Neuroblastoma is the most common solid tumor of infancy, accounting for 15% of all cancer cell deaths in children. Expression of the anti-apoptotic protein survivin in these tumors correlates with poor prognostic features and resistance to therapy. The mammalian target of rapamycin (mTOR) protein is being explored as a potential therapeutic target in patients with this disease. The objective of this study was to test the hypothesis that rapamycin regulates survivin expression and function in neuroblastoma cells. To explore this hypothesis, we treated two different neuroblastoma lines (NB7, NB8) and a well-characterized control lung cancer cell line, A549, with varying doses of rapamycin (0.1-10µM) for serial time points (2-48 hours). RNA and protein expression levels were then evaluated by quantitative RT-PCR and western blotting, respectively. Cell proliferation and apoptosis were assayed by WST-1 and Annexin V. The results showed a rapamycin-dependent increase in survivin mRNA and protein levels in the neuroblastoma cell lines in a dose- and time-dependent fashion, while a decrease in these levels was observed in control cells. Rapamycin inhibited cell proliferation in both A549 and neuroblastoma cells however neuroblastoma cells had less apoptosis than A549 cells (9% vs. 20%). In summary, our results indicate that rapamycin induces expression of the anti-apoptotic protein survivin in neuroblastoma cells which may protect these cells from programmed cell death. Induction of survivin by rapamycin could therefore be a potential mechanism of neuroblastoma tumor cell resistance and rapamycin may not be an effective therapeutic agent for these tumors.

Keywords: Survivin, neuroblastoma, rapamycin, mTOR, HSP90
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mTOR has been shown to play an important role in cellular proliferation and protein synthesis and is frequently altered in human cancers, including neuroblastoma [12, 13]; mTOR forms two different complexes through unique accessory proteins: (i) regulatory-associated protein of mTOR (RAPTOR) and (ii) rapamycin-insensitive companion of mTOR (RICTOR), resulting in the mTORC1 and mTORC2 complexes, respectively [12], both of which are involved in tumorigenesis. mTORC1 regulates the initiation of mRNA translation and thus controls the rate of protein synthesis through its downstream proteins human p70 S6 kinase (pS6K) and eIF4E [12]. Activation of mTORC1 induces tumorigenesis by suppressing autophagy and upregulating numerous genes, such as \( \text{HIF1}\alpha \), that increases angiogenesis [14]. mTORC2 is upregulated in multiple cancer subtypes, including brain gliomas and breast cancer [15, 16]. mTOR inhibitors are being used in clinical trials for leukemia [17], lung [18] and breast cancer [19]. They are also in Phase 1 trials for refractory or recurrent pediatric solid tumors [20] that include neuroblastoma because of recent evidence that PI3K/AKT/mTOR signaling pathway activation is a common event in this disease [21, 22]. The efficacy of inhibiting mTOR in the treatment of various types of cancer is still being evaluated. Previous studies examined the role of rapamycin in apoptosis and evaluated the effect of rapamycin on the anti-apoptotic protein survivin in different types of cancer, showing that it induces cell cycle arrest during the G1 phase and leads to a decrease in survivin expression [23]. No studies to date have investigated the effects of mTOR inhibitors on survivin pathways in neuroblastoma.

In contrast to those studies demonstrating a decrease in survivin expression in response to rapamycin [24-26], here we show that rapamycin induces survivin in neuroblastoma tumor cells by a molecular mechanism that includes stabilization of survivin mRNA, and that rapamycin-treated neuroblastoma cells are relatively protected from the cell death effects observed in other tumor cell types. These data suggest that rapamycin should be cautiously considered as potential therapy for this disease as it may be inducing rather than inhibiting some tumor survival pathways.

**Material and methods**

**Cells and culture**

The neuroblastoma cell lines NB7 and NB8 were a kind gift from Dr. Jill Lahti at St. Jude Children’s Research Hospital. Adenocarcinomic human alveolar basal epithelial cells A549 were a gift from Dr. Jeffrey Hasday at University of Maryland Medical Center. All cell lines were grown in complete Dulbecco’s modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin in a 37 °C incubator maintained at 5% CO2.

**Western blotting, immunoprecipitation and quantitative PCR**

NB7, NB8 and A549 cells were treated with 1nM to 10 \( \mu \)M rapamycin (LC laboratories, Woburn, MA) for 0 to 48 hours. Cells were harvested at the indicated time points and whole cell lysates prepared in RIPA buffer. 40 \( \mu \)g of protein per lane was separated on a 12% SDS-PAGE. Gels were transferred to PVDF membranes then blotted with rabbit polyclonal anti-Survivin (Santa Cruz Biotechnology, Santa Cruz, CA sc-10811, 1:1000), rabbit polyclonal anti-pS6K (Cell Signaling Technology, Beverly, MA, 1:1000), and mouse monoclonal anti-GAPDH (Chemicon International Inc., Temecula, CA NAB-374, 1:10000) in 5% non-fat dry milk, followed by secondary HRP anti-rabbit (GE healthcare, 1:2000) and HRP anti-mouse-secondary antibodies (GE Healthcare, Piscataway, NJ 1:2000), according to the primary antibody species. For the immunoprecipitation assays, lysates were precleared with the appropriate control IgG and protein A-agarose (EMD Chemicals, Gibbstown, NJ) prior to incubation with primary antibodies and protein A Plus-agarose. Immunocomplexes were resolved by SDS-PAGE. Immunoprecipitations were performed with rabbit polyclonal anti-Survivin (Santa Cruz Biotechnology, Santa Cruz, CA). PVDF membranes were probed with mouse
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monoclonal anti-HSP90 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-Survivin (Santa Cruz Biotechnology, Santa Cruz, CA). For RNA analyses, A459 and NB7 cultures were treated with 10 μM rapamycin for 2, 4, and 8 hours. RNA was extracted using the Qiagen RNeasy kit (Qiagen, Inc, Valencia CA). cDNA was produced with the Qiagen Omniscript kit (Qiagen, Inc, Valencia CA). Quantitative PCR was performed using an Applied Biosystems 7900 HT qPCR machine and Applied Biosystems Power SYBR Green PCR master mix reagent (Applied Biosystems, Carlsbad, CA). The relative abundance of cDNA was calculated using the relative standard curve method. Samples were assayed in triplicate.

Proliferation and apoptosis assays

For the proliferation assay, 5x10^4 NB7, NB8, and A549 cells were cultured in triplicate in 96 well, flat bottom cell culture microplates, in a final volume of 100 μl/well culture medium and incubated overnight. Cells were then treated with 10 μM rapamycin or DMSO as a control for 24 hours. 10 μl/well WST-1 reagent was added. Cells were incubated in a 37 °C incubator maintained at 5% CO2 for 2 hours, then were shaken thoroughly for 1 min. Absorbance of the samples was measured using a microplate reader at 480 nm.

Apoptosis assays were performed using an Annexin-V-Fluos staining kit, according to manufacturer guidelines (Roche Diagnostics). NB7 and A549 cells were cultured and incubated in a 37 °C incubator and maintained at 5% CO2 overnight. Cells then were washed twice with PBS, then co-stained with Annexin-V-FLUOS (green) and propidium iodide (red) diluted 1:50 in Annexin V binding buffer for 15 min at room temperature. Apoptosis was evaluated by counting the number of apoptotic cells by fluorescence microscopy using an excitation wavelength of 480 nm and detection in the range of 515-565 nm. The percentage of apoptotic cells versus the total number of cells counted was calculated.

Results

Rapamycin induces survivin expression in neuroblastoma cells

We chose the high-risk neuroblastoma cell lines NB7 [27] and NB8 [28], with the molecular characteristics of NMYC gene amplification and chromosome 1p deletion, for these studies. For a control, we chose human alveolar basal epithelial cells (A549), originally developed through cultures of lung cancer tissue [29], because the effects of rapamycin in these cells have been well-characterized [30]. Cells were treated with dose-escalating concentrations of rapamycin (1 nM, 100 nM, 1 μM and 10 μM) in complete media for 24 hours. Expression of survivin and a known molecular target of rapamycin, human p70 S6 kinase (pS6K), were examined by Western blot analysis. Results showed that the control A549 cells had a significant decrease (10-fold) in survivin expression following rapamycin treatment and that pS6K was inhibited (Figure 1A), as previously reported [30]. By contrast, treatment of NB7 and NB8 cells with rapamycin resulted in a significant increase (5-fold) in survivin expression that was noted at the 1 to 10 μM concentration range (Figure 1A). To examine the timing of onset of these effects, we performed a serial time course from 0 to 48 hours after treatment with 10 μM rapamycin. Rapamycin treatment of A549 cells resulted in a decrease in survivin expression noted at the 24 hour time point, while treatment of NB7 cells resulted in an increase in survivin expression after 6 hours (Figure 1B). These data suggest that rapamycin leads to a rapid induction of survivin in neuroblastoma cells by a mechanism unlike that observed in other cell types.

Rapamycin increases survivin mRNA levels in neuroblastoma cells

To examine the effects of rapamycin at the mRNA level, cells were treated with and without rapamycin, harvested for RNA at 2, 4, and 8 hours after treatment then cDNA levels were quantified by real-time PCR. In the A549 cells, a decrease in mRNA levels was noted within 2 hours of treatment however in the NB7 cells mRNA levels were slightly increased and remained elevated in the presence of rapamycin (Figure 2A). Together, these results suggest that rapamycin increases the steady-state levels of survivin message which likely leads to an increase in survivin protein levels in NB7 cells while in other cell types rapamycin leads to degradation of both the message and protein.

Rapamycin stabilizes survivin - HSP90 binding

To determine a mechanism where rapamycin
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Figure 1. Rapamycin induces survivin expression in MYCN amplified neuroblastoma cell lines. (A) Neuroblastoma (NB7, NB8) and control lung cancer epithelial cells (A549) were treated with varying concentrations of rapamycin, as indicated. Proteins were harvested at 24 hours and western blots assayed for survivin, the rapamycin target, pS6K, and Gapdh loading control. (B) NB7 and A549 cells were treated with 10 μM rapamycin for the time course indicated. Survivin, pS6K, and Gapdh levels were determined by western blotting.

Figure 2. Rapamycin stabilizes survivin mRNA and induces binding to HSP90 in neuroblastoma cells. (A) A549 and NB7 cells were treated with 10 μM rapamycin or DMSO control for the indicated time periods. cDNA was measured by quantitative PCR and compared to β-actin control. Means and standard deviations of the mean were calculated from triplicate experiments. (B) A549 and NB7 cells were treated with 10 μM rapamycin for 24 hours then lysates were immunoprecipitated (IP) with anti-Survivin or IgG control and immunoblots (IB) were performed with the indicated antibodies.

might stabilize survivin protein levels, we hypothesized that it might also affect an interaction with heat shock protein 90 (HSP90). HSP90 is a molecular chaperone that is central to the cellular stress response [31]. In HeLa cells, disrupting the survivin-HSP90 interaction destabilizes survivin protein, leading to its degradation [32]. To determine a potential involvement of HSP90, NB7 and A549 cells were treated with rapamycin for 24 hours, following which cells were harvested for protein and immunoprecipitated with anti-Survivin or IgG control, followed by immunoblotting with anti-HSP90 or anti-Survivin. Results showed that rapamycin treatment led to a loss of survivin binding to HSP90 in A549 cells however in NB7 cells the interaction between survivin and HSP90 was increased (Figure 2B). These data suggest that rapamycin may stabilize the interaction between survivin.
Rapamycin induces anti-apoptotic protein survivin in neuroblastoma cells, perhaps contributing to survivin protein stability.

**Rapamycin induces growth arrest in neuroblastoma cells**

As an increase in survivin protein levels could potentially enhance neuroblastoma cell survival by stimulating proliferation or by inhibiting apoptosis, we evaluated if the observed increase in survivin protein in cells treated with rapamycin affected cell growth or cell death rates. To examine the effects of rapamycin on proliferation, NB7, NB8 and A549 cells were treated with 10 μM rapamycin over a period of 72 hours and daily cell growth was determined using the WST-1 assay [33]. A significant, time-dependent inhibition of cell proliferation was observed in all three cell lines (Figure 3A), indicating that during this time frame, increases in survivin expression in the rapamycin-treated neuroblastoma cells do not by themselves influence the induced cell cycle arrest. To assess the potential effects on cell death, NB7 and A549 cells were treated with 10 μM rapamycin or DMSO for 24 hours then co-stained with Annexin-V-FLUOS (green) and the DNA stain propidium iodide (red) then analyzed by fluorescence microscopy. The percentage of apoptotic cells compared with the total cell number was quantified. Experiments were performed in triplicate. Error bars indicate standard deviation of the mean.

![Figure 3. Rapamycin effects on cell proliferation and cell death. (A) For the proliferation assay, NB7, NB8 and A549 cells were treated with 10μM of rapamycin (RPM) or DMSO control and cell proliferation was quantified at 24 hour intervals using the WST-1 assay. Experiments were performed in triplicate. Error bars indicate standard deviation of the mean. (B) For the apoptosis assay, NB7 and A549 cells were treated with 10 μM rapamycin (RPM) or DMSO control for 24 hours, immunostained with Annexin-V-FLUOS (green) and the DNA stain propidium iodide (red) then analyzed by fluorescence microscopy. Images were acquired using a Nikon fluorescence microscope. The percentage of apoptotic cells compared with the total cell number was quantified. Experiments were performed in triplicate. Error bars indicate standard deviation of the mean.](image_url)
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mycin-treated cells could indicate that an increase in survivin levels was protective of the rapamycin-induced cell death effects in neuroblastoma cells.

**Discussion**

Neuroblastoma is an embryonal tumor of the autonomic nervous system, with an incidence of 10.2 cases per million children under the age of 15 years [34]. Despite major advances in diagnosis and treatment, the increase in cure rates is primarily observed among patients with low-risk disease; high-risk patients have shown only modest improvements in their outcomes over the last 10 years [35]. Current therapies include intensive chemotherapy and radiation followed by autologous bone marrow transplant [36], 13-cis-retinoic acid [37], and anti-GD2 immunotherapy [38]. One of the major challenges in high-risk neuroblastoma is to find an effective targeted therapy that could potentially improve the current poor outcomes.

One of the emerging class of drugs that have been under consideration for neuroblastoma therapy are mTOR inhibitors, based on previous studies showing that phospho-mTOR, and its downstream proteins, phospho-p70S6K and phospho-4EBP1 are highly expressed in this disease [21]. Although there are conflicting data as to whether mTOR inhibitors downregulate MYCN protein expression [22, 39], mTOR inhibitors have been shown in multiple in vitro studies to induce apoptosis and inhibit proliferation in neuroblastoma cells [22, 39]. Our observations that rapamycin inhibits proliferation in the aggressive NB7 and NB8 cells are in agreement with these studies.

Since most high-risk neuroblastoma patients initially respond to therapy, including conventional chemotherapeutic agents, but ultimately relapse, it is likely that these tumors have high rates of acquired drug resistance [35]. The exact molecular mechanisms of drug resistance in neuroblastoma tumor cells are still under investigation. One hypothesis suggested by our data is that increases in survivin expression levels, potentially induced by chemotherapeutic agents, may ultimately contribute to drug-resistance in these tumors. Increases in survivin expression have been shown to increase chemotherapy resistance in other tumor cell types including breast cancer [40], thyroid cancer [41], non-small cell lung cancer [42], prostate cancer [43], and chondrosarcoma [44]. Other studies also showed that survivin can induce radiation-resistance in colorectal cancer [45] and in glioblastoma [46]. Our finding that rapamycin increases survivin levels in neuroblastoma cells, but not in lung cancer cells suggests that rapamycin may play a role in increasing chemotherapy- and radiation therapy-resistance in neuroblastoma. Future *in vitro* studies using this drug in combination with other chemotherapeutic agents and/or radiation in neuroblastoma cells and in xenograft models would be helpful to confirm this hypothesis.

Our results provide a potential mechanism by which rapamycin enhances survivin levels by increasing steady-state mRNA levels and enhancing binding to HSP90. HSP90 is a conserved molecular chaperone that participates in stabilizing and activating several proteins including transcription factors, protein kinases, and co-chaperones [47] and is a facilitator of cancer cell survival [48]. In HeLa cells, inhibition of HSP90 enhanced survivin protein degradation, leading to cell cycle arrest and cell death [32]. Currently, there is growing evidence of the benefits of HSP90-inhibition in neuroblastoma, as it increases p53 expression and destabilizes MYCN gene expression in these tumors [49]. Considering our observation that rapamycin may increase survivin protein levels in neuroblastoma by increasing HSP90-survivin binding, a potential therapeutic choice might be to combine the use of mTOR inhibitors with HSP90 inhibitors, in order to gain the full benefits of the mTOR inhibitors without inducing survivin expression, and subsequently promoting chemotherapy resistance.

In conclusion, rapamycin induces survivin expression in MYCN-amplified neuroblastoma cell lines which may contribute to a loss of the rapamycin-induced cell death effects of this drug in these cells. Further induction of survivin in these tumors suggests that rapamycin may not be effective and could potentially enhance chemotherapy-resistance. When considering mTOR inhibitors for the treatment of neuroblastoma, combination therapy with HSP90 inhibitors may enhance their beneficial effects and decrease their potential adverse effects.

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