IGF-I Receptor Inhibition Combined with Rapamycin or Temsirolimus Inhibits Neuroblastoma Cell Growth

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Abstract. *Background: Neuroblastoma is the third most common solid tumor in children. Treatment continues to be challenging. The pathogenesis of neuroblastoma has been related to expression of the type 1 insulin-like growth factor receptor (IGF1R) and to transcription factor MYC-N amplification. Previous studies have shown that MYC-N expression is disrupted by blockade of the IGF1R with a specific monoclonal antibody, αIR3. Inhibition of IGF1R signaling can be accomplished by other agents, including rapamycin or temsirolimus, which target mTOR (mammalian target of rapamycin). Materials and Methods: BE-2(c) and IMR–32 neuroblastoma cell lines were treated with varying concentrations of αIR3, rapamycin and temsirolimus alone or in combination and the viable cells were counted. Results: Blockade of IGF1R signaling significantly inhibited cell growth as compared to untreated controls ($p<0.05$), and a combination of agents was more effective than each agent alone. Conclusion: The combination of rapamycin or temsirolimus with αIR3 blocks the IGF1R signaling pathway and has an antiproliferative effect on neuroblastoma cells warranting further investigations using inhibitors of IGF1R signaling as novel combination therapy for neuroblastoma.*

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% (1). MYC-N, a transcription factor found in neuroblastoma that portends a poor prognosis, is often amplified in these children. A better understanding of neuroblastoma growth may lead to better treatment approaches.

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Key Words: IGF-I receptor, MYC-N, mTOR, neuroblastoma, rapamycin, temsirolimus.

Epidemiological studies suggesting a correlation between high birth weight and the occurrence of neuroblastoma (2) support the hypothesis that a factor(s) capable of augmenting somatic growth plays a role in the pathogenesis of this tumor (3, 4). 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 (5). 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 MYC-N expression (4).

The IGF1R is a membrane tyrosine kinase receptor which transduces signals through both the ras/MAPK pathway (proliferative growth) and the phospho-inositol (PI)-3 kinase/Akt signaling cascade (anti-apoptosis) (6). Binding of IGFs to the IGF1R is modulated through the actions of IGF binding proteins (IGFBP) 1 through 6 (6). The IGF1R can be blocked by a specific monoclonal antibody, αIR3, which in turn can disrupt MYC-N expression (4). Inhibition of a portion of the IGF1R signaling pathway can also be accomplished by other therapies, including rapamycin that targets mTOR (mammalian target of rapamycin), a downstream element in the PI3 kinase/Akt pathway. Rapamycin, which is approved for use in children, is a macrolide fungicide and a member of the PI3-kinase related kinase family. It has *in vitro* cytostatic activity (7) against a broad range of malignancies occurring in children and adolescents, including rhabdomyosarcoma, glioblastoma, T-cell acute lymphoblastic leukemia (8) and osteosarcoma (9). Rapamycin acts primarily by binding the 12 kd FK506 binding protein (FKBP12) (10) which in turn directly binds and inhibits mTOR. Subsequently there is a decrease in cyclin-dependent kinase (cdk) inhibitor turnover, inhibition of retinoblastoma protein (Rb) phosphorylation, and increased cyclin D1 turnover, ultimately resulting in cell cycle arrest at the intersection of the G1 and S phases (11).

Analogues of rapamycin with improved pharmacological characteristics have been developed including temsirolimus a rapamycin prodrug available in both intravenous and oral

Table I. *PCR primers used in RT-PCR analyses to identify expression of human IGF system RNAs.*

Gene	GenBank accession #	Primers	Product size
IGF-I	NM_000618	5'-GCTTTTGTGATTTCTTGAAGGTGAA-3' (nt 210 - nt 234 in exon 1)	
		5'-TCCCTCTACTTGCGTTCTTCAAAT-3' (nt 599 - nt 576 in exon 4)	389 bp
IGF-II	NM_000612	5' GTGCTTCTCACCTTCTTGGCCTTC 3' (nt 783 - nt 806 in exon 2)	
		5' CCTCAACGCCTCGAGCTCCTT 3' (nt 1202 - nt 1182 in exon 4)	419 bp
IGF1R	NM_000875	5' GCTGACCTCTGTTACCTCTCCACT3' (nt 489 - nt 512 in exon2)	
		5' GCAAGGACCTTCACAAGGGAT3' (nt 1034 - nt 1014 in exon 3)	545 bp
IGFBP-1	NM_000596	5' GTGCAGGAGTCTGACGCCTCCGCT 3' (nt 606 - nt 629 in exon 1)	
		5' GGTGACATGGAGAGCCTTCGA 3' (nt 794 - nt 774 in exon 2)	188 bp
IGFBP-2	NM_000597	5' GAGAAGCGCCGGGACGCCGAGTAT 3' (nt 523 - nt 546 in exon 1)	
		5' GGGGGTGGTCGCAGCTTCTT 3' (nt 794 - nt 775 in exon 2)	271 bp
IGFBP-3	NM_000598	5' GTCAACGCTAGTGCCGTCAGCCGC 3' (nt 475 - nt 498 in exon 1)	
		5' ATATTCTGTCTCCCGCTTGGA 3' (nt 762 - nt 742 in exon 2)	287 bp
IGFBP-4	NM_001552	5' GAGCTGGCGGAGATCGAGGCCATC 3' (nt 616 - nt 639 in exon 1)	
		5' CGCCCCATTGACCTTCATCTT 3' (nt 795 - nt 775 in exon 2)	179 bp
IGFBP-5	NM_000599	5' GAGAAGATGGTGTTGCTCA3' (nt 768 - nt 786 in exon 1)	
		5' GCTCACGGGAGTCTCTCTCGA 3' (nt 1130 - nt 1110 in exon 2)	332 bp
IGFBP-6	NM_002178	5'CTCAGTGCTGCAGCAACTCCAG 3' (nt 569 - nt 571 in exon 3)	
		5' CAGCGACCCCAAGCAGAGCTTTATTG 3' (nt 966 - nt 941 in exon 4)	397 bp

formulations. In Phase II clinical trials, temsirolimus has been given on a weekly schedule to adult patients with metastatic endometrial cancer, recurrent glioblastoma and advanced breast cancer or renal cell carcinoma (12). Adverse effects have included fatigue, rash, mucositis, anorexia, nausea, diarrhea, thrombocytopenia, leucopenia, anemia and metabolic effects that have been managed by dose reduction (12). Temsirolimus was approved by the Food and Drug Administration (FDA) in May, 2007, for the treatment of advanced renal cell carcinoma (13). Rapamycin is currently used therapeutically in children, and dosing data and side effect profiles are established. There are no current reports of the use of temsirolimus in children.

Inhibition of IGF1R signaling at the IGF1R and at the downstream element mTOR might be expected to result in additive or synergistic anti-proliferative effects on human neuroblastoma. The findings on the effects of inhibition of IGF1R and mTOR signaling on proliferation in two neuroblastoma-derived cell lines are reported. The concentrations of rapamycin that were chosen corresponded to serum levels achievable with doses approved for use in children after renal transplantation and are well tolerated without major side-effects (14).

Materials and Methods

Cells and reagents. The cryopreserved mycoplasma-negative cell lines IMR–32 and BE–2(c) were purchased from the Lineberger Comprehensive Cancer Center Tissue Culture Facility (University of North Carolina, Chapel Hill, NC, USA). Both cell lines were derived from human neuroblastoma tissue and are maintained by the American Type Culture Collection (ATCC). The BE–2(c) line contains 123 copies of the MYC-N gene (15), and the IMR–32 cell line contains 25 copies of the MYC-N gene (16).

Rapamycin and temsirolimus were purchased from Wyeth Pharmaceuticals (Madison, NJ, USA). The monoclonal antibody, αIR3, was a gift from Dr. Judson J. Van Wyk, University of North Carolina at Chapel Hill, North Carolina, USA. Dilutions of stock were prepared in sterile PBS prior to all experiments. Alpha-MEM, fetal calf serum, and penicillin/streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) were purchased from the UNC Lineberger Comprehensive Cancer Center Tissue Culture Facility, University of North Carolina.

RNA preparation. The total RNA was prepared from log phase IMR–32 and BE–2(c) cell cultures using Trizol reagent (Invitrogen Corporation) a modification of the single step method by Chomczynski and Sacchi (17). The RNA was stored frozen at –80˚C until used.

Oligodeoxynucleotides. Oligodeoxynucleotides (oligos) were purchased from the Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility (University of North Carolina). The oligos used in semi-quantitative reverse transcription-PCR (RT-PCR) of the IGF system are shown in Table I. An 18S ribosomal RNA primer set purchased from Ambion Incorporated (Austin, TX, USA) was used as a normative positive control in the semi-quantitative RT-PCR analyses. All the oligos were designed to span exons so that any contaminating genomic DNA in the RT-PCR would be distinguishable from the product derived from RNA by its large size.

Reverse transcription-polymerase chain reaction (RT-PCR). The reverse transcription of RNA prepared from the IMR–32 and BE–2(c) cell cultures was performed at 37˚C for 1 hour, as described by Frohman *et al*. (18). Following first strand synthesis, 1 μl of the reverse transcription reaction product was amplified by PCR using the oligo pairs for the IGF system, the 18S control or a reaction with no input cDNA to serve as a negative control. The PCR products were run in Tris-borate EDTA (TBE) agarose gels, stained with ethidium bromide, and visualized under UV light and photographed using a Polaroid PhotoMaxPro digital camera (Best Buy, Durham, NC, USA).

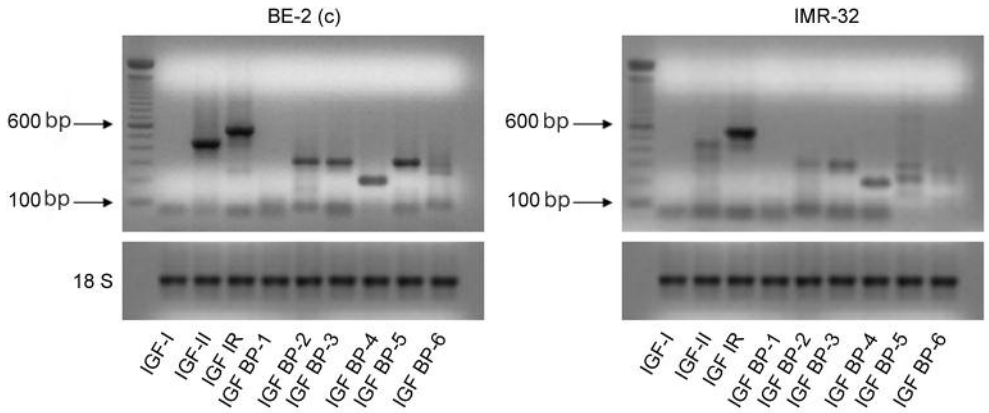


Figure 1. *IGF system expression in BE-2(c) and IMR-32 cell lines, analyzed by RT-PCR of RNA extracted from the cells. Neither IGF-1 or IGFBP-1 were amplified. The other IGF system genes studied were amplified and were of the predicted sizes in both cell lines: IGF-II, 419 bp; IGF1R, 545 bp; IGFBP-2, 271 bp; IGFBP-3, 287 bp; IGFBP-4, 179 bp; IGFBP-5, 332 bp; IGFBP-6, 397 bp. Note the presence of an increased amount of IGF-II RNA in the BE–2 (c) cell line. Replicons were subjected to sequencing (University of North Carolina Automated Sequencing Facility) to confirm their identity. 18 S: ribosomal RNA, positive control.*

Anti-proliferative assays. The cells were grown at 37˚C, 5% CO_2 in Alpha MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (control medium, CM). The cells were seeded in 96 well plates (Costar, Corning, NY, USA) at 1×10^4/ml or 1×10^5/ml, and untreated triplicate specimens for each experimental group were serum starved for twenty-four hours prior to treatment, and then allowed to grow for 48 hours in one of the following media: CM (experimental control), CM plus 0.5 μg/ml monoclonal antibody directed against human myoglobin (IgG) was included in all the αIR3 blocking experiments to serve as an isotypic control antibody, CM plus 0.5 μg/ml αIR3 (19), CM plus 1, 10 or 100 ng/ml rapamycin, CM plus 1, 10 or 100 ng/ml temsirolimus, CM plus rapamycin and αIR3 (1 ng/ml and 0.5 μg/ml, respectively) and CM plus temsirolimus and αIR3 (10 ng/ml and 0.5 μg/ml, respectively). The viable cells were counted in triplicate every 24 hours using trypan blue exclusion.

Propidium iodide (PI) assay. The cells were plated in triplicate in 96 well plates (Costar) at a density of 1×10^4/well and incubated for 24 hours with 0.3 μg/ml PI (20) and CM plus 0.5 μg/ml IgG, CM plus 0.5 μg/ml αIR3, CM plus rapamycin (1 ng/ml), CM plus temsirolimus (10 ng/ml), CM plus rapamycin and αIR3 (1 ng/ml and 0.5 μg/ml, respectively) or CM plus temsirolimus and αIR3 (10 ng/ml and 0.5 μg/ml, respectively). The cells were examined using fluorescent microscopy and light microscopy at one and 24 hours. Propidium iodide is a fluorescent phenanthridine dye that intercalates into DNA (20) and is impermeable to living cells. All the fluorescent (dead) and non-fluorescent (live) cells were counted at each time-point, and the percentage of dead cells was calculated.

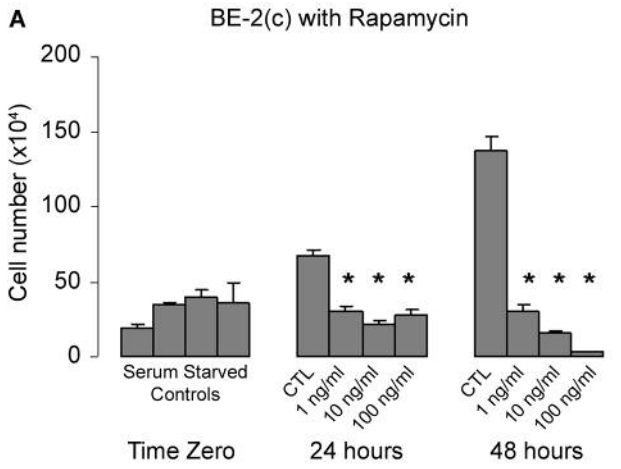
Statistical analyses. Statistical analyses including one-way ANOVA and Student's *t*-test, were performed using the Sigma Stat program, version 1 (Jandel Scientific, San Rafael, CA, USA).

Results

Expression of the IGF system components in BE-2(c) and IMR-32 cells. The RT-PCR analyses revealed that both the cell lines expressed RNAs encoding IGF1R and IGF-II, as well as IGFBPs 2 through 6 (Figure 1). Each replicon was the predicted size as indicated in Table I. Neither IGF-I nor IGFBP-1 transcripts were detected. IGF-II was more abundantly expressed in the BE–2(c) cells than in the IMR–32 cells.

Effect of inhibition of mTOR on neuroblastoma cell number. Dose response curves were completed for both rapamycin (Figures 2A and 3A) and temsirolimus (Figure 2B and 3B) in both the cell lines. When serum-starved BE–2(c) cells were incubated with rapamycin for 24 or 48 hours cell number did not increase over time compared to the untreated control cultures (Figure 2A). In fact, the higher concentrations of rapamycin (10 ng/ml and 100 ng/ml) caused the cell number to decrease below the levels at time zero. Similar results were seen after treatment with temsirolimus, including a decrease in cell numbers by 48 hours to below the levels at the hour zero time-point (Figure 2B). When the serum–starved IMR–32 cells were incubated with rapamycin or temsirolimus for 24 or 48 hours similar results were obtained with the exception of the treatment group that received the lowest drug concentration (Figure 3). The groups, which received 1 ng/ml of rapamycin or 1 ng/ml of temsirolimus, did not show a significant difference in cell number after 24 hours of incubation, although cytotoxicity was demonstrable at 48 hours.

Effect of inhibition of IGF1R alone or in combination with mTOR blockade. When serum-starved cells were incubated with 0.5 μg/ml of αIR3, alone or in combination with 1 ng/ml rapamycin for 24 or 48 hours, the cell number did not increase compared to the untreated control cultures (Figure



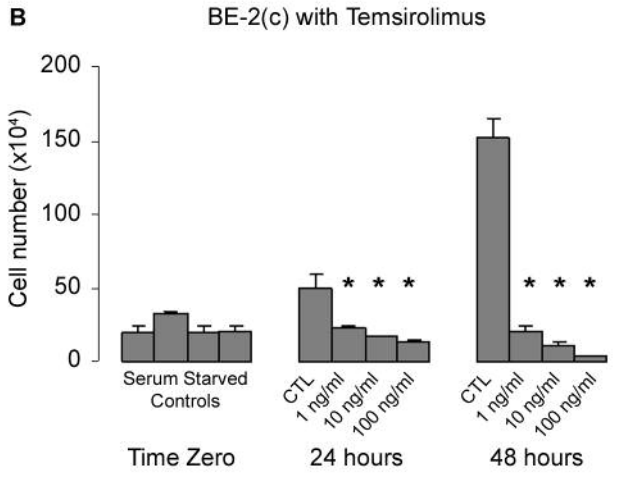
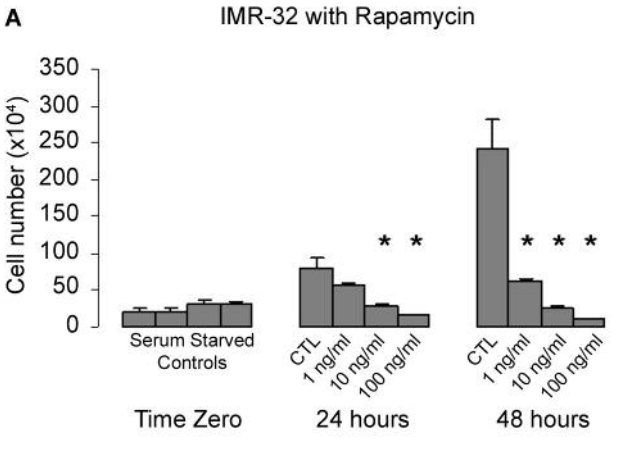


Figure 2. *Dose response of BE–2(c) cells to rapamycin and temsirolimus. Cell number in control and treated cells plated at 10^4 cells per well. Time zero represents serum starved untreated cells from each experimental group prior to treatment. A) Absolute cell counts are shown after 24 and 48 hours in control medium (CM) (CTL) or CM plus 1, 10 or 100 ng/ml rapamycin. *p<0.05 compared to CM (CTL). B) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL), CM (CTL) or CM plus 1, 10, or 100 ng/ml temsirolimus. *p<0.05 compared to CM (CTL).*



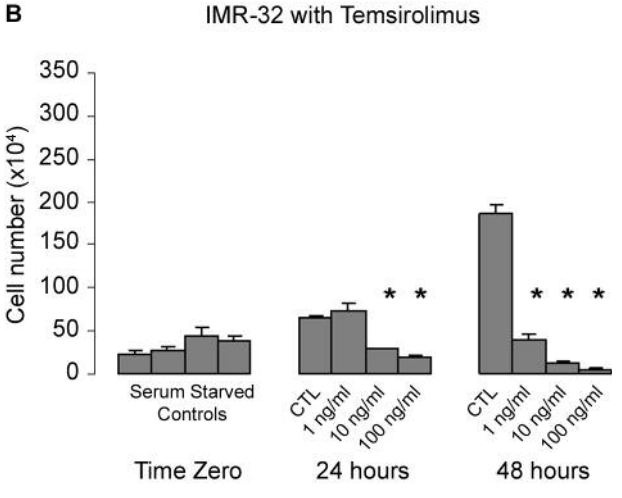


Figure 3. *Dose response of IMR-32 cells to rapamycin and temsirolimus. Cell number in control and treated cells plated at 10^4 cells per well. Time zero represents serum starved untreated cells from each experimental group prior to treatment. A) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL) or CM plus 1, 10 or 100 ng/ml rapamycin. *p<0.05 compared to CM (CTL). B) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL) or CM plus 1, 10 or 100 ng/ml temsirolimus. *p<0.05 compared to CM (CTL).*

4A and Figure 5A). Similar results were found after incubation with 10 ng/ml temsirolimus, alone or in combination with 0.5 μg/ml of αIR3 (Figures 4B and 5B), with the exception of the 0.5 μg/ml αIR3 treatment group in the BE–2(c) cell culture (Figure 4B). This treatment group did not show a statistically significant difference in the cell number when compared to the control.

A combination of both agents showed a statistically significant reduction in cell number when compared to untreated controls in both cell types (Figures 4 and 5). Furthermore, following the combination treatment, the total cell number in the BE-2(c) and IMR–32 cultures at 24 and 48 hours declined to levels below those at the zero time-point, indicating that cell survival deteriorated.

Blockade of mTOR in combination with IGF1R inhibition and propidium iodide fluorescence. mTOR blockade, alone or in combination with IGF1R inhibition with αIR3 increased PI fluorescent cells (dying cells) in the BE–2(c) and IMR–32 cell cultures (Figure 6). The combination of

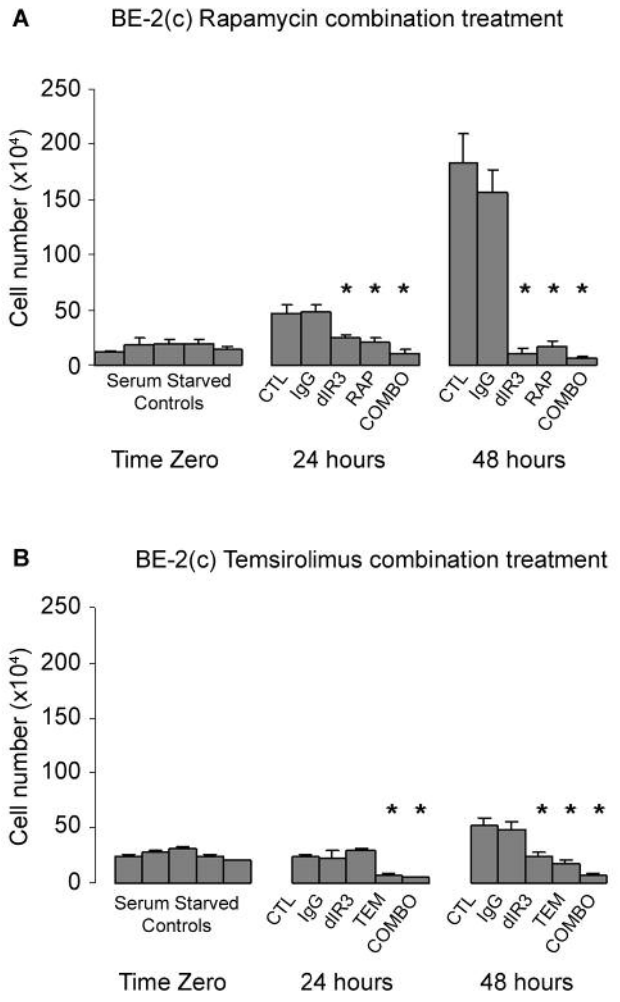


Figure 4. *Combination of rapamycin or temsirolimus and αIR3 treatment of BE–2(c) cells. Cells were plated at 10^4 cells per well. Time zero represents serum starved untreated cells from each experimental group prior to treatment. A) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL), CM plus 0.5 μg/ml IgG (IgG), CM plus 0.5 μg/ml αIR3 (αIR3), CM plus 1 ng/ml rapamycin (RAP), CM plus 1ng/ml rapamycin plus 0.5 μg/ml αIR3 (COMBO). *p<0.05 compared to CM (CTL). B) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL), CM plus 0.5 μg/ml IgG (IgG), CM plus 0.5 μg/ml αIR3 (αIR3), CM plus 10 ng/ml temsirolimus (TEM), CM plus 10 ng/ml temsirolimus plus 0.5 μg/ml αIR3 (COMBO). *p<0.05 compared to CM (CTL).*

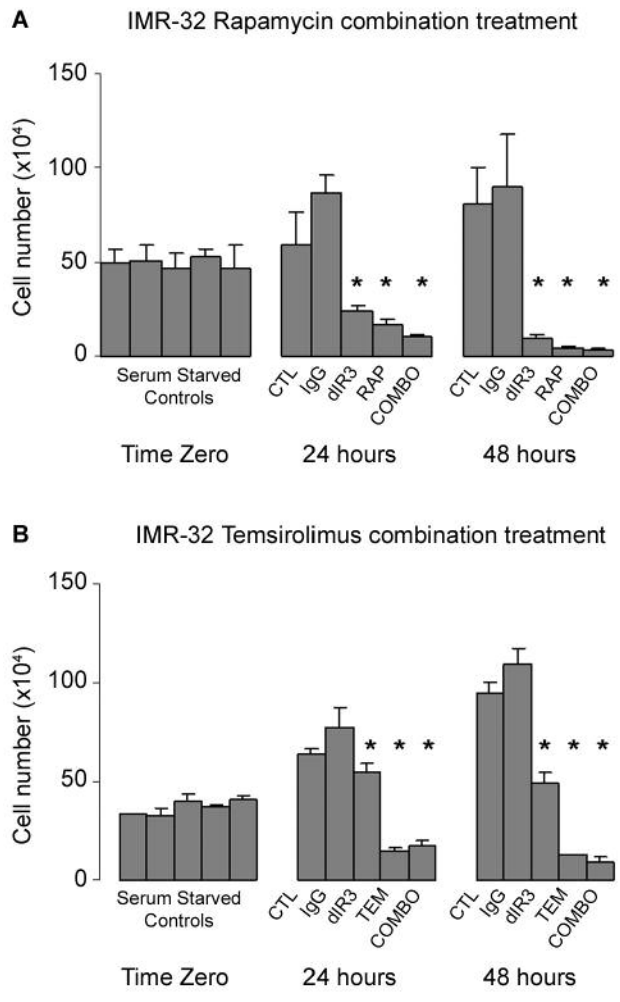


Figure 5. *Treatment of IMR-32 cells with rapamycin or temsirolimus and αIR3. Cells were plated at 10^5 cells per well. Time zero represents serum starved untreated cells from each experimental group prior to treatment. A) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL), CM plus 0.5 μg/ml IgG (IgG), CM plus 0.5 μg/ml αIR3 (αIR3), CM plus 1 ng/ml rapamycin (RAP) or CM plus 1 ng/ml rapamycin plus 0.5 μg/ml αIR3 (COMBO). *p<0.05 compared to CM (CTL). B) Absolute cell counts are shown 24 and 48 hours after treatment with CM (CTL), CM plus 0.5 μg/ml IgG (IgG), CM plus 0.5μg/ml αIR3 (αIR3), CM plus 10 ng/ml temsirolimus (TEM) or CM plus 10 ng/ml temsirolimus plus 0.5 μg/ml αIR3 (COMBO). *p<0.05 compared CM (CTL).*

αIR3 with rapamycin or temsirolimus appeared to induce greater cell death than αIR3, rapamycin, or temsirolimus alone.

Discussion

The BE–2(c) and IMR-32 neuroblastoma-derived cell lines synthesized a number of IGF system components including IGF-II, IGF1R and IGFBPs 2-6. These results were expected and agree with previously published data for other neuroblastoma-derived cell lines (21). IGF-II has been indicated as both an autocrine and paracrine growth factor for neuroblastoma, and has also been found to induce the production of MYC-N (21). Because the MYC-N gene copy number is 5-fold greater in BE–2(c) cells than in IMR-32 cells, the more abundant expression of IGF-II in the BE-2(c)

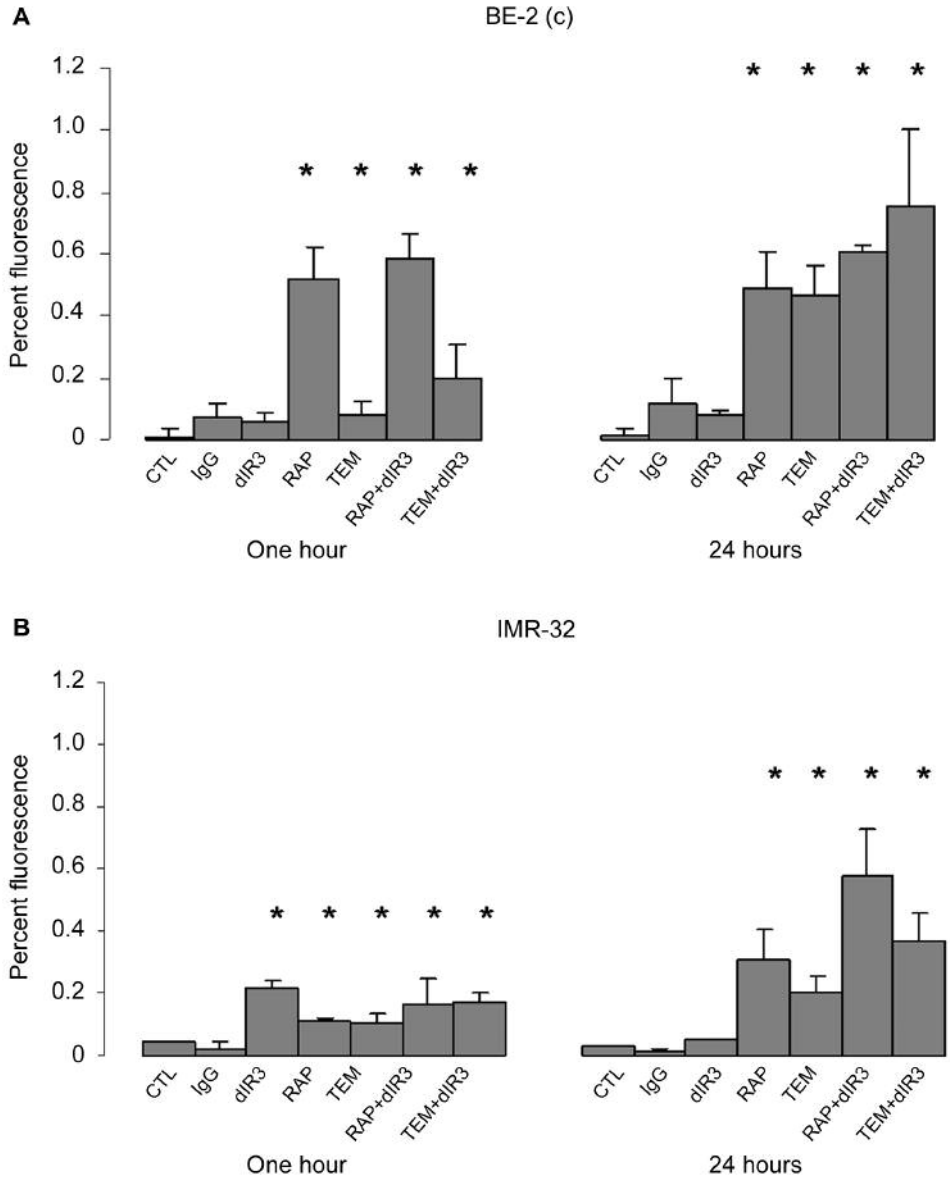


Figure 6. *Propidium Iodide staining of treated BE–2(c) and IMR–32 cells. A) BE–2(c) cells and B) IMR-32 cells grown in media containing propidium iodide and treated with CM (CTL), CM plus 0.5 μg/ml IgG (IgG), CM plus 0.5 μg/ml αIR3 (αIR3), CM plus 1 ng/ml rapamycin (RAP), CM plus 10 ng/ml temsirolimus (TEM), CM plus 1 ng/ml rapamycin plus 0.5 μg/ml αIR3 (RAP + αIR3), and CM plus 10 ng/ml temsirolimus plus 0.5 μg/ml αIR3 (TEM + αIR3). *p<0.05 compared to control.*

cells was consistent with the evidence that IGF-II induces MYC-N (21).

The mTOR blockade treatment of the BE–2(c) cells with rapamycin or temsirolimus decreased the overall cell number in a dose dependent manner. Similar results were seen for the IMR–32 cell line, with the exception of the lowest concentration of either rapamycin or temsirolimus (1 ng/ml) at 24 hours. The failure of the lower concentrations to inhibit cell growth at 24 hours may have been due to the slower growth rate of the IMR–32 cells (24 hours) when compared to the BE–2(c) cells (18 hours), and therefore a delay in metabolizing the drug. This possibility was supported by data showing a statistically significant decrease of cell number in the IMR–32 line 48 hours after incubation with 1 ng/ml of either rapamycin or temsirolimus.

The monoclonal antibody αIR3 which acts as an IGF1R antagonist (22) also abrogated the increase in cell number stimulated by serum addition to serum starved cells at 48

hours, consistent with blockage of IGF1R signaling effectively blocking proliferation. The αIR3 antibody has been shown to decrease proliferation *in vitro* in a broad range of tumor cell lines, including neuroblastoma (4). The significant decreases in cell numbers which resulted from treatment with αIR3 in the present study also were in line with prior studies of IGF-I pro-survival action (4). These data were consistent with arrest at the G1/S-phase checkpoint of the cell cycle resulting from an absence of IGF1R signaling.

When αIR3 and rapamycin or temsirolimus were used in combination, the decline in cell number was greater than when each agent was used alone. Furthermore, the cell number decreased to levels below the zero time-point. Although cell growth was not as robust in the temsirolimus treated BE-2(c) cells (Figure 4B), the control groups, CTL and IgG, doubled in number by 48 hours while the number of cells in the combination treated group (COMBO) decreased significantly below the level of the serum starved group. The reduction in cell number was greater than could be accounted for by cell cycle arrest, and suggested the occurrence of cell death. Clear evidence of increased numbers of dying cells in cultures treated with rapamycin, temsirolimus, or a combination of either agent with αIR3 was shown by propidium iodide staining (Figure 6). The combination of αIR3 and rapamycin or temsirolimus was more effective in inducing cell death at 24 hours than rapamycin or temsirolimus alone. This finding was consistent with both agents acting through the same pathway. The data were also in agreement with other studies which have shown that combined mTOR and IGF1R inhibition in rhabdomyosarcoma cell lines resulted in additive cytotoxicity (23).

In conclusion, neuroblastoma cells express multiple components of the IGF system, and a combination of rapamycin or temsirolimus with αIR3 is able to block the IGF1R signaling pathway more effectively and has more anti-proliferative effect than either agent alone. The present study represents the first use of temsirolimus in pediatric tumors in the medical literature. Studies evaluating the mechanism of cell death induced by these agents, and the possible impact of IGF1R blockade on MYC-N protein expression are currently underway. It will be necessary to understand the regulation of IGF1R signaling and the complex interplay of the IGF1R and mTOR signaling pathways before their role in neuroblastoma growth can be elucidated, and hopefully the development of novel therapeutics for neuroblastoma can be initiated.

Acknowledgements

This research was supported in part by grants from the St. Baldrick's Foundation, the UNC Medical Alumni Fund, and the Lindy Gupton Fund (UNC Children's Hospital). We gratefully acknowledge Dr. Judson J. Van Wyk (1920-2003) for the gift of the anti-IGF-I receptor antibody and for his contributions to the IGF-I receptor signaling field through years of basic growth factor research which made the experiments reported here possible.

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Received December 21, 2007
Revised March 3, 2008
Accepted March 14, 2008