IFN-\(\beta\) sensitizes neuroblastoma to the antitumor activity of temozolomide by modulating \(O^6\)-methylguanine DNA methyltransferase expression

Shannon F. Rosati, Regan F. Williams, Lindsey C. Nunnally, Mackenzie C. McGee, Thomas L. Sims, Lorraine Tracey, Junfang Zhou, Meiyun Fan, Catherine Y. Ng, Amit C. Nathwani, Clinton F. Stewart, Lawrence M. Pfeffer, and Andrew M. Davidoff

Departments of 1Surgery and 2Pharmaceutical Sciences, St. Jude Children’s Research Hospital; Departments of 3Surgery and 4Pathology, University of Tennessee College of Medicine, Memphis, Tennessee and 5Department of Hematology, University College London, London, United Kingdom

Abstract

Although temozolomide has shown clinical activity against neuroblastoma, this activity is likely limited by the DNA repair enzyme \(O^6\)-methylguanine DNA methyltransferase (MGMT). We hypothesized that IFN-\(\beta\) could sensitize neuroblastoma cells to the cytotoxic effects of temozolomide through its ability to down-regulate MGMT expression. \textit{In vitro} proliferation of three neuroblastoma cell lines treated with IFN-\(\beta\) and temozolomide alone or in combination was examined. Antitumor activity was assessed in both localized and disseminated neuroblastoma xenografts using single-agent and combination therapy, with continuous delivery of IFN-\(\beta\) being established by a liver-targeted adeno-associated virus-mediated approach. Two neuroblastoma cell lines (NB-1691 and SK-N-AS) were found to have high baseline levels of MGMT expression, whereas a third cell line (CHLA-255) had low levels. Temozolomide had little effect on \textit{in vitro} proliferation of the neuroblastoma cell lines with high MGMT expression, but pretreatment with IFN-\(\beta\) significantly decreased MGMT expression and cell counts (NB-1691: 36 ± 3% of control, \(P = 0.0008\); SK-N-AS: 54 ± 7% control, \(P = 0.003\)). \textit{In vivo}, NB-1691 tumors in CB17-SCID mice treated with the combination of IFN-\(\beta\) and temozolomide had lower MGMT expression and a significantly reduced tumor burden, both localized [percent initial tumor volume: 2,516 ± 680% (control) versus 1,272 ± 330% (temozolomide), \(P = 0.01\); 1,348 ± 220%, \(P = 0.03\) (IFN-\(\beta\)); 352 ± 110%, \(P = 0.0001\) (combo)] and disseminated [bioluminescent signal: control (1.32e\(10\) ± 6.5e\(6\)) versus IFN-\(\beta\) (2.78e\(8\) ± 3.09e\(6\)), \(P = 0.025\), versus temozolomide (2.06e\(9\) ± 1.55e\(8\)), \(P = 0.1\), versus combination (2.13e\(7\) ± 7.67e\(6\)), \(P = 0.009\)]. IFN-\(\beta\) appears to sensitize neuroblastoma cells to the cytotoxic effects of temozolomide through attenuation of MGMT expression. Thus, IFN-\(\beta\) and temozolomide may be a useful combination for treating children with this difficult disease. [Mol Cancer Ther 2008;7(12):3852–8]

Introduction

Neuroblastoma is an aggressive malignancy of the sympathetic nervous system and is the most common solid extracranial tumor of childhood (1). Whereas disease with favorable clinical and biological features is usually curable with surgery alone, disease with a high-risk phenotype rarely is, with long-term survival being <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 have shown promise in treating a variety of solid tumors including neuroblastoma. Temozolomide 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). Temozolomide has been approved for treatment of glioblastoma (3) and has shown some activity in phase II trials for neuroblastoma (4, 5). Temozolomide 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 T:G 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 temozolomide (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; ref. 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). Thus,
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, to realize the maximal cytotoxic activity of temozolomide against neuroblastoma, MGMT activity needs to be suppressed. O6-benzylguanine is one such agent that has been shown to inactivate MGMT in neuroblastoma cell lines, thereby increasing the cytotoxicity of temozolomide (11). O6-benzylguanine, 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 antitumor activity, would be a logical adjuvant to temozolomide (10).

Type I IFNs are regulatory cytokines that have been found to have clinical use in the treatment of various types of malignancies. Their pleiotropic antitumor 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 temozolomide 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 temozolomide for the treatment of neuroblastoma. We hypothesized that IFN-β should sensitize MGMT-expressing neuroblastoma cells to the cytotoxic effects of temozolomide, thus possibly increasing the treatment response to combination therapy in high-risk neuroblastoma patients.

Materials and Methods

Cell Lines

The human neuroblastoma cell lines NB-1691, provided by Dr. P. Houghton (St. Jude Children’s Research Hospital) and SK-N-AS, purchased from the American Type Culture Collection, were maintained in RPMI 1640 (Hyclone). The human neuroblastoma cell line CHLA-255, provided by Dr. C. Patrick Reynolds (Children’s Hospital of Los Angeles), was maintained in DMEM (Cellgro; Mediatech). All culture media were supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies), and 2 mmol/L l-glutamine (Life Technologies). NB-1691 was modified to constitutively express the enzyme luciferase as described previously (14).

In vitro Effects of IFN-β and Temozolomide on Neuroblastoma Proliferation

Tumor cells were plated in 24-well plates and then treated with either vehicle control (0.1% DMSO; Sigma-Aldrich), temozolomide (in 0.1% DMSO; LKT Labs), recombinant human IFN-β (rhIFN-β; Avonex; Biogen), or a combination of temozolomide and rhIFN-β. After 48 h of exposure to the drug(s), cells were counted and statistical analysis was done. Temozolomide was used at a final concentration of 100 μmol/L. rhIFN-β was used at a final concentration of 50 IU/mL. Fluorescence-based cell counts were also done using the Guava PCA and viacount reagent (Guava Technologies) to confirm results. Cells were then evaluated by flow cytometry for cell cycle distribution (DNA content) and apoptosis (Annexin V).

A MTS cytotoxicity assay was done to determine the effect of temozolomide alone on each cell line. NB-1691, CHLA-255, and SK-N-AS cells were plated at 5,000 per well in a 96-well plate and allowed to attach overnight. Temozolomide was added at a dose ranging from 1 mmol/L to 62.5 μmol/L. Vehicle-only controls and blank wells containing medium only were included. Cells were incubated with temozolomide for 72 h and cell viability was determined by adding 20 μL CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) to each well and measuring absorbance at 490 nm after 2 h incubation at 37°C. All treatments were done in duplicate, and each experiment was done in at least triplicate.

Adeno-Associated Virus Vector Preparation

Construction of the pAV2-CAGG-hIFN-β vector plasmid has been described previously (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). Recombinant adeno-associated virus (AAV) 2 vectors pseudotyped with serotype 8 capsid were generated with a triple plasmid transfection method as described previously (15). These AAV2/8 vectors were purified using ion-exchange chromatography (17).

Murine Tumor Models

S.c. human neuroblastoma xenografts were established in male CB17-SCID (The Jackson Laboratory) mice via right flank injection of 3 × 106 NB-1691 or SK-N-AS cells. Growth of the s.c. tumors was monitored by measurements in two dimensions with calipers, and volumes were calculated as width2 × length × 0.5. When the tumors were an average volume of 0.2 cm3, ~3 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 × 1010 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-rhIFN-β-treated cohorts received 2.5 mg temozolomide via oral gavage daily for 5 consecutive days. Suspension of temozolomide 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 percent initial tumor volume was calculated as tumor volume at each time point divided by tumor volume at day 0 multiplied by 100%. Mice were sacrificed on day 17.

Disseminated neuroblastoma was established by injecting 2 × 106 NB-1691 luc tumor cells via tail vein. Three weeks after initial tumor cell injection, tumor burden in the mice was then matched based on the intensity of the bioluminescent signal (photons/s), and mice were placed...
into four treatment groups as described for those with s.c. 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 (15 mg/mL; Xenogen) in sterile PBS was injected i.p., 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/s. Tumor weight in the liver on day 17 was also determined as the weight of each tumor-bearing liver minus the average weight of a normal, disease-free liver (1.3 g). Bone marrow was collected from the femurs of each mouse, from which RNA was isolated using the TEL-TEST RNA-STAT-60 protocol (TEL-TEST).

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

Quantitative PCR
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. cDNA was generated by reverse-transcription and then amplified with primer and probe sets for human MYCN, GAPDH, and murine HPRT (Hs00232074_m1, Hs9999905_m1, and Mm00446968_m1, respectively; Applied Biosystems). 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. Relative MYCN content was determined by the ratio of MYCN concentration to the mHPRT concentration and expressed as the average of two measurements.

Human IFN-β Immunoassay
Quantification of systemic AAV-mediated hIFN-β expression was done on mouse plasma using a commercially available sandwich immunoassay (ELISA; TRB, Fujirebio). 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 protein lysis solution buffer (25 mmol/L Tris HCl, 150 mmol/L HCl, 0.5%. NP40, 0.5% sodium deoxycholate, 0.2% SDS, 1.0 mg Pefabloc SC, and 1 protease tablet; Boehringer Mannheim) per plate or 1.0 g tissue. The lysate was then combined from each sample, placed into a sterile tube, incubated on ice for 30 min, and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatants were then combined, 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 Immobilon-Polyvinyldene difluoride membranes and then blocked overnight. Membranes were incubated with the MGMT antibody (clone MT3.1; Lab Vision) and an appropriate secondary antibody. MGMT was detected using chemiluminescence (ECL Plus; Amersham). Membranes were subsequently stripped and incubated with GAPDH (Millipore) as a positive control to confirm equal loading of protein.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay
Apoptosis in s.c. tumors was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (TUNEL) method using a commercially available in situ apoptosis detection kit (Serologicals). Densities of apoptotic cells were determined at ×400 by light microscopy (Olympus U-SPT light microscope) by counting three high-power fields of view per sample (both the number of TUNEL-positive cells and the total number of cells per high-power field) and calculating the average number of TUNEL-positive cells per 1,000 cells per sample.

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

Statistical Analyses
Results are reported as mean ± SE. The Sigma plot program (SPSS) was used to analyze and graphically represent the data. An unpaired Student’s t test was used to analyze the statistical differences between treatment groups. P < 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 h 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, whereas CHLA-255 cells had no detectable expression. IFN-β treatment resulted in a significant decrease in expression of MGMT in both NB-1691 and SK-N-AS cells.

Effects of IFN-β and Temozolomide In vitro
Single-agent temozolomide had little effect on the in vitro proliferation of NB-1691 or SK-N-AS cells (102 ± 13% of control, P = 0.904, and 87 ± 15% of control, P = 0.44, respectively). The IC50 of temozolomide for both these cell lines was >1 mmol/L. Exposure of the neuroblastoma cell lines to 50 IU/mL rhIFN-β for 48 h inhibited the proliferation of both NB-1691 cells (61 ± 9% of control,
with both cell lines, the combination of IFN-β and temozolomide further inhibited proliferation when compared with control (NB-1691, 36 ± 3% of control, \( P = 0.0008 \); SK-N-AS, 54 ± 7% of control, \( P = 0.003 \)) and 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 temozolomide in vitro.

When using a neuroblastoma cell line that does not express MGMT (CHLA-255), both temozolomide and IFN-β were able to significantly restrict the proliferation of the cells in vitro (Fig. 2C). The temozolomide IC\(_{50}\) ± SE for the CHLA-255 cell line was estimated to be 467.28 ± 25 µmol/L from five independent experiments and is significantly lower than the IC\(_{50}\) for either NB-1691 or SK-N-AS, which had an IC\(_{50}\) above the maximal achievable concentration in vitro (\( P = 0.000004 \)). After treatment with rhIFN-β, the cell count was 51 ± 4% of control (\( P = 0.019 \)), and after treatment with temozolomide, the cell count was 57 ± 4% of control (\( P = 0.03 \)). Treatment with the combination of rhIFN-β and temozolomide 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, \( P = 0.009 \); temozolomide, \( P = 0.003 \)).

These results were confirmed using a fluorescent-based automatic cell counter, which measures both cell viability and cell count. In the NB-1691 cell line, IFN alone, temozolomide alone, or the combination treatment had no significant effect on cell viability or increase in Annexin V-positive cells (data not shown). Thus, the decrease in cell count can be attributed to a decrease in cellular proliferation. Although low-dose IFN had no significant effect on cell cycle arrest, temozolomide alone caused a slight increase percent of cells in G\(_0\)-G\(_1\) cell cycle (70.32 ± 0.23% versus 67.8 ± 0.25% in the control, \( P = 0.002 \)) with a consequent drop in the percent of cells in S phase (20.7 ± 0.61% versus 25.5 ± 0.33% in the control, \( P = 0.002 \)). However, the combination therapy had a
much greater effect on cell cycle with a pronounced increase in percent of cells in G<sub>0</sub>-G<sub>1</sub> phase (74.5 ± 0.66% versus 67.8 ± 0.25% in the control, \( P = 0.0006 \)) and reduced number of cells in S phase (17.73 ± 0.72% versus 25.5 ± 0.33% in the control \( P = 0.0006 \)).

Effects of IFN-\( \beta \) and Temozolomide in vivo against Localized Neuroblastoma

AAV vectors encoding hIFN-\( \beta \) were used to achieve continuous, systemic delivery of hIFN-\( \beta \). 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-\( \beta \) from the liver (15). When s.c. NB-1691 tumors were treated with temozolomide or AAV2/8-hIFN-\( \beta \) alone, a significant effect on tumor growth was seen at day 17 (Fig. 3A) when compared with the untreated controls [percent initial tumor volumes: 2,516 ± 680% (control) versus 1,272 ± 330% (temozolomide), \( P = 0.01 \); 1,348 ± 220%, \( P = 0.03 \) (IFN-\( \beta \))]. Because temozolomide showed little direct tumor cell toxicity in vitro, its significant antitumor activity in vivo is a little surprising. The activity may be the result of an indirect effect on the tumor cells achieved by targeting surrounding, supportive host microenvironment.

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 [352 ± 110% versus 2,516 ± 680% (control), \( P = 0.0001 \)] and monotherapy with either AAV-hIFN-\( \beta \) or temozolomide (\( P = 0.05 \) and 0.02, respectively). Systemic levels of IFN-\( \beta \) at the time of sacrifice in mice receiving AAV-hIFN-\( \beta \) averaged 90.5 ng/mL (range, 54-127 ng/mL).

Evaluation by TUNEL staining of treated NB-1691 tumors revealed that the cohort receiving combination therapy had the greatest number of apoptotic cells (40.39 ± 4.41 per 1,000 cells). Combination therapy induced significantly more tumor cell apoptosis than control or temozolomide (6.91 ± 0.99 per 1,000 cells) and IFN-\( \beta \) (22.36 ± 4.9 per 1,000 cells) monotherapy (Fig. 4).

Similar effects of combination therapy with AAV-hIFN-\( \beta \) and temozolomide were seen with SK-N-AS xenografts, although neither agent when used as monotherapy had a significant effect on tumor growth (Fig. 3B). The combination therapy group had the lowest relative tumor volume overall (combo: 4.8 ± 0.8 versus control: 22.5 ± 10.3, \( P = 0.07 \), versus IFN-\( \beta \): 17.0 ± 3.6, \( P = 0.003 \), versus temozolomide: 19.3 ± 8.0, \( P = 0.07 \)).

In vivo MGMT Expression

Day 17 NB-1691 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 temozolomide-treated tumor samples produced bands ~25 kDa, representative of MGMT expression, whereas tumors treated with IFN-\( \beta \) either alone or in combination with temozolomide had either absent or significantly reduced MGMT expression.

Effects of IFN-\( \beta \) and Temozolomide in vivo against Disseminated Neuroblastoma

This tumor-sensitizing effect of IFN-\( \beta \) to temozolomide was also shown in a disseminated tumor model. All cohorts (untreated control, single-agent AAV-hIFN-\( \beta \), single-agent temozolomide, and combination of AAV-hIFN-\( \beta \) and temozolomide) were initially matched for disease burden based on the intensity of the bioluminescent signal (in photons/s). At day 17 after the initiation of therapy, there was a difference in bioluminescent signal for all groups compared with the control group [(1.32e10 ± 6.5e6) versus IFN-\( \beta \) (2.78 e3 ± 3.09 e3), \( P = 0.025 \), versus temozolomide (2.06e9 ± 1.55e9), \( P = 0.1 \), and versus combination (2.13 e2 ± 7.67 e2), \( P = 0.009 \), respectively].

Effects of IFN-\( \beta \) and Temozolomide in vivo against Disseminated Neuroblastoma

Figure 4. Tumor cell apoptosis. A, TUNEL-stained sections of NB-1691 xenografts (×400). a, untreated tumor; b, temozolomide-treated tumor; c, AAV-IFN-\( \beta \)-treated tumor; d, AAV-IFN-\( \beta \) + temozolomide-treated tumor. The TUNEL-positive cells are stained brown. B, quantitation of TUNEL-positive cells per 1,000 cells for each treatment group. \( P \) values relative to the untreated control group. \( P = 0.0702 \), AAV-IFN-\( \beta \) alone versus AAV-IFN-\( \beta \) temozolomide.

Figure 5. Western blot analysis of MGMT expression in s.c. NB-1691 tumor xenografts.
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 temozolomide alone uniformly had obvious gross disease. The untreated control cohort had an average tumor burden in the liver of 1.37 ± 0.71 g, whereas the cohort treated with temozolomide had an average tumor burden in the liver of 1.07 ± 0.81 g (\(P = 0.87\); Fig. 6B). Mice that received IFN-β alone had no gross evidence of disease in the liver and no increase in liver weight compared with naive 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 temozolomide 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). To further quantify the difference in tumor burden in the liver of IFN-β-treated mice compared with those treated with combination therapy, quantitative PCR for human MYCN was done on DNA extracted from the livers. Tumor burden in the livers of mice treated with combination therapy had lower levels of hMYCN (standardized to mHPRT) when compared with mice treated with IFN-β alone (0.93 ± 0.7 vs. 13.67 ± 5.0, \(P = 0.02\)).

Bone marrow from the mice with disseminated NB-1691luc was harvested at the time of sacrifice. Quantitative PCR was then done 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- to 3-fold log reduction was seen with both IFN-β alone and combination therapy when compared with 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 temozolomide. 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, whereas CHLA-255 cells have a low level of MGMT expression. The level of MGMT expression inversely

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**Figure 6.** Effect of temozolomide and IFN-β on disseminated neuroblastoma (NB-1691luc). A, bioluminescent signals on day 17 (day of sacrifice) are shown with representative images. *, \(P = 0.10\); **, \(P = 0.025\); ***, \(P = 0.009\), compared with control on day 17. \(P = 0.025\), IFN-β versus combo on day 17. B, tumor weight in livers of mice with disseminated disease. Tumor weight = weight of diseased liver - weight of liver in a non-tumor-bearing mouse. Quantitative PCR for MYCN expression in the livers of mice treated with IFN-β alone and combination therapy. C, H&E sections of livers from in vivo disseminated NB-1691luc model (×20). a, untreated control; b, temozolomide; c, AAV-IFN-β; d, AAV-IFN-β + temozolomide.
correlated with the cytotoxic effect of temozolomide therapy on each of the cell lines. Pretreatment with IFN-β down-regulated MGMT expression both in vitro and in vivo enhancing the effect of temozolomide against neuroblastoma cell lines with high MGMT expression.

Aside from the ability to down-regulate MGMT, IFN also has an independent antitumor 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 x 10^5 IU/d i.p. This established appreciable systemic levels of IFN-β (10.7–25.7 ng/mL), but 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 although 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 before temozolomide 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 temozolomide to be extremely effective. Not only did our treatment slow the progression of disseminated disease, but also tumor burden was nearly eradicated as shown 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 and liver samples both showed a dramatic reduction in disseminated tumor burden.

We have shown previously 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 used in this experiment, which was able to down-regulate MGMT, but the low dose did not affect delivery of temozolomide 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 temozolomide 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

Molecular Cancer Therapeutics

IFN-β sensitizes neuroblastoma to the antitumor activity of temozolomide by modulating O6-methylguanine DNA methyltransferase expression

Shannon F. Rosati, Regan F. Williams, Lindsey C. Nunnally, et al.


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