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# Interferon-β Sensitizes Neuroblastoma to the Anti-Tumor Activity of Temozolomide by Modulating O⁶-Methylguanine-DNA Methyltransferase Expression

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## Abstract

**Purpose—**Although temozolomide (TMZ) has shown clinical activity against neuroblastoma, this activity is likely limited by the DNA repair enzyme O⁶-methylguanine DNA methyltransferase (MGMT). We hypothesized that interferon-beta (IFN-β) could sensitize neuroblastoma cells to the cytotoxic effects of TMZ through its ability to down regulate MGMT expression.

**Methods—**In vitro proliferation of three neuroblastoma cell lines treated with IFN-β and TMZ alone, or in combination, was examined. Anti-tumor activity was assessed in both localized and disseminated neuroblastoma xenograft models using single agent and combination therapy, with continuous delivery of IFN-β being established by a liver-targeted AAV-mediated approach. MGMT expression was also measured.

**Results—**Two neuroblastoma cell lines (NB-1691, SK-N-AS) were found to have high baseline levels of MGMT expression while a third cell line (CHLA-255) had low levels. TMZ had little effect on in vitro proliferation of the neuroblastoma cell lines with high MGMT expression, but pretreatment with IFN-β significantly decreased MGMT expression and cell counts. In vivo, tumor bearing mice treated with the combination of IFN-β and TMZ had lower MGMT expression and a significantly reduced NB-1691 tumor burden in models of localized and disseminated disease when compared to untreated control mice and those treated with either agent alone.

**Conclusions—**IFN-β appears to sensitize neuroblastoma cells to the cytotoxic effects of TMZ through attenuation of MGMT expression. Thus, IFN-β and TMZ may be a useful combination for treating children with this difficult disease.

### Keywords

interferon-β; temozolomide; neuroblastoma; MGMT

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## Introduction

Neuroblastoma is an aggressive malignancy of the sympathetic nervous system and is the most common solid extra-cranial tumor of childhood.(1) While disease with favorable clinical and biologic features is usually curable with surgery alone, disease with a high-risk phenotype rarely is, with long-term survival being less than 40%.(1) Patients with relapsed, high-risk disease are essentially incurable. Therefore, new treatment strategies are needed for these patients.

Alkylating agents such as temozolomide (TMZ) have shown promise in treating a variety of solid tumors including neuroblastoma. TMZ can be administered orally, has a bioavailability of almost 100% and can penetrate into all body tissues, including the brain, due to its ability to cross the blood brain barrier.(2) TMZ has been approved for treatment of glioblastoma(3) and has shown some activity in Phase II trials for neuroblastoma.(4,5)TMZ can cause cell death by binding to DNA, most frequently methylating the $O^6$ position of guanine.(6) The creation of this $O^6$-methylguanine causes the incorporation of a thymine residue opposite $O^6$-methylguanine instead of the normal cytosine residue, resulting in a G:C to G:T transition mutation. This mutation is repetitively repaired by the mismatch repair pathway, but eventually leads to the generation of a chronic strand break condition that elicits an apoptotic response in the cell.(7,8) Therefore, the extent of DNA methylation has been shown to correlate well with both the therapeutic activity and the toxicity of TMZ.(9)

The methyl group on the $O^6$ position of guanine can be removed, however, by the suicide DNA repair protein $O^6$-methylguanine DNA methyltransferase (MGMT).(2) This enzyme transfers the methyl group to an active cysteine residue within its own sequence in a reaction that returns the DNA to its previously intact state, inactivating one MGMT molecule for each mutation repaired.(10) Thua, the action of MGMT inhibits the otherwise lethal cross-linking between adjacent strands of DNA, conferring resistance to alkylating agents.(2,10) Tumors that contain high levels of MGMT are, therefore, likely to be resistant to alkylating agents.(8) MGMT is widely expressed in primary neuroblastoma tumors and established cell lines.(11) Thus, in order to realize the maximal cytotoxic activity of TMZ against neuroblastoma, MGMT activity needs to be suppressed. $O^6$-benzylguanine (BG) is one such agent that has been shown to inactivate MGMT in neuroblastoma cell lines, thereby increasing the cytotoxicity of TMZ. (11) BG, however, does not itself have significant cytotoxic activity. A drug with the capability of attenuating the function of MGMT, as well as possessing some direct anti-tumor activity, would be a logical adjuvant to TMZ.(10)

Type I interferons (IFN) are regulatory cytokines that have been found to have clinical use in the treatment of various types of malignancies. Their pleiotropic anti-tumor effects include direct tumor cell cytotoxicity and indirect activity through immunomodulation and inhibition of angiogenesis. (12) IFN-β has also recently been noted to be a potent sensitizer of glioma cell lines to the cytotoxic activity of TMZ through the down regulation of MGMT expression. (13) The ability of IFN to down regulate MGMT, along with its known cytotoxic effects makes it an appealing choice to combine with TMZ for the treatment of neuroblastoma. We hypothesized that IFN-β should sensitize MGMT-expressing neuroblastoma cells to the cytotoxic effects of TMZ.

## Materials and methods

### Cell lines

The human neuroblastoma cell lines NB-1691, provided by Dr. P Houghton (St. Jude Children's Research Hospital, Memphis, TN) and SK-N-AS, purchased from the American Type Culture Collection (Manassas, Virginia) were maintained in RPMI-1640 culture media

(Hyclone, Logan, UT). The human neuroblastoma cell line CHLA-255, provided by Dr. C. Patrick Reynolds (Children's Hospital of Los Angeles, L.A., CA), was maintained in DMEM medium (Cellgro, Mediatech Inc., Herndon, VA). All culture media was supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 units/mL penicillin, 100 μg/mL streptomycin (GIBCO BRL, Grand Island, NY) and 2 mM L-glutamine (GIBCO). NB-1691 was modified to constitutively express the enzyme luciferase, as previously described.(14)

### In vitro effects of IFN-β and TMZ on neuroblastoma proliferation

Tumor cells were plated in 24-well plates and then treated with either vehicle control [0.1% dimethylsulphoxide, (DMSO), Sigma-Aldrich Company, St. Louis, MO)], TMZ (in 0.1% DMSO), recombinant human IFN-β (rhIFN-β), (Avonex®, Biogen Inc, Cambridge, MA) or a combination of TMZ and rhIFN-β. After 48 hours of exposure to the drug(s), cells were counted and statistical analysis performed. TMZ [LKT Labs, (St. Paul, MN)] was used at a final concentration of 100 μM. Recombinant human IFN-β was used at a final concentration of 100 IU/ml for CHLA-255 cells, a dose previously shown to be effective,(13) but was decreased to 50 IU/ml for NB-1691 and SK-N-AS cells, as these cell lines were more sensitive to IFN. Furthermore, fluorescence based cell counts were performed using the Guava PCA and viacount reagent (Guava Technologies Inc. Hayward, CA) to confirm results.

### AAV vector preparation

Construction of the pAV2-CAGG-hIFN-β vector plasmid has been previously described.(16) This vector plasmid includes the cytomegalovirus immediate-early enhancer, β-actin promoter, a chicken β-actin/rabbit β-globulin composite intron, and a rabbit β-globulin polyadenylation signal mediating the expression of the cDNA for hIFN-β (Invitrogen,Carlsbad, CA). Recombinant AAV2 vectors pseudotyped with serotype 8 capsid were generated with a triple plasmid transfection method as previously described.(15) These AAV2/8 vectors were purified using ion exchange chromatography.(17)

### Murine Tumor Models

Subcutaneous human neuroblastoma xenografts were established in male C.B.-17 SCID (Jackson Laboratory, Bar Harbor, ME) mice via right flank injection of $3 \times 10^6$ NB-1691 or SK-N-AS cells. Growth of the subcutaneous tumors was monitored by measurements in two dimensions with calipers, and volumes were calculated as width$^2$ x length x 0.5. When the tumors were an average volume of 0.2 cm$^3$, approximately three weeks after tumor cell injection, mice were separated into four size-matched cohorts containing five mice each. Two cohorts initially (day 0) received no treatment and two cohorts received $5 \times 10^{10}$AAV2/8-CAGG-hIFN-β vector genomes/mouse via tail vein injection. Forty-eight hours later, one of the untreated cohorts and one of the AAV-hIFN-β treated cohorts received 2.5 mg TMZ via oral gavage daily for five consecutive days. Suspension of TMZ for in vivo administration was prepared by mixing in a 1:1 solution of sterile water and carboxymethylcellulose, at 100 mg/kg. Tumor growth was monitored, and relative tumor volume was calculated as tumor volume at each time point divided by tumor volume at day zero. Mice were sacrificed on day 17.

Disseminated neuroblastoma was established by injecting $2 \times 10^6$ NB-1691luc tumor cells via tail vein. Three weeks after initial tumor cell injection, tumor burden in the mice was then size matched based on the intensity of the bioluminescent signal (photons/second), and mice were placed into four treatment groups as described for those with subcutaneous tumors. Progression of disseminated neuroblastoma was monitored with bioluminescent imaging on days 0 (defined as the day of initial treatment), 7, 12, and 17. D-luciferin (Xenogen Corporation, Alameda, CA) 15 mg/ml in sterile PBS was injected intraperitoneally (IP), after which images were obtained with an IVIS Imaging System 100 Series (Xenogen). These images were analyzed with Living Image Software version 2.50 (Xenogen) and expressed as photons per second.

Tumor weight in the liver on day 17 was also determined as the weight of each tumor-bearing liver - the average weight of a normal, disease-free liver (1.3 g). Bone marrow was collected from both femurs of each mouse, from which RNA was isolated utilizing the TEL-TEST RNA-STAT-60 protocol (TEL-TEST Inc., Friendswood, TX).

All murine experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

### Quantitative Polymerase Chain Reaction (QPCR)

To measure tumor burden in the bone marrow, total RNA was isolated from the intrafemoral marrow of mice with disseminated disease. Tumor burden in the liver was quantitated by isolating RNA from frozen liver samples of disseminated mice. Complimentary DNA was generated by reverse-transcription and then amplified with primer and probe sets for *MYCN* and *GAPDH* (Hs00232074_m1, Hs9999905_ml, respectively; Applied Biosystems, Foster City, CA). Standard curves were constructed in each PCR run with 10-fold dilutions of NB-1691 cells in mouse leukemic cells (YAC). The dosages of the target genes in each sample were interpolated using these standard curves. The *MYCN* copy number was determined by the ratio of the *MYCN* Ct to the *GAPDH* Ct. Copy numbers were expressed as the average of two measurements.

### Human IFN-β immunoassay

Quantification of systemic AAV-mediated hIFN-β expression was performed on mouse plasma utilizing a commercially available sandwich immunoassay (ELISA, TRB INC, Fujirebio INC, Tokyo, Japan). The sensitivity range for this assay is 250 to 10,000 pg/mL.

### Protein Extraction

Protein lysates were made from cell pellets or tumors using 1 mL of protein lysis solution buffer (25 mM Tris HCl, 150 mM HCl, 0.5%. NP40, 0.5% sodium deoxycholate, 0.2% SDS, 1.0 mg Pefabloc SC and 1 protease tablet [Boehringer Mannheim, Indianapolis, IN]) per plate or 1.0 g of tissue. The lysate was then collected from each sample, placed into a sterile tube, incubated on ice for 30 minutes, and then centrifuged at 10,000 x g for 10 minutes at 4° C. The supernatants were then collected, centrifuged, collected, and frozen at -80° C for later use. Protein lysates were quantified using the Bradford Assay (Bio-Rad) and the Beckman DU-600 system.

### Western Blot Analysis

Protein extracts (200 μg) were separated by gel electrophoresis and transferred to Bio-Rad Immun-Blot PVDF membranes, then blocked overnight. Membranes were incubated with the MGMT antibody (clone MT3.1, Lab Vision Corporation) and an appropriate secondary antibody. MGMT was detected using chemiluminescence (ECL Plus, Amersham, England). Membranes were subsequently stripped and incubated with GAPDH (Millipore, Billerica, MA) as a positive control to confirm equal loading of protein.

### Tdt-mediated dUTP nick end labeling (TUNEL) assay

Apoptosis in subcutaneous tumors was determined by the TUNEL method using a commercially available in situ apoptosis detection kit (Serologicals, Norcross, GA). Densities of apoptotic cells were determined at 400X light microscopy (Olympus U-SPT light miscroscope) by counting three high-powered fields of view per sample (both the number of TUNEL positive and the total number of cells per high-powered field) and calculating the average number of TUNEL positive cells per 1000 cells per sample.

### TMZ plasma and tumor concentrations

TMZ and MTIC (3-methyl-(triazen-1-yl)imidazole-4-carboxamide) concentrations were measured in murine plasma and neuroblastoma tumor samples as previously described. (18)

### Statistical Analyses

Results are reported as mean +/ - SE. The Sigma plot program (SPSS Inc, Chicago, Il) was used to analyze and graphically represent the data. An unpaired student *t* test was used to analyze the statistical differences between treatment groups. A P value of less than 0.05 was considered to be statistically significant.

## Results

### MGMT Expression in vitro

MGMT levels in NB-1691, SK-N-AS, and CHLA-255 cells treated for 48 hours with 50 IU/ml rhIFN-β were determined by Western immunoblot analysis (Fig 1). Untreated NB-1691 and SK-N-AS were found to have significant levels of MGMT expression while CHLA-255 cells had no detectable expression. IFN-β treatment resulted in a significant decrease in expression of MGMT in both the NB-1691 and SK-N-AS cells.

### Effects of IFN-β and TMZ in vitro

Single agent TMZ had little effect on the in vitro proliferation of NB-1691 or SK-N-AS cells (102 +/- 13% of control, p = 0.904, 87 +/-15% of control, p=0.44, respectively). Exposure of the neuroblastoma cell lines to 50 IU/mL rhIFN-β for 48 hrs inhibited the proliferation of both NB-1691 cells (61 +/- 9% of control, p = 0.035, Fig 2A) and SK-N-AS cells (69 +/-4% of control, p=0.003, Fig 2B). With both cell lines, the combination of IFN-β and TMZ further inhibited proliferation when compared to control (NB-1691, 36 +/- 3% of control, p = 0.0008; SK-N-AS, 54 +/-7% of control, p=0.003), and to single agent IFN-β although the difference did not reach statistical significance for the SK-N-AS cell line. Thus, the addition of IFN-β appeared to sensitize the two cell lines to the antitumor activity of TMZ in vitro.

When using a neuroblastoma cell line that does not express MGMT (CHLA-255), both TMZ and IFN-β were able to significantly restrict the proliferation of the cells in vitro (Fig 2C). After treatment with rhIFN-β, the cell count was 51 +/- 4% of control (p = 0.019), and after treatment with TMZ, the cell count was 57 +/- 4% of control (p = 0.03). Treatment with the combination of rhIFN-β and TMZ resulted in an even lower final cell count, 33 +/- 1% of control (p = 0.006). This restriction in cell proliferation was also significantly greater than with either monotherapy (IFN-β, p=0.009 and TMZ, p=0.003).

These results were confirmed using a fluorescent based automatic cell counter which measures both cell viability and cell count. No difference was observed in viability amongst different treatment groups although the same trend in cell number was observed (data not shown).

### Effects of IFN-β and TMZ in vivo against localized neuroblastoma

AAV vectors encoding hIFN-β were used to achieve continuous, systemic delivery of hIFN-β. Because these vectors were pseudotyped with serotype 8 capsid, tail vein administration of vector resulted primarily in hepatocyte transduction, with subsequent prompt, continuous production of hIFN-β from the liver.(15) When subcutaneous NB-1691 tumors were treated with TMZ or AAV2/8-hIFN-β alone, a significant effect on tumor growth was seen at day 17 (Fig 3A) when compared to the untreated controls [relative tumor volumes: 25.16 +/- 6.8 (ctrl) vs. 12.72 +/- 3.3 (TMZ), p= 0.01, 13.48 +/- 2.2, p=0.03 (IFN-β)]. The effect of IFN-β on tumor size was seen in vitro with a decrease in cell counts with IFN-β monotherapy; however, the

reduction in tumor volume secondary to TMZ was not apparent in vitro. Therefore, TMZ may be affecting the host environment, thus reducing tumor size without a direct cytotoxic effect on the tumor cells.

Although single agent therapy was able to slow the growth of the NB-1691 tumors, combination therapy began to cause a decrease in tumor size, with tumor volumes at day 17 being significantly smaller than untreated control tumors [3.52 +/- 1.1 vs. 25.16 +/- 6.8 (ctrl), p=0.0001], and monotherapy with either AAV-hIFN-β or TMZ (p=0.05 and p=0.02, respectively). Systemic levels of IFN-β at the time of sacrifice in mice receiving AAV-hIFN-β averaged 90.5 ng/ml (range 54 - 127 ng/ml).

Similar effects of combination therapy with AAV-hIFN-β and TMZ were seen with SK-N-AS xenografts although neither agent when used as monotherapy had a significant impact on tumor growth (Fig 3B). The combination therapy group had the lowest relative tumor volume overall (combo: 4.8 +/- 0.8 vs. control: 22.5 +/- 10.3, p = 0.07; vs. IFN-β: 17.0 +/- 3.6, p = 0.003; vs. TMZ: 19.3 +/- 8.0, p = 0.07).

Evaluation by TUNEL staining of treated tumors revealed that the cohort receiving combination therapy had the greatest number of apoptotic cells (40.39 +/- 4.41 per 1000 cells). Combination therapy induced significantly more tumor cell apoptosis than control or TMZ (6.91 +/- 0.99 per 1000 cells) and IFN-β (22.36 +/- 4.9 per 1000 cells) monotherapy (Fig 4).

### In Vivo MGMT Expression

Day 17 tumor samples were collected and snap-frozen in liquid nitrogen. Protein was extracted from each sample, and MGMT levels were determined by Western immunoblot analysis (Fig 5). Both the control tumor samples and the TMZ treated tumor samples produced bands around 25 kDa, representative of MGMT expression, while tumors treated with IFN-β either alone or in combination with TMZ had either absent or significantly reduced MGMT expression.

### Effects of IFN-β and TMZ in vivo against disseminated neuroblastoma

This tumor sensitizing effect of IFN-β to TMZ was also demonstrated in the disseminated tumor model. All cohorts (untreated control, single agent AAV-hIFN-β, single agent TMZ, and combination of AAV-hIFN-β and TMZ) were initially matched for disease burden based on the intensity of the bioluminescent signal (in photons/second). At day 17 after the initiation of therapy, there was a difference in bioluminescent signal for all groups when compared to the control group [(1.32 $e^{10}$ +/- 6.5 $e^{9}$) vs. IFN-β (2.78 $e^{8}$ +/- 3.09 $e^{8}$), p=0.025, vs. TMZ (2.06 $e^{9}$ +/- 1.55 $e^{9}$), p=0.1 and vs. combination (2.13 $e^{7}$ +/- 7.67 $e^{6}$), p=0.009, respectively)] and between combination and monotherapy with IFN-β (p=0.025) (Fig 6A). Systemic levels of IFN-β at the time of sacrifice in mice receiving AAV-hIFN-β averaged 53.4 ng/ml (range 2.8 - 131 ng/ml).

Livers of control mice with disseminated disease or those treated with TMZ alone uniformly had obvious gross disease. Quantitative PCR performed on liver samples from disseminated mice showed a decrease in tumor burden with combination therapy (Fig 6B). Mice that received IFN-β alone had no gross evidence of disease in the liver and no difference in weight of naïve livers, but malignant cells were present in small, scattered, focal areas throughout the normal liver tissue on histologic evaluation. Mice that had received the combination AAV-hIFN-β and TMZ also had no grossly evident disease in the livers which were of normal weight and had only few scattered individual malignant cells present on microscopic evaluation (Fig 6C).

Bone marrow from the mice with disseminated NB-1691luc was harvested at the time of sacrifice. Quantitative PCR was then performed on RNA extracted from bone marrow flushed from the femurs of these mice to further assess marrow involvement in this murine model of

disseminated disease. A 2-3 fold log reduction was seen with both IFN-β alone and combination therapy when compared to controls (data not shown). No significant difference in tumor burden was seen in the marrows of mice in these two treatment groups.

## DISCUSSION

High risk neuroblastoma is a difficult disease to treat, and recurrent disease is generally resistant to all chemotherapeutic agents. Overexpression of MGMT is one mechanism by which tumors become resistant to alkylating agents such as TMZ. We evaluated three different neuroblastoma cell lines to determine MGMT levels. NB-1691 and SK-N-AS were found to have high levels of MGMT expression while CHLA-255 cells have a low level of MGMT expression. The level of MGMT expression inversely correlated with the cytotoxic effect of TMZ therapy on each of the cell lines. Pre-treatment with IFN-β down regulated MGMT expression both in vitro and in vivo enhancing the effect of TMZ against neuroblastoma cell lines with high MGMT expression.

Aside from the ability to down regulate MGMT, IFN also has an independent anti-tumor effect on neuroblastoma. Treatment with rhIFN-β decreased cell counts in vitro. Due to the short half life of rhIFN-β, we used an AAV vector in our murine model to generate prolonged expression of IFN-β. In an additional experiment, we treated mice with recombinant IFN-β protein at a dose of $2 \times 10^5$ IU/day given intraperitoneally. This established appreciable systemic levels of IFN-β (10.7-25.7 ng/ml), although because of the very short half life of IFN-β, these levels were not maintained. Nevertheless, recombinant IFN-β decreased the size of localized tumors though not to the extent of treatment with AAV-hIFN-β which established continuous, systemic delivery of IFN-β. The level of MGMT expression was not decreased in these tumors when they were treated with rhIFN-β (data not shown).

When tumors were treated with AAV-hIFN-β prior to TMZ dosing, the combined effect was significantly greater than either monotherapy. Combination therapy showed improved effectiveness in localized tumors resulting in much smaller relative tumor volumes than controls or either monotherapy. TUNEL staining exhibited an increase in apoptosis in treated tumors with the largest number of apoptotic cells being seen in the combination group.

Because many patients present with widespread metastatic disease, it is important to assess the effects of combination treatment in a disseminated model. As shown previously in localized tumors, we found the combination of IFN and TMZ to be extremely effective. Not only did our treatment slow the progression of disseminated disease, but tumor burden was nearly eradicated as demonstrated by the statistically significant decreased bioluminescence signal. This difference in bioluminescent signal intensity suggests a significant difference in tumor burden as this measure has been shown to correlate with extent of disease in murine models of neuroblastoma.(14) Additionally, the gross and microscopic examination of diseased livers and quantitative PCR of bone marrow both showed a dramatic reduction in disseminated tumor burden.

We have previously shown that combination therapy with AAV-hIFN-β improves tumor perfusion thus increasing the delivery of chemotherapy to the tumor itself which might explain the improved effect of combination therapy.(19) However, a lower dose of AAV-IFN-β was utilized in this experiment which was able to down regulate MGMT, but the low dose did not affect delivery of TMZ to the tumor itself when tumor and plasma levels were measured (data not shown). Therefore, the effect of combination therapy is most likely related to overcoming tumor resistance by down regulating MGMT with IFN-β.

Based on the encouraging results of this study, the combination of IFN-β and TMZ appears to be an effective treatment option for neuroblastoma. The cytotoxic effect of IFN-β and its ability

to down regulate MGMT allow the two agents to work together to decrease disease burden. As neuroblastoma is known to be resistant to a variety of treatments, it is important to use a multimodal approach to eradicate disease. The effectiveness of combination therapy in murine models is encouraging; therefore, use of this combination therapy should be considered in clinical trials for patients with high risk neuroblastoma.

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## Abbreviations

TMZ, Temozolomide; MGMT, $O^6$-methylguanine DNA methyltransferase; IFN-β, interferon-beta; AAV, Adeno-associated virus; BG, $O^6$-benzylguanine; DMSO, dimethylsulphoxide; rhIFN-β, recombinant human IFN-β; CMC, carboxymethylcellulose; QPCR, quantitative polymerase chain reaction; TUNEL, Tdt-mediated dUTP nick end labeling.

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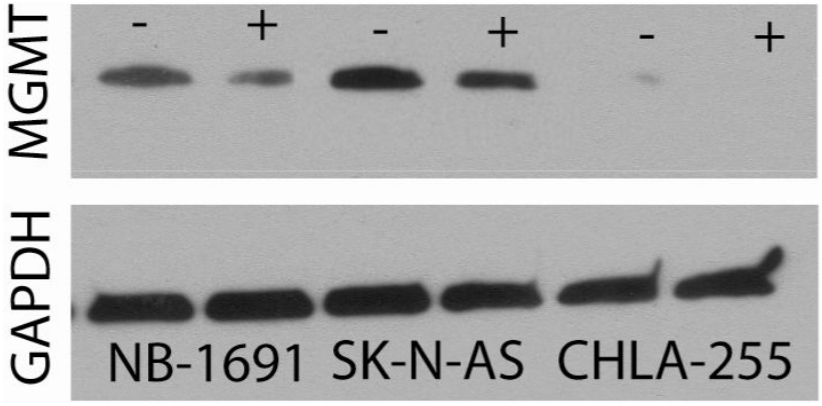


**Fig. 1.**

MGMT expression as detected by western blot analysis in three neuroblastoma cell lines (NB-1691, SK-N-AS, CHLA-255) in vitro. Cells were treated in vitro with PBS (-) or 50 units/ml of rIFN-β (+) for 72 hours.

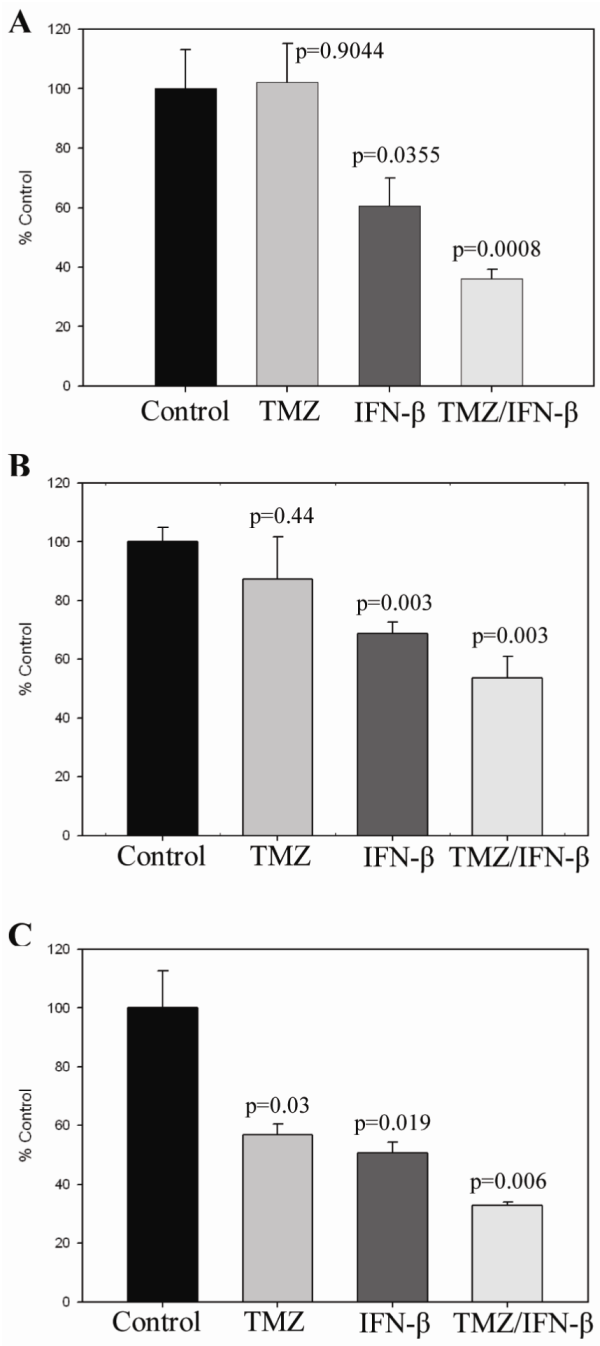


**Fig. 2.**

Effect of TMZ and IFN-B on cellular proliferation in vitro**. A.** NB1691, p values shown are compared to control. The p value of the single agent IFN-β group vs. the combination group= 0.0332. **B.** SK-N-AS, Combination group vs IFN-β, p = 0.11. **C.** CHLA-255, combination vs IFN-β, p = 0.009, combination vs TMZ, p = 0.003

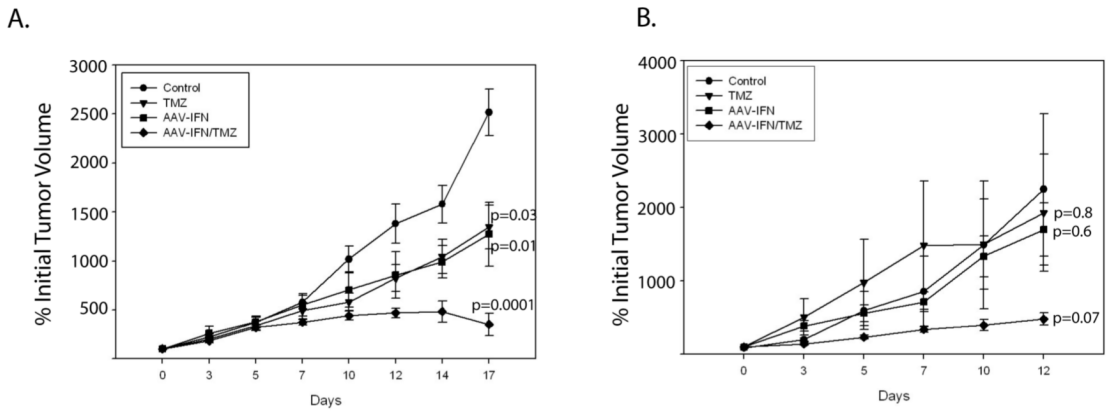


**Fig. 3.**

Effect of TMZ and IFN-B on the growth of subcutaneous neuroblastoma xenografts. P values in the figure are the comparison of relative tumor size to control on day of sacrifice. **A.** NB1691, AAV-IFN-β+TMZ vs AAV-IFN-β alone, p = 0.05. **B.** SK-N-AS, AAV-IFN-β+TMZ vs AAV-IFN-β alone, p = 0.003.

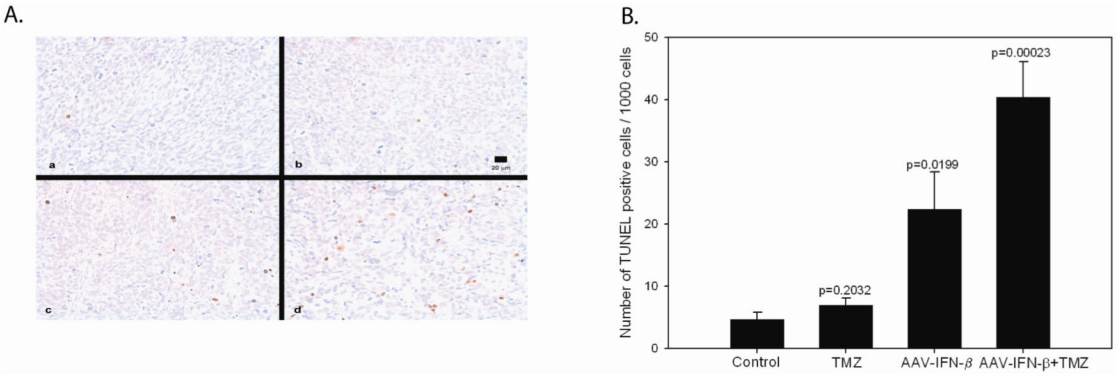


**Fig. 4.**

Tumor cell apoptosis. **A.** TUNEL stained sections of NB-1691 xenografts (400X). *a* - untreated tumor, *b* - TMZ treated tumor, *c* - AAV-IFN-β treated tumor, *d* - AAV-IFN-β+TMZ treated tumor. The TUNEL positive cells are stained brown. **B.** Quantitation of TUNEL positive cells per 1000 cells for each treatment group. P values are relative to the untreated control group. AAV-IFN-β alone vs. AAV-IFN-β+TMZ, p = 0.0702.

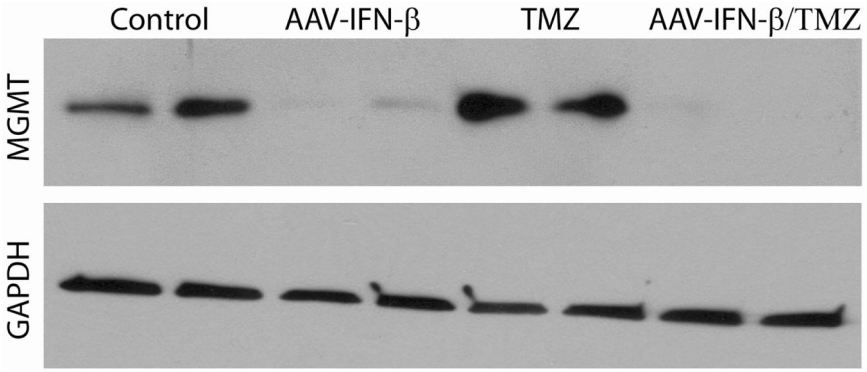


**Fig. 5.**
Western blot analysis of MGMT expression in subcutaneous NB-1691 tumor xenografts.

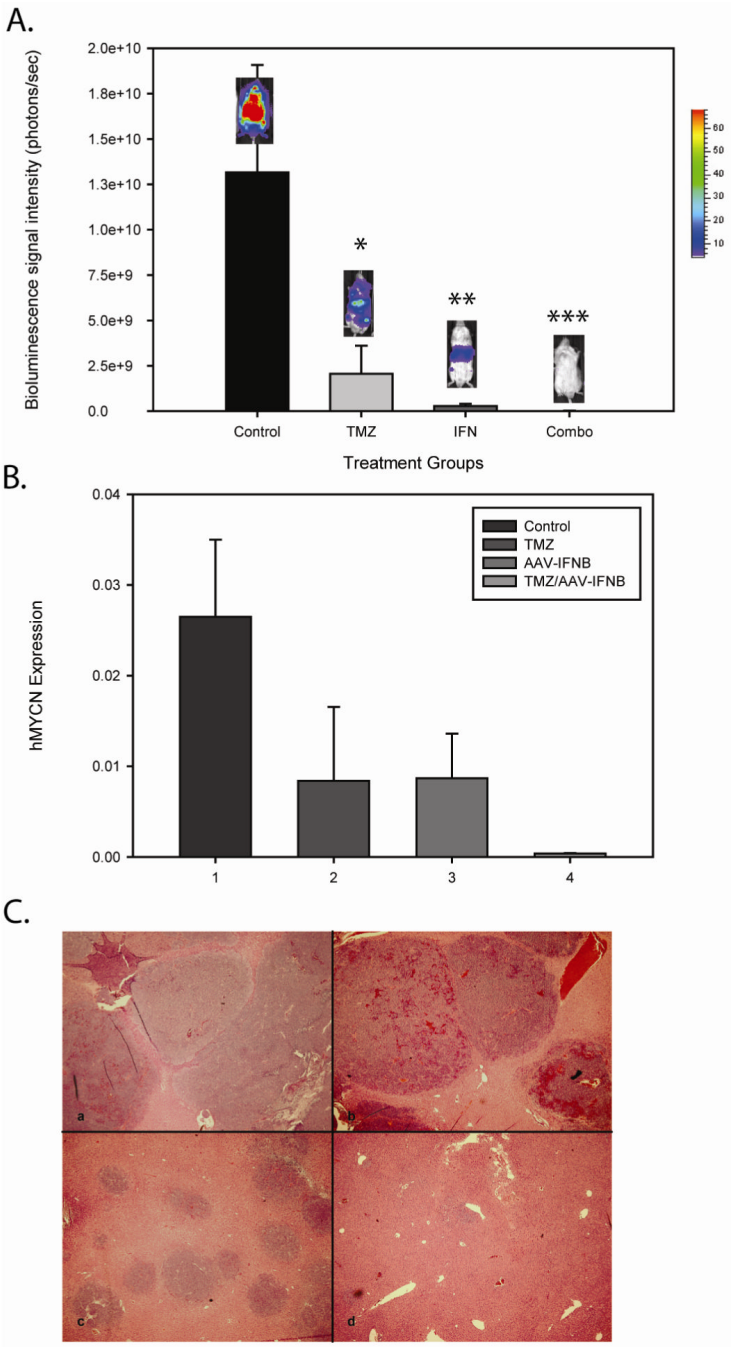


**Fig. 6.**

Effect of TMZ and IFN-B on disseminated neuroblastoma (NB-1691luc). **A.** Bioluminescent signals on day 17 (day of sacrifice) are shown with representative images. P values on Day 17 compared to control are: *= 0.10, **= 0.025, ***= 0.009, while the p value for IFN-β vs combo on Day 17 = 0.025. **B.** Expression of human MYCN in liver samples from mice with disseminated neuroblastoma **C.** H & E sections of livers from in vivo disseminated NB-1691luc model (20X). *a* - untreated control; *b* - TMZ; *c* - AAV-IFN-β; *d* - AAV-IFN-β + TMZ.