



## **Hyundai Hope on Wheels Hyundai Scholar Research**

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Metastatic neuroblastoma remains a difficult disease to treat with survival rates of less than 40 % despite aggressive therapy. N-MYC, a transcription factor found in neuroblastoma that portends a poor prognosis, is often amplified in these children. The treatment of recurrent or refractory disease includes myeloablative chemotherapy, radiation therapy, and stem cell transplant. This treatment plan is highly toxic, and has remained relatively unchanged for a number of years. A better understanding of the mechanisms regulating neuroblastoma growth and N-MYC activity is likely to lead to better approaches to treatment.

The observation that neuroblastoma occurs more frequently in children with high birth weights has led to the speculation that growth factors such as IGF-I may be important in the pathogenesis of neuroblastoma. IGF-I is a small peptide that signals through a specific membrane tyrosine kinase receptor, the insulin-like growth factor-I receptor (IGF-IR), and has an important role in cell survival and proliferation. Signals are transduced through either the ras/MAPK or PI3 kinase/Akt pathways. The mammalian target of rapamycin (mTOR) is a downstream element of the PI3 kinase/AKT pathway that plays an important role in the regulation of cell growth, proliferation, motility, and survival. Given that cell survival and proliferation are key aspects of malignant cells, the IGF-I signaling pathway and mTOR present intriguing avenues for neuroblastoma research.

mTOR can be blocked by rapamycin, a commercially available drug that is already approved for use in children. Analogs of rapamycin exist, including temsirolimus, which is currently being investigated in a number of adult cancers. My previous work has been through collaboration with Dr. Billie Moats-Staats at the University of North Carolina at Chapel Hill investigating the impact of mTOR blockade in neuroblastoma. We inhibited mTOR signaling in neuroblastoma cell cultures by treating them with rapamycin or temsirolimus and found that this decreased both neuroblastoma cell number and N-MYC activity. This work has resulted in two publications in the medical literature.

One of the ways these drugs may have inhibited cell growth is a process called autophagy, a process where cells begin digesting themselves from within and eventually die. The inhibition of mTOR with rapamycin or temsirolimus is one way to increase autophagy in cells, but other methods of inducing autophagy have been described. Valproic acid, a histone deacetylases inhibitor, is a drug approved to treat children with seizures that has been shown to increase autophagy in cells without blocking mTOR.

We plan to evaluate the induction of autophagy in human neuroblastoma cell cultures through mTOR-dependent and mTOR-independent mechanisms. **We hypothesize that rapamycin, temsirolimus and valproic acid will induce autophagy in neuroblastoma cell cultures, and that combinations of rapamycin or temsirolimus with valproic acid will result in additive cytotoxicity.** We plan to evaluate neuroblastoma cell cultures for multiple protein markers of autophagy, which may provide further potential targets for investigation.

## **SPECIFIC AIMS**

1) Study the *in vitro* effects of temsirolimus, rapamycin, and/or valproic acid on induction of autophagy in neuroblastoma cells. Measures to be used include:

- a. Western analyses to determine the changes in expression of components of the PI3K/Akt and ras/MAPK signaling cascades, and the phosphorylation status of N-MYC.
- b. Western analyses to determine changes in expression of autophagy markers including components of the mTOR complexes 1 (mTORC1) and 2 (mTORC2), Beclin 1 (also known as ATG6) and ATG 1
- c. Western analyses analysing the phosphorylation states of the autophagy markers to determine induction and activation states resulting from drug treatments.

## **SIGNIFICANCE**

Neuroblastoma is the most common extra-cranial solid tumor of childhood. For the 70% of patients who present with metastatic disease, prognosis remains poor with an overall survival of under 40%. Multiple clinical and biological factors have been utilized to assess the prognosis of children with neuroblastoma. For example, amplification of the nuclear transcription factor N-MYC in these tumors portends a poor prognosis. A better understanding of the mechanisms regulating neuroblastoma growth and N-MYC activity is likely to lead to better approaches to treatment.

Epidemiological studies suggesting a correlation between high birth weight and the occurrence of neuroblastoma support the hypothesis that a factor(s) capable of augmenting somatic growth plays a role in the pathogenesis of this tumor. Insulin-like growth factor I (IGF-I), a peptide growth factor of the insulin

family, is known to stimulate proliferation, cell survival, and motility in a wide range of normal and malignant cell types. The pathogenesis of neuroblastoma has been related to the expression of the type 1 insulin-like growth factor receptor (IGF1R; the receptor that primarily mediates IGF-I actions) and IGF1R signaling can induce N-MYC expression.

The IGF1R is a membrane tyrosine kinase receptor which transduces signals through both the ras/MAPK and PI3 kinase/Akt signaling pathways. The IGF1R signaling can be blocked by a specific monoclonal antibody,  $\alpha$ IR3, which in turn can decrease N-MYC expression. Inhibition of elements in IGF1R signaling pathways can also be accomplished by a variety of agents. For example, mTOR (mammalian target of rapamycin), a downstream target of the PI3 kinase/Akt pathway, can be inhibited by rapamycin.

Rapamycin, which is approved for use in children, is a macrolide fungicide and a member of the PI3K-related kinase family. It has *in vitro* cytostatic activity against a broad range of cancers occurring in children and adolescents, including rhabdomyosarcoma, glioblastoma, T-cell acute lymphoblastic leukemia, and osteosarcoma. Rapamycin acts primarily by binding the FK506 binding protein (FKBP12) which in turn directly binds and inhibits mTOR. Subsequently there is a decrease in cdk inhibitor turnover, inhibition of Rb phosphorylation, and increased cyclin D1 turnover, ultimately resulting in cell cycle arrest at the intersection of G1 and S phases. Rapamycin analogs, such as temsirolimus, are currently being investigated in Phase II Clinical Trials in adult cancers.

Previously, we have shown that blockade of the IGF-I receptor with a monoclonal antibody,  $\alpha$ IR3, combined with mTOR signaling inhibition with rapamycin decreases neuroblastoma growth *in vitro*. The results of these experiments were published in *AntiCancer Research* in 2008. We have also shown that treatment with rapamycin or temsirolimus when combined with  $\alpha$ IR3 decreases N-MYC protein, increases N-MYC phosphorylation, and increases apoptosis in neuroblastoma cell cultures. A manuscript detailing these results has been accepted by *AntiCancer Research*, and will be published later this year.

Death by apoptosis may not be the only mechanism whereby rapamycin or temsirolimus impact the growth potential of neuroblastoma. An emerging area of interest in cell biology focuses on autophagy, a process where cells destroy themselves by digesting proteins and organelles. Autophagy is controlled in part by mTOR, and the inhibition of mTOR has been shown to increase autophagy in a number of different cell types. Other mechanisms of inducing autophagy exist, including lowering inositol and IP3 with valproic acid.

Valproic acid is a commercially available, oral antiepileptic agent that has been used in children since 1970. Over recent years, it has been discovered that valproic acid can have potent anti-tumor effects. Although the mechanism of valproic acid's actions are not clearly understood, it has been shown that valproic acid

induces autophagy through an mTOR independent pathway. In neuroblastoma valproic acid stimulated a marked dose-dependent morphological and biochemical differentiation, accompanied by a marked dose-dependent growth inhibition. Furthermore, valproic acid has been shown to decrease the amount of N-MYC protein in human neuroblastoma cultures. Valproic acid is an intriguing drug to study in children, since like rapamycin, it is already approved for use and has established side-effect profiles.

Given that dosing curves have been completed for valproic acid in neuroblastoma cell cultures, including BE – 2 (c), we propose to investigate the effects of combining valproic acid with mTOR blockade. It is our hope that focusing on oral agents that are already approved for use in children will increase the translational potential of our research.

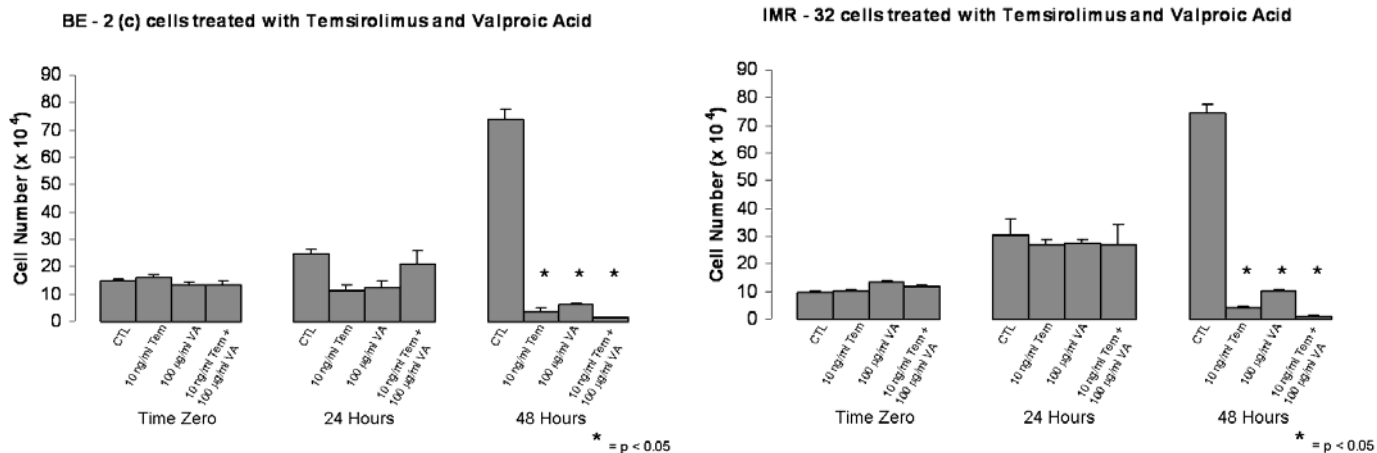
Given the data reviewed above, we plan to investigate the effects of inhibition of mTOR activity with rapamycin or temsirolimus combined with valproic acid in human neuroblastoma cells. Specifically, we plan to examine changes in protein levels of N-MYC and a number of different protein markers of autophagy after treatment with each agent. We will also investigate the anti - proliferative effects of each agent alone or in combination. Furthermore, given that IGF1R signaling regulates N-MYC phosphorylation, we plan to investigate the phosphorylation status of the N – MYC protein after treatment with rapamycin, temsirolimus, or valproic acid.

## **PRELIMINARY STUDIES**

### **Treatment of neuroblastoma cell cultures with temsirolimus and/or valproic acid decreases proliferation**

Preliminary experiments were performed to determine the effect of treatment of human neuroblastoma cell cultures BE-2 (c) and IMR – 32 with temsirolimus, valproic acid, and a combination of both agents. Cell number in control and treated BE-2(c) cells after 24 and 48 hours in culture are shown. Cells were plated at  $10^4$  cells per well. Time zero represents serum starved cells from each treatment group. Absolute cell counts are shown 24 and 48 hours in control media (CTL; Alpha MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin), control media plus 10 ng/ml temsirolimus (10 ng/ml TEM), control media plus 100 µg/ml valproic acid (100 µg/ml VA), and control media plus 10 ng/ml temsirolimus and 100 µg/ml valproic acid (10 ng/ml Tem + 100 µg/ml VA). \* is  $p < 0.05$  compared to CM.

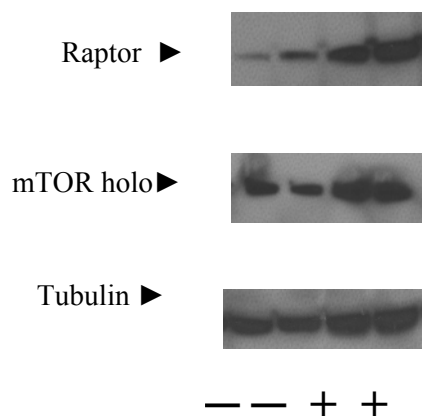
### **Figure 1.**



These data demonstrate that temsirolimus (10 ng/ml), valproic acid (100 µg/ml), and the two treatments in combination cause a statistically significant decrease in cell number at 48 hours as compared to control.

### BE-2(c) cell cultures treated with rapamycin express components of the mTORC1 signaling pathway

A preliminary experiment was performed to determine the effect of treatment of BE-2(c) cells with rapamycin. Cells were plated at  $10^6$  cells per well, serum starved for 24 hours then released into serum-containing media with or without 1 ng/ml rapamycin for 24 hours. Protein lysates were made and analyzed by Western blotting for expression of Raptor (mTORC1), mTORholo (mTORC1 and 2), and a tubulin loading control.



**Figure 2.** Western analyses showing expression of Raptor, mTORholo, and tubulin. "—" indicates cells grown in media without rapamycin and "+" indicates cells grown in media plus rapamycin.

These preliminary Western analyses demonstrate that BE-2(c) cells express the mTOR signaling pathway and that rapamycin treatment of cell cultures increases expression of raptor, a marker of autophagy.