Gray (Gy), of craniospinal radiation therapy in standard risk medulloblastoma patients even though it has been successfully reduced from 36 Gy (Leary & Olson 2012).

Even though the majority of medulloblastoma occur sporadically from cerebellar granule neuron precursors that experience immense proliferation and migration immediately after birth, there are three known inherited syndromes associated with medulloblastoma (Packer et al., 1999; Hallahan et al., 2004). Gorlin’s syndrome is characterized by a deregulation of the Sonic Hedgehog (Shh) pathway. Basal cell nevus syndrome patients have also displayed Shh mutations. Turcot syndrome is associated with a deregulation of the WNT gene (Leary & Olson 2012). The Li Fraumeni syndrome results in a familial cancer predisposition due to p53 mutations. These genetic predispositions for medulloblastoma seem to not affect twins, as it is rarely reported in either monozygous or dizygous twins. Medulloblastoma as a whole affects predominately males. The understanding of these pathways has inspired the creation of transgenic mouse models characterizing the SHH and WNT pathways (Hatton et al., 2010). These models provide a background for the patient derived xenograph mouse models we used.

**Medulloblastoma vs. SPNET**

Both supratentorial primitive neuroectodermal tumors (SPNET) and medulloblastoma are primitive neuroectodermal tumors. Initially it was thought that all PNETs shared a common origin from primitive neuroectodermal cells (Inda et al., 2006).
Primitive cells from which these tumors arise grow in sheets or cords of dense cellularity, and are characterized under a microscope by massive nuclei in comparison to the amount of cytoplasm present (Inda et al., 2006). This allows for both types of tumor to appear under the microscope as small, round, poorly differentiated, cells with large nuclei.

Despite the histological similarities some studies suggest medulloblastoma and SPNETs are genetically dissimilar. Isochromosome 17q is the result of a transverse centromere split during mitosis or meiosis that alters the genetic material. It is also the most frequent abnormality seen in medulloblastoma. Although SPNET genetics have not been comprehensively studied, isochromosome 17q is a rarity in SPNETs (Burnett et al., 1997). Another example is the hypermethylation of the CDKN2A locus observed in medulloblastoma with a 64% frequency and absent in SPNET. Fourteen percent of SPNETs had a loss of 14q. The same loss of 14q was not observed in medulloblastoma. In turn, the patched gene located at 9q encodes a tumor suppressor protein that regulates the Shh pathway; mutations of the gene have been found in patients with basal cell nevus syndrome, who have an increased risk of medulloblastoma (Burnett et al., 1997). Russo et al. noted through comparative genetic hybridization that a loss of genetic material was more common than a gain in SPNET. But gain of genetic material was more common in medulloblastoma (1999). Even though the genetic studies of SPNETs are quite limited, these examples of genetic difference are a small component of fully understanding medulloblastoma and SPNET as entirely separate conditions. Research suggests that medulloblastoma, not SPNET, arise from cerebellar granule cells (Pomeroy et al., 2002). The question arises as to whether or not small cell embryonal tumors in the CNS should be categorized together as PNET, or whether medulloblastoma, SPNET, etcetera should
be considered independent diseases with different molecular pathways (Inda et al., 2006). Judging by the comparative analysis, SPNET and medulloblastoma can be divided into two biologically distinct tumor types.

**Supratentorial Primitive Neuroectodermal Tumors**

Although histologically similar to the medulloblastoma, SPNETs are in fact distinct entities. SPNETs are a rare childhood brain tumor comprising only 2.5-6.6% of all pediatric brain tumors, and are extremely rare in adults with only 57 cases ever reported worldwide (Raghuram et al., 2012; Ohba et al., 2008). These tumors were characterized in the 1990s and have not been extensively studied. They present clinically similar to medulloblastoma with the exception of the location of the solid, heterogeneous, tumor with respect to the tentorial: medulloblastoma is always infratentorial. The tentorial is the line that separates the cerebellum from the cerebrum. If a tumor is said to be infratentorial then it lies in the cerebellum. SPNETs are divided into two categories: pineal and non-pineal tumors. Pineal SPNETs affect the pineal gland. (Biswas et al., 2009). An SPNET is thought to be less curable than the medulloblastoma because only 20-30% of diagnosed patients reach the 5-year survival mark. One explanation of this difference is the inability to completely resect supratentorial lesions (Russo et al., 1999). In addition to the poor 5-year survival rate, SPNET patients have been reported to have 30-100% relapse after standard therapy; no standard treatment exists for relapsed SPNETs and their outcome is extremely poor (Butturini, 2009). The current meager SPNET overall survival data created a need for this research.
Standard therapy does not exist for SPNETs. Packer et al. set the standard therapy for medulloblastoma in 2000, and since then this chemotherapy regimen has been adopted to treat other embryonal brain tumors such as SPNET, but to no avail (2000; Leary & Olson, 2012). They are treated identically to the high risk medulloblastoma patients except for the higher dose of craniospinal radiation of 39 Gy to treat high risk individuals, patients>3 years of age (Reddy et al., 2000). Nevertheless several authors have reported SPNETs responding to conventional treatments: for example high-dose chemotherapy with autologous bone marrow or stem cell rescue (Chintagumpla et al., 2009). It is imperative that SPNETs undergo further study so as to establish a standard therapy. A xenograft model was utilized to test novel therapies on SPNETs.

The biological distinction between medulloblastoma and SPNET along with the astounding difference in patient survival created a need this study. We searched for an alternative method of treatment for SPNETs in hopes of significantly improving the patient survival and quality of life following treatment. The recent successes with novel therapies utilizing kinase inhibitors in other forms of cancer such as breast, prostate, and non-Hodgkin’s lymphoma has directed our curiosity to the potential application of these types of treatment to embryonal brain tumors and specifically SPNETs. (Ramakrishnan et al., 2011; Nam et al., 2005; Yu et al., 2001).

**Targeted Kinase Inhibition**

Cell growth is a highly regulated process, in which a cell increases it mass, duplicates it DNA and then splits into two daughter cells. This progression is known as the cell cycle and it is regulated kinase cascades (Darnell, 1990). A kinase is an enzyme
that transfers a phosphate group from a donor to an acceptor; in general the ATP acts as
the donor (Voet et al., 2008). The result of kinase interactions with receptors creates a
cascade of cellular events, potentially involving multiple pathways. These cascades
regulate the cell cycle progression. Tumors form as a result of the disregulation of the cell
cycle due to genetic mutations. Activated genes can ultimately increase the frequency of
cell division causing tumors to grow. Conversely deactivated genes can halt cell division
and induce programmed cell death, apoptosis, thus causing tumors to shrink (Darnell,
1990).

Cell signaling allows cells to communicate and coordinate the growth, death, and
metabolism of groups of cells. Extracellular chemicals synthesized and released by the
signaling cell are used to communicate. When these signals are transported to, and
received by the target cell they cause response triggered by the receptor-signaling
molecule complex. This response can affect one or multiple pathways in a cascade of
cellular events leading to a metabolic change. A common theme is the use of a kinase as a
receptor protein waiting to elicit a response triggered by a phosphate group. The rate of
the cell cycle can be affected by a disregulation in a regulatory kinase leading to tumor
metastasis. The web of regulatory pathways is vast and highly intertwined. One pathway
can elicit a variety of responses from multiple pathways (Darnell, 1990). This makes
malignant cells difficult to target and kill.

The traditional chemotherapy methodology is to utilize a general cytotoxic agent
to eradicate malignant cells. A cytotoxic drug is any compound that inhibits cell
proliferation within the body. A therapy is defined as cytotoxic when the targeted
cancerous cells along with the majority of naturally occurring, rapidly dividing cells are
also affected. Well-known side effects of chemotherapy, such as hair loss and nausea, are due to the use of general cytotoxins (Mukherjee, 2010). Taking advantage of the understanding that malignancy is due to regulation errors in the cell cycle, kinase inhibitors possess the ability to halt malignant cell growth in either the tumor or surrounding blood vessels. If the overactive kinase induces malignant cell growth, a specific kinase inhibitor can act as a cytostatic agent, suppressing the growth of cells. This inhibitor could prevent the tumor cells from growing or prevent angiogenesis and cause the tumor to shrink due to a lack of nutrients.

Dasatinib

Src family kinases (SFK) have elevated activity levels in numerous human cancer cell lines (Nam et al., 2005). SFKs phosphorylate tyrosine residues resulting in the activation of oncogenic signal transduction pathways. These pathways regulate tumor cell’s adhesion, migration, and invasion abilities (Nam et al., 2005). Dasatinib [N-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide] is a synthetic, orally available, small molecule inhibitor of Src Bcr-Abl family kinases (Tokarski et al., 2006). Its mechanism of action is based on its ability to disrupt the oncogenic signal transduction pathways (Nam et al., 2005). Dasatinib is FDA approved to treat Philadelphia Chromosome positive patients presenting with Acute Lymphoblastic Leukemia and Chronic Myelogenous Leukemia along with currently being studied to treat a variety of other cancers such as prostate; it has never been used to treat medulloblastoma in humans. Dasatinib was chosen as an agent because of its FDA approval, its ability to cross the blood-brain barrier, and its availability at the pharmacy at Seattle Children’s (Porkka et al., 2008).
Sorafenib

Vascular endothelial growth factors (VEGFs) and disregulation of Raf pathways are vital to angiogenesis and tumor growth. Raf kinases are serine/threonine-specific protein kinases that regulate tumor growth and survival (Shiota et al., 2009). In contrast, VEGFs affect the cell migration and spreading of tumors. Sorafenib [4-[4-[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-N-methyl-pyridine-2-carboxaminde] is a small molecule inhibitor of VEGF kinases and the Raf kinase. It has exhibited anticancer properties in pancreatic, renal, ovarian, and nonsmall cell lung cancer cell lines, but has never been used to treat medulloblastoma (Ramakrishnan et al., 2011). Akt is another serine/threonine kinase that inhibits apoptosis. It is also involved in glucose metabolism, cell migration, and transcription. Sorafenib has also shown Akt inhibition by an unknown mechanism in Acute Lymphoblastic Leukemia (Schult et al., 2010). When bevacizumab, a single agent VEGF inhibitor, was tested in Non-Hodgkins Lymphoma, where VEGFs are over expressed, only a 2% overall response rate was reported. This suggests that treatments targeting other pathways along with VEGFs must be utilized in combination with VEGF specific therapies (Ramakrishnan et al., 2011).

Thus Sorafenib with its broad antiangiogenic properties and ability to cross the blood-brain barrier becomes appealing, especially considering its Raf inhibition as well (Lemasson et al. 2010). With one study goal of ours being to select agents for further combination studies in SPNETs, Sorafenib becomes an ideal therapeutic due to its broad anticancer properties.
Erlotinib

Epidermal growth factors receptors (EGFRs) are regulated by tyrosine kinases (Ng et al., 2002). Inactive EGFRs exist in a monomer state and consequently dimerize after ligand binding (Voet et al., 2008). This activates the tyrosine kinase intracellular domain of the receptor, instigating a cascade of intracellular events (Ciardiello & Tortora, 2001). The autophosphorylation of the tyrosine kinases is crucial to the activation of many EGFRs, even in the absence of an extracellular signal. This is advantageous because many different pathways can be affected by a single signal (Voet et al., 2008).

Malignant cells secrete Epidermal growth factor; and over-expression of EGFR has been shown to contribute to a malignant phenotype in cells (Ng et al., 2002). EGFR signaling is crucial for cell cycle progression from G1 to S phase. Further studies have shown EGFR-mediated signals are active in angiogenesis, metastatic spread, and the inhibition of apoptosis. Over-expression of EGFR has been associated with many different solid human cancers such as breast, cranial, gastric, prostate, bladder, ovarian, and glioblastomas (Ciardiello & Tortora, 2001). By inhibiting EGFR and the consequential cascade of events that follow, it would be possible to specifically prevent the proliferation of and even kill the malignant cells (Voet et al., 2008).

Erlotinib [16,7-bis(2-methoxy-ethoxy)-quinazolin-4yl-(3-ethynylphenyl)amine], formerly referred to as OSI-774] reversibly inhibits EGFR kinase activity (Ciardiello & Tortora, 2001; Ng et al., 2002). Erlotinib’s affect on EGFR autophosphorylation in intact tumor cells has been shown to induce apoptosis in human colon cancer cells (Ng et al., 2002). Previous success against colon and lung tumors has been achieved with Erlotinib and cisplatin in human tumor xenographs, and it is currently in a handful of clinical trials
one example being pancreatic cancer (Ciardiello & Tortora, 2001; Troiani et al., 2012).

Although gene expression studies of SPNETs have not been conducted to measure epidermal growth factors, the previous efficacy of Erlotinib against a wide variety of solid tumor cancers was the basis for its inclusion in this study (Momota et al., 2008; Pomeroy et al., 2002).

**Rapamycin**

Rapamycin [(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,01,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2[1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]-oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone] is a natural product isolated from the bacterium *Streptomyces hygroscopicus* indigenous to Easter Island. It is a macrolide antibiotic but has been shown to possesses anti-fungal, immunosuppressive, and anti-tumor properties (Yu et al., 2001). Rapamycin allosterically inhibits the mammalian target of Rapamycin (mTOR), a kinase (Yang et al., 2011). Cell cycle progression is regulated by the mTOR protein due to its enhancement of the translation initiation complex’s interactions with the mRNA 5´ cap (Yu et al., 2001). Rapamycin has not had overwhelming anti-tumor clinical success due to its instigation of feedback-activated survival signaling pathways such as Akt phosphorylation (Yang et al., 2001). This effect bypasses the turned off mTOR protein, and is a result of the cascade of cellular events induced by Rapamycin. In order for Rapamycin and its derivative to achieve complete success, mTOR inhibition must be coupled with Akt inhibition, caused by Sorafenib, due to the parallel nature of the pathways (Schult et al., 2010). Similar to
the targets of the other kinase inhibitors previously mentioned, the mTOR-signaling pathway has been found expressed in many human tumors (Yang et al., 2011). This makes Rapamycin a potentially successful agent in our study, especially if combination studies are implemented.

Due to the differences between medulloblastoma and SPNET, we hypothesized that in order for the long-term patient survival for SPNET to improve it is possible a novel therapy must be utilized. In this study patient-derived xenograft models of SPNET were treated with single agent kinases inhibitors in hopes of taking the necessary initial steps toward improving long-term patient survival and quality of life for patient diagnosed with SPNET.

Materials and Methods

Patient Derived Xenograph Establishment

All mice were of the Nu/Nu strain obtained from JAX laboratories and maintained in accordance with the NIH Guide for the Care and Use of Experimental Animals. All experiments and animal handling were done with the approval of the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Animal Care and Use Committee (IACUC) (IR#1697). Nu/Nu mice are immunocompromised, decreasing the likelihood the transplant will be rejected by the mouse (Khor et al., 2006). Human tissue samples were obtained from Seattle Children’s Hospital after they were resected in a surgical procedure while the patient was under general anesthesia. The Institutional Review Board (IRB) approved the use of resected tumors. The samples were transported to FHCRC packed in solid CO₂; they arrived within two hours of the completion of the surgery and were
immediately taken to tissue culture. Once under the cell culture hood, the tumor was placed in 2-3ml of Dulbecco’s Modified Eagle Medium (DMEM; Gibco (Invitrogen) New York) on a 10cm diameter plate. Using a pair of scalpel blades the tumor was gently chopped into smaller pieces. Two of these pieces were saved for future analysis; one was placed in 10% paraformaldehyde and the other in an Eppendorf tube, which was snap frozen at -70°C. The tip of a filtered p1000 pipette tip was cut so the diameter equaled 0.7~0.8mm. A normal pipette tip is too narrow to be used to separate the tumor without damaging an unacceptable percentage of the viable cells. The DMEM solution and tumor pieces were pipetted up and down with the cut tip for about 40 cycles. The tumor dissociated easily and fell into a single cell suspension. The fibrous tumor portions were left intact. A cell strainer (BD Bioscience Maryland, 70μm-100μm) was placed in another 50ml conical tube. The cell suspension including any large pieces were pipetted onto the cell strainer. An additional 2-3ml cold DMEM was used to wash the cell strainer. The remnants in the strainer were discarded. Using a 10:1 dilution (900μl DMEM, 100μl cell suspension) the viable cells in suspension were counted using a Vicell flow cytometer (Beckman Coulter California). If the percentage of viable cells was acceptable (>20%) a centrifuge was used and the cell suspension was spun at 1000rpm for 5 minutes. Afterwards the supernatant was removed. The remaining pellet of cells was resuspended at a concentration of 10x10^6 cells/ml in cold, serum free, DMEM. Serum-free media was used because the recipient mouse provided the necessary serum. The final concentration was 5x10^6 cells/ml. The equation to calculate the resuspension volume is as follows:

\[
\frac{ViableCells}{ml} \cdot \frac{TotalVolumeCellSuspension(ml)}{5 \times 10^7 (cells/ml)} \cdot 1000 (cells/ml)
\]
surgery Nu/Nu mice were anesthetized with 600μl of Avertin (Sigma-Aldrich, St. Louis, MO). Each eye was treated with eye ointment to keep the eye hydrated easing the post-procedure recovery. Surgery began when the mouse no longer responded to a test foot pinch. The head and neck were swabbed with an alcohol swab prior to incision. Starting between the ears the incision ended at the base of the skull. It was kept as small as possible to expedite the healing. Using a drill designed for dental procedures, a small burr hole was drilled through the bone. Using a p20 pipette with a 2μl tip, 2μl of the cell suspension were injected into the cortex of the cerebrum. Once the pipette tip was removed a small piece of SurgiFoam (Seattle Children’s Hospital) was placed into the burr hole. As the scalp-tissue was pulled back together Tissuemend II (Veterinary Products Laboratory, Columbus, Ohio) was placed over the incision to seal it. The mouse was placed on a heat mat to help maintain its body temperature during recovery. Additionally 500μl of saline was injected subcutaneously between the shoulder blades to keep the animal hydrated. The animal was monitored each hour until consciousness was regained; it was then placed back into the cage. Three unique patient derived xenograph lines were maintained; the subjects for this study were drawn from each of the three lines based upon the timing of the appearance of tumors.

SPNET Flank Allograft

Once a brain tumor was present in a patient derived xenograft mouse, the mouse was euthanized using CO₂ inhalation. Tumors usually arose after 8 weeks, and the animals were sacrificed before symptoms became severe enough to impede the animal’s quality of life. The tumor was removed and placed in 3-5ml of cold DMEM (or PBS) in a 50ml conical tube that was placed on ice for transfer to the cell culture facilities. The
tissue culture procedure was identical to the xenograft establishment methodology, with
the exception being an equal volume of Matrigel (BD Bioscience Maryland), a substance
that solidifies at room temperatures, was added to the cell suspension to keep the cells in
a matrix once injected, improving the percentage of successful injections. This was added
as a final step. The final concentration was 5x10^6 cells/ml. Using a 1ml syringe and a 30
gauge needle the solution was drawn from the conical tube and the syringe placed on ice
until injection. This increased the survival time of the viable cells and prevented the
Matrigel from solidifying. Five recipient Nu/Nu mice were anesthetized prior to injection
with isoflorane inhalant. In the right rear flank of each mouse a subcutaneous injection of
200μl (1x10^6 cells/ml) was administered (Hallahan et al., 2004; Hatton et al., 2010;
Hatton et al., 2006).

Drug Delivery

Once the tumor was growing and measurable (>0.5cm), the mouse was enrolled
into one of the five study arms, including four treatment arms and one vehicle control
arm. A rolling enrollment was used starting with treatment group “A” (Sorafenib)
followed by group “B” (Rapamycin) and so forth until all the study arms had one mouse.
When the sixth tumor was large enough for enrollment, group “A” received its second
animal. This continued until all groups had a sample size of 3. Each mouse was given
drug by daily gavage therapy for four weeks. Doses were based on FDA approved dosing
in adult humans and converted to mouse dosing using the NCI dose calculator assuming
mouse weight of 20 grams. To calculate the dose all mice were assumed to weight 20
grams. The animals were weighed upon enrollment into the study in order to determine
their healthy weight. The same dose was given to every adult mouse independent of his or her body weight.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Daily Gavage Dose</th>
<th>Dose Administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorafenib</td>
<td>1.2mg/kg</td>
<td>0.24mg</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>0.1mg/kg</td>
<td>0.02mg</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.8mg/kg</td>
<td>0.36mg</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>0.5mg/kg</td>
<td>0.10mg</td>
</tr>
</tbody>
</table>

Table 1: Kinase Inhibitors and their respective doses based on the NCI dose calculator (Kang et al., 2011)

Each dose is based on the NCI calculator, and calculated with the assumption that every mouse weighed 20 grams. Mice were weighed weekly to monitor health but these measurements did not affect the dose administered.

Approximately 10 doses of each drug were weighed into an Eppendorf tube (refer to concentrations above) labeled “Drug A, Drug B, etc.” including a vehicle control. The nondescript labels were used to blind the observer who delivered the drug because of his or her other role measuring tumor size. Tubes were stored in -20°C. 1% Methyl cellulose 0.1% Tween 80 solution, the vehicle for drug administration, was added to each tube, approximately 1ml, until the desired concentration of the drug was reached. Due to insolubility, sonication and vortexing was used to create a suspension. All the drugs are soluble in DMSO and ethanol but have very poor solubility in water due to their non-polar properties. The drug would fall out of suspension and pellet in the bottom of the tube. Therefore prior to administration the tubes were vortexed until the pellet was grossly resuspended. They were then placed in a sonicater for 1 hour. Once in the
procedure room the tubes were vortexed immediately before drawing the drug into the syringe. A 1ml syringe was used to "slurry" the solution prior to injection. A gavage needle was used to administer a 100μl oral dose to the mouse. The mice were given daily oral gavage treatment for 28 days. Every third day the tumor was measured with a pair of calipers. To calculate tumor volume the equation \( \frac{(Length \times Width^2)}{2} \) was used. The reason for squaring the width is to provide an estimate for the depth of the tumor into the tissue.

**Tumor Resection and Fixation**

At the conclusion of the 4-week treatment period the mice were euthanized with CO\(_2\) inhalation. Tumors were resected from the flank for further analysis. Half of the tumor was fixed with 10% paraformaldehyde, and the other half was snap frozen at -70°C. Tissue blocks were paraffin embedded, cut into 4-μm sections, and then stained with hematoxylin and eosin using standard methods (Hallahan et al., 2004). An outside observer blinded from the treatment data evaluated the pathology.

**Statistical Analysis**

The recorded tumor volumes were analyzed using NCSS 2007 (NCSS Statistical Software Utah). A Bonferroni (with control) multiple comparison test with an alpha=0.05 was used. A Dunnett’s Upper and Lower one-sided multiple comparison test were also used. Graphs were made using Microsoft Excel (Microsoft, Seattle, WA) and R (Lucent Technologies, New Jersey, NJ).
Results

In order to test the efficacy of novel oral kinase inhibitors in SPNET xenografts, 15 mouse flank tumors from three different patient-derived SPNET xenografts were enrolled into one of the five treatment arms. The tumors were treated with the kinase inhibitors (Erlotinib, Dasatinib, Rapamycin, and Sorafenib) or the vehicle control for 28 days via daily gavage.

Throughout the entire study period the vehicle control was found to be statistically significant from the other treatment groups (p<0.05) (Fig. 1). The Sorafenib, Rapamycin, and Dasatinib groups were not found to differ from each other significantly; however, the Erlotinib treatment arm did differ significantly from Dasatinib, Rapamycin, and Sorafenib (p=0.05) (Fig. 1, 2). All analysis comparing groups was reported using the Bonferroni and Dunnett's comparison tests. The proportional tumor volume on the final day of treatment exemplifies the difference between the vehicle control and kinase inhibitors (Fig. 2). The kinase inhibitor treatment groups showed a slower growth rate than the vehicle control throughout the study period (Fig. 1). This was attributed to smaller proportional tumor volumes on the final day for the kinase inhibitor groups (Fig. 2).

The mouse xenografts did resemble human SPNET histologically due to the presence of round, blue cells with large nuclei when stained with hemotoxylin and eosin (Fig. 3). The multiple mitotic events in both the vehicle control and Erlotinib treatment group also represent a similarity between the xenograft model and SPNET in humans (Fig. 3A, 3B). Apoptosis noted in the Erlotinib treatment group is consistent with
previous tumor response and the induction of apoptosis by Erlotinib (Fig. 3B). The tumors seemed to be outgrowing their nutrient supply due to the necrosis seen in both kinase inhibitor treated and vehicle control groups (Fig. 3C, 3D).

Thus it appears a marked difference was observed between the kinase inhibitor therapy and the vehicle control. It suggests Erlotinib, Rapamycin, Sorafenib, and Dasatinib warrant further study in SPNET. All kinase inhibitors are potentially successful agents to be utilized in combination studies especially the combination of Sorafenib and Rapamycin. As a single agent, Erlotinib seems most promising.
Figure 1: Growth of treated SPNET tumors throughout entirety of study period

Proportional tumor growth of each treatment arm throughout the entire treatment period with n=3 for each treatment arm. Tumor volumes have been normalized to themselves to show a proportional growth curve. R-squared values for the above regression lines are: Sorafenib (0.7585); Rapamycin (0.9576); Erlotinib (0.0897); Dasatinib (0.9412); Vehicle Control (0.9306).
Figure 2: SPNET Tumor volumes as measured on final study day group into treatment arms

The size of each tumor is plotted based on its proportional size compared to the day it was enrolled into the study. They are grouped based upon the treatment arm they belonged to. Only tumors that received treatment for the entire period are shown.
Figure 3: Pathology of sPNET xenograft tumors after study period stained with hematoxylin and eosin (H&E).

Lighter stained areas of the 10x and 4x images are dead cells bordering the dark live tumor cells.
Discussion

The flank xenograft model that has been established for the pediatric patient-derived brain tumors is a useful model for evaluation of novel therapeutics. This is due to the successful transplantation and treatment that replicates the previous success with medulloblastoma xenografts (Hallahan et al., 2012). The Nu/Nu immunocompromised mice did not reject the flank tumor. Matrigel was used by Rossi et al. in order to increase efficacy of flank xenograft injections, identical to the method of flank xenograft establishment used in this study (2010). The initial transplant into the cerebrum had been previously established; this model is what the xenograft in this study was based upon (Hatton et al., 2006). A major difference is the acquisition of tumors. Hallahan et al. used a mouse modified in the SmoA1 gene that spontaneously generated medulloblastoma, whereas a direct transplant of patient-derived cells into the cerebrum was used in this study (2004). Simultaneously Hallahan et al. translated a successful treatment achieved by Notch pathway inhibition in the spontaneous medulloblastomas into clinical trials by confirming the role of Notch signaling in human medulloblastoma by using RT-PCR on the Notch gene (2004). Based on these previous results it can be assumed that the results seen in the SPNET xenograft model can be translated to human patients presenting with SPNET. Hallahan et al. can be used as a reference in directly adapting therapeutic successes seen in future xenograft studies to clinical trials; therefore, this model has potential for use in evaluation of other pediatric brain tumors with novel therapeutics (2004).
Due to the marked response seen in the treatment groups, Erlotinib, Sorafenib, Dasatinib, and Rapamycin warrant further study in SPNET. Their statistical difference from the vehicle control treatment arm justifies their use in further combination studies. The reactivation of the Akt pathway previously seen to limit the Rapamycin’s efficacy could potentially be solved by combination therapy with Sorafenib (Yu et al., 2001). A synergistic response has been observed due to a reduction in Akt activation and angiogenesis through treatment with Sorafenib (Ibrahim et al., 2012; Ramakrishnan et al., 2012). Erlotinib’s unique response from the other treatments could be correlated to the apoptosis seen in the pathology (Fig. 3). The R-squared value of 0.0897 for the Erlotinib group suggests nonlinear growth (Fig. 1). Although the pathology has not been quantified, Erlotinib does have history of inducing apoptosis in colon cancer cells (Fig. 3; Ng et al., 2002). If this is seen consistently in all Erlotinib-treated SPNETs it could be in response to EGFR over-expression. Sorafenib has also been known to induce apoptosis but the histology has not been completed for the Sorafenib treatment group (Rossi et al., 2010). Thus, Erlotinib and its relationship to EGFR over-expression should be prioritized when developing combination therapies along with gene expression analysis. Also a combination of Rapamycin with Sorafenib unquestionably warrants further study due to the previous success seen when combined.

Obviously the ultimate goal in a study of this nature is to make the cancer itself disappear. Thus far we have only shown a reduction in tumor growth rates. In previous studies kinase inhibitors have had limited success due to other pathways that run parallel to the targeted pathway. An example of this is Rapamycin and the Akt pathway it triggers via feedback activation (Yu et al., 2001). Based upon the assumption that more of these
pathways exist without our knowledge, complete success lies in the area of combination studies. By combining the single agents, especially Erlotinib, with the other drugs we hope to observe complete tumor disappearance in the future.

As the flank tumors presented themselves from the three xenograft lines they were assigned to a study group starting with group “A” for the first tumor to present itself; group “B” for the second tumor, and so forth. This style of randomization was utilized to expedite the pace of the study, allowing for each treatment group to obtain a full cohort of three animals as rapidly as possible. An issue presented by this style of randomization is group “A” theoretically could have obtained the first and sixth fastest growing tumors; group “B” the second and seventh fastest, etc. These potential differences in growth rates could have affected the perceived efficacy of the treatment agents. A simple random sampling with a tumor from each xenograft line having the same chance of being selected would have been ideal. But due to the period of time required for tumors to present themselves this was not feasible in terms of time.

Erlotinib, Rapamycin, Dasatinib and Sorafenib have previously been used to treat mouse xenograft models. A major difference seen in this study when compared to others is the dose concentrations. Sorafenib was administered via oral gavage in 200μl doses (Yuen et al., 2011). The 40mg/kg dose used by Yuen et al. is much larger when compared to the 0.5mg/kg dose used in this study (2011). Even though a response was seen, the dose of Sorafenib could be increased in further studies in accordance with the IACUC. Dasatinib was administered twice daily via oral gavage at a dose of 25mg/kg (Rossi et al., 2010). The vehicle used by Rossi et al. is not mentioned, but similar to Sorafenib the dose can be increased in future studies (2010). Dasatinib was conversely administered via
intraperitoneal (i.p.) injection, but in order to mimic human patients Dasatinib was administered orally in this study (Rossi et al., 2010). Even though Dasatinib treatment only continued for 7 days, necrosis was also noted when tumors were resected for histology (Rossi et al., 2010). Erlotinib has also been used to treat mice via oral gavage at 100mg/kg, with a vehicle of delivery not noted (Abraham et al., 2011). This does is much larger than the dose used in this study but there is no mention of a “slurry” technique or vehicle. Once solubility issues are overcome the Erlotinib dose could be increased in accordance with IACUC. Rapamycin has previously been used to treat mice at 4mg/kg by i.p. injection (Wang et al., 2011). Once again this dose is higher than the Rapamycin dose used in this study. Overall the oral drug doses were administered at conservative concentrations. If needed all the doses could be increased in future studies.

The methodology of drug delivery poses another error source. All of the agents are insoluble in H₂O or other polar solvents such as saline. We found that all agents were completely soluble in 100% DMSO. Other studies avoid this issue by using i.p. injections but oral gavage was used in order to mimic clinical trials as closely as possible (Wang et al., 2011; Rossi et al., 2010). Due to the known effects of DMSO on DNA at low concentrations it was decided not to use DMSO as a vehicle for delivery (Jia, 2010). In other studies Rapamycin was dissolved in 5% polyethylene glycol (PEG) (Sigma) and Tween 80 in PBS (Wang et al., 2011). Sorafenib was administered in a 30% Captisol vehicle (Yuen et al., 2011). Captisol is a β-cyclodextrin known as SBE-CD; its hydrophilic ring structure and slightly hydrophobic interior make it ideal for drug delivery (Yuen et al., 2011). Captisol could have been an option but instead the “slurry” method was developed with obvious flaws using a vehicle similar to Wang et al. with
Tween 80 in PBS (2011). The most obvious flaw was the inability to know the exact concentration of drug delivered in each treatment. The agents were dosed into tubes based on the quantities of 10 daily doses. Vortexing and sonication were utilized to break the drug into the smallest particles. During the intensive vortexing and repeated delivery using the same tube it is possible some of the active agent was rendered ineffective. The assumption was made that over a period of 10 doses the animal would receive an average of the desired dose over the 10-day period. But it is possible a portion of the active agent was stuck to the sides of the tube and never entered the gavage needle. In future studies if Captisol can solve the solubility issue these kinase inhibitors present, it would be possible to eliminate the "slurry" method. All of the agents are normally administered to humans in pill form.

It should be noted that the use of digital calipers represents the largest consistent source of error. When the calipers are placed around the tumor the dimensions will vary between two different measurers. The difference comes from how tightly the measurer places the calipers around the tumor. The measurer was consistent throughout the entire study period with the exception of a few weekend days. Yuen et al. is one example of other studies that used calipers to measure tumor volume with these same restrictions, but attempted to correct for them by measuring every two days instead of bi-weekly as in this study (2011). Another option that depends on funding would be to use bi-weekly MRI or CT scans to measure the tumor. This method would place the mice under the added stress of being anesthetized twice a week. The calipers were placed around the tumor as tight as possible without altering the natural shape of the tumor. But the correct fit of the calipers
around the tumor is subjective to each measurer's own personal opinion. This would affect the volume calculation ever so slightly.

Upon the completed treatment of all subjects the tumors were removed after the animal was sacrificed. The pathological review of these samples still needs to be completed. Quantification of the mitotic events and apoptotic bodies seen in all treatment groups is needed. This data will allow for comparisons between groups that can evaluate correlation and, potentially in combination with gene expression analysis, causation. The analysis of the snap-frozen tissue samples has not been started. The RNA analysis is critical in evaluating the link between the efficacy of the agents and their known pathways. For example if an over-expression of EGFR is seen in the snap frozen samples it proves causation between Erlotinib and the apoptotic bodies seen.

Successfully curing solid tumors in an animal model unfortunately does not always translate into success in human patients. Eventually the goal is to develop future clinical trials based upon the successes that we hope will be achieved with the combination studies. This study was the first step in successfully obtaining the cure for one type of pediatric cancer that has been eluding physicians for centuries.
Bibliography


