Targeting Methylguanine-DNA Methyltransferase in the Treatment of Neuroblastoma

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Abstract

Purpose: The combination of temozolomide and irinotecan has preclinical schedule-dependent synergy against neuroblastoma but is not curative for relapsed high-risk patients. We hypothesized that the DNA repair protein methylguanine-DNA methyltransferase (MGMT) is an important resistance factor, and that inactivation of MGMT would sensitize neuroblastoma cells to these agents.

Experimental Design: MGMT protein expression was assessed in 74 primary neuroblastoma tumors. Growth inhibition assays were done to determine the IC50 and the extent of synergy observed with various concentrations of temozolomide, irinotecan, and the MGMT-inactivating agent O6-benzylguanine, using cultured syngeneic neuroblastoma cells with either low or high levels of MGMT expression. We then assessed efficacy in a mouse xenograft model of metastatic neuroblastoma.

Results: MGMT was expressed by all 74 tumors evaluated. Pretreatment of neuroblastoma cells with O6-benzylguanine reduced the IC50 of temozolomide by 10-fold regardless of level of MGMT expression, and pretreatment with BG followed by temozolomide + irinotecan further reduced the IC50 in cells with high MGMT expression another 10-fold, to well below clinically achievable concentrations. The combination index was 0.27 to 0.30 for all three drugs in both cell lines, indicating strong synergy. Survival at 100 days for mice with metastatic neuroblastoma was 56% with three-drug treatment, compared with untreated controls (0%, P < 0.001) or temozolomide + irinotecan (10%, P = 0.081).

Conclusions: MGMT is widely expressed in primary neuroblastoma tumors, and is a relevant therapeutic target. Both in vitro and in vivo studies suggest inactivation of MGMT with O6-benzylguanine may increase the activity of temozolomide and irinotecan against neuroblastoma.

Neuroblastoma is the most common extracranial solid tumor of childhood, and accounts for 15% of all pediatric cancer deaths. Fewer than half of all children diagnosed with high-risk neuroblastoma will survive 5 years after diagnosis (1). The identification of new active agents or drug combinations is essential to improving the prognosis for these patients. One potentially promising regimen is the combination of the DNA methylating agent temozolomide with the topoisomerase I inhibitor irinotecan. Both drugs have single-agent activity against neuroblastoma (2, 3), and preclinical studies have shown that administration of TEM followed by IRN results in schedule-dependent synergy against mouse models of this disease (4). Based on encouraging preliminary reports of this combination in children with relapsed solid tumors (5–7), the Children’s Oncology Group is now conducting a national phase II trial of this drug pair to define its activity for neuroblastoma in first relapse. It is unlikely, however, that this two-drug combination will be curative for the majority of patients with high-risk disease at relapse due to drug resistance.

One recognized mechanism of TEM resistance is a high level of expression of methylguanine-DNA methyltransferase (MGMT), a DNA repair protein which removes methyl adducts placed on DNA by TEM, thus negating the cytotoxic effects of this drug (8). Sensitivity to TEM seems to be inversely related to levels of MGMT expression, which can be assessed directly at the protein level by immunohistochemistry analysis of tumor tissue (9, 10), or indirectly by evaluating methylation of the promoter that regulates MGMT expression because hypermethylation silences the expression of this gene (11). MGMT expression has been most thoroughly analyzed in brain tumors, and the reproducibility of these findings has led to suggestions for stratification of the treatment of malignant glioma patients based on their MGMT phenotype (12), given that patients whose tumors express relatively high levels of this protein would be expected to fail TEM therapy.
TEM has recently been evaluated as a single-agent for neuroblastoma (3, 13). Similar to the case with high-grade glioma, responses and prolonged stable disease are seen in a subset of patients. However, for most patients, benefits are either short-lived or absent, suggesting the presence of one or more resistance factors in a majority of tumors. To date, systematic investigation of MGMT protein expression in primary neuroblastoma tumors has not been reported. However, based on the experience from patients with brain tumors and preliminary results from neuroblastoma trials, we hypothesized that MGMT expression would be readily detectable in a majority of high-risk neuroblastomas.

A second known mechanism of resistance to TEM is decreased or absent expression of the mismatch repair proteins MSH-2 and MLH-1 (14, 15). In the presence of methylating agents such as TEM, it is thought that these repair enzymes engage in repeated futile attempts to repair DNA lesions, generating a chronic strand break condition that induces apoptosis. Thus, the presence of an intact mismatch repair system is thought to be necessary for TEM cytotoxicity. This mechanism can be evaluated directly by immunohistochemical analysis of the levels of MSH-2 and MLH-1 proteins, or indirectly by assessing microsatellite instability. Previous reports suggest that this mechanism of resistance is uncommon in neuroblastoma (15, 16), but the number of tumors previously examined is limited.

If widely expressed in neuroblastoma, MGMT would be a potentially relevant therapeutic target, given the availability of the MGMT-inactivating agent O\(^{\beta}\)-benzylguanine (BG). This drug has been shown in single-agent clinical studies to effectively inactivate MGMT activity in tumor tissue (17, 18). In preclinical experiments, pretreatment with BG significantly improved the activity of TEM against different tumors with a range of MGMT activity due to the depletion of MGMT protein by this “decoy alkylating agent” (reviewed in ref. 19). Phase II clinical trials in adults and children are now under way to evaluate the clinical activity of BG combined with TEM in patients with brain tumors.

Because of the schedule-dependent synergy seen with both BG → TEM and also TEM → IRN, Friedman et al. investigated the three-drug combination of BG → TEM → IRN in a murine model of malignant glioma which expressed MGMT (20). These investigators observed that the three-drug combination delayed tumor growth by a median of >150 days, compared with median delays of 37 days for TEM → IRN and <37 days for each drug as a single agent.

Based on the above data, tumors expected to be most sensitive to TEM would express little or no MGMT but retain MLH-1 and MSH-2. For tumors with high expression of MGMT coupled with a proficient mismatch repair system, pretreatment with BG should provide sensitization to TEM, and the addition of IRN to this regimen would be predicted to further increase the antitumor response. Therefore, we first evaluated MGMT, MSH-2, and MLH-1 expression in a tissue microarray containing primary neuroblastoma tumors to investigate whether MGMT would represent a rational therapeutic target. We also assessed the contribution of MGMT expression to the cytotoxicity of TEM with and without BG pretreatment using neuroblastoma cell lines, and evaluated the efficacy of the combination of BG + TEM + IRN in a preclinical model of metastatic neuroblastoma.

### Materials and Methods

**Primary neuroblastoma tumor tissue microarray.** A tumor tissue microarray comprised of pretreatment primary neuroblastoma tumor samples was obtained from the Children’s Oncology Group. This array contains three replicate 0.6 mm cores from each of 90 paraffin-embedded, distinct neuroblastoma tumor samples in addition to control tissues. Samples were obtained at presentation and represent the following tumor stages: 16 stage I, 16 stage II, 15 stage IVS, 15 stage III, and 28 stage IV.

**Immunohistochemical assessment of MGMT, MSH-2, and MLH-1.** Immunohistochemistry was done according to published methodology (9). Briefly, 5-μm-thick paraffin sections underwent prolonged deparaffinization in three changes of xylenes over a period of 4 h. Endogenous peroxidase was blocked in 1.85% of H\(_2\)O\(_2\) in methanol. Antibiot retrieval was accomplished by microwave heating (Panasonic NN-S666) at medium power for a total of 10 min in Biogenex AR-10 Buffer (Biogenex), followed by cooling at room temperature for 30 min. Blocking of nonspecific protein binding was accomplished with 5% normal goat serum for 15 min. After the removal of excess normal goat serum by blotting around the tissues, slides were incubated overnight at 40°C with the primary antibodies MLH-1 (clone 14, 10 μg/mL; Oncogene Research Products), MSH-2 (clone FE11, 1 μg/mL; Oncogene Research Products), and MGMT (clone MT3.1, 5 μg/mL). Primary antibody was detected in the tissues with Biogenex Super-Sensitive Multi-Link HRP detection system and 3,3′-diaminobenzidine (D-5637; Sigma Chemical Company) substrate and used according to the instructions of the manufacturer. Counterstaining was achieved with Harris modified hematoxylin (Fisher Scientific). Tonsil was used as the normal tissue control. Mouse immunoglobulin IgG\(_\lambda\) was substituted for the primary antibody at appropriate dilutions on serial sections of each case and run concurrently as the negative control. Immunoreactivity was quantified by counting staining tumor cell nuclei in at least 400 cells and determining the percentage of positive reactivity on a decile basis. Cytoplasmic-only reactivity and granular nuclear reactivity were considered negative.

Endothelial cells served as the internal positive controls. Samples were interpreted by a neuropathologist (R.E. McLeod), and the highest percentage score for each tumor was reported.

**Drugs.** The clinical formulation of IRN (CPT-11, Camptosar) was obtained from the St. Jude Hospital Pharmacy, and was diluted with saline immediately prior to use. IRN was administered i.v. TEM powder was obtained from the National Cancer Institute/NIH Developmental Therapeutics Program and was dissolved in DMSO as a stock solution of 100 mmol/L. The stock solution was diluted with water prior to use in vitro or in vivo. TEM was administered to mice by oral gavage. BG was obtained as a powder from Sigma Chemical Co. and suspended in water prior to use, and was administered i.p. to mice.

**Cell lines.** NB-1643 and NB-1691 cells were obtained from the Pediatric Oncology Group Cell Bank. SK-N-AS cells were purchased from American Type Culture Collection. Cell lines were propagated in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO\(_2\). To evaluate the effect of different levels of MGMT on the sensitivity of tumor cells to the proposed three-drug combination, we constructed isogenic neuroblastoma cell lines by transfecting NB-1643 cells with the control plasmid pRESneo (Promea) or with this plasmid containing the cDNA encoding human MGMT. The transfected population of cells was selected by exposure to G418. The use of this plasmid circumvented the need for isolating multiple clones. The MGMT cDNA was a gift from Dr. Thomas Brent (St. Jude Children’s Research Hospital), and was inserted into the EcoRI multiple cloning site following the internal ribosomal entry site sequence of pRESneo. Expression of MGMT by the transfected cells was detected by standard immunoblotting methods, using MT3.1 antibody (Chemicon International, Inc.).

**Growth inhibition assays.** These assays were done by standard methods using a Coulter counter to determine cell number and IC\(_{50}\)s were calculated using GraphPad Prism software, as reported previously.

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The degree of synergy, additivity, or antagonism of drug combination was determined using CalcuSyn software, with adherence to protocol design as detailed in the user’s manual for this software (Biosoft).

Mouse model of metastatic disease. This mouse model of disseminated neuroblastoma has been characterized extensively (22–25). In brief, 1 million NB-1691 cells were injected i.v. per tail vein into Es1e/SCID mice. This dose produces disseminated neuroblastoma tumors in multiple anatomic locations, necessitating euthanasia of 100% of mice by 60 to 85 days after injection due to tumor progression. The strain of mice, also characterized extensively (26), was used for this study because the plasma carboxylesterase activity of these mice is similar to that in human plasma. This aspect of the model is relevant to studies described herein because carboxylesterases activate the prodrug IRN. In this model, at the doses of IRN used, plasma levels of the active form of IRN (SN-38) are within the range of those tolerated by pediatric patients (2, 25).

Preclinical therapeutic protocol. Previous pharmacokinetic studies have shown that 66 mg/kg of TEM given to mice results in a drug exposure similar to the maximum tolerated dose of single-agent TEM in humans (200 mg/m²/d; ref. 27). The dose of TEM selected to be given in combination with BG for this study was 40% of 66 mg/kg (25 mg/kg). This dose was based on the clinical observation that the addition of BG to TEM results in significant myelosuppression and requires a similarly reduced dose of TEM (28), presumably because BG inactivates residual levels of MGMT expressed in some hematopoietic progenitor cells and further enhances toxicity (29). To give “full-dose” TEM to mice also receiving BG may therefore not be as relevant for clinical trials, even if tolerated by the mice.

As discussed above in the section describing the Es1e/SCID mouse model, IRN given at a dose of 15 mg/kg to these mice produces a systemic exposure of SN-38 similar to that seen in pediatric patients who receive 20 mg/m²/d on the [d × 5 (×2)] schedule, which is the maximum tolerated dose as a single-agent. Because clinical trials use half-dose protracted IRN when combined with TEM (5), we chose to use an IRN dose of 7.5 mg/kg for these experiments.

Assessment of toxicity and response. In vivo preclinical experiments were designed to assess the effect on survival afforded by adding BG to a clinically relevant regimen of TEM and IRN. Mice were followed until they were judged to be in discomfort by animal caretakers, when they were euthanized and the day of euthanasia recorded as the day of death. The method of Kaplan and Meier was used to estimate survival distributions. SEs were calculated using the method of Peto and Pike. Survival distributions were compared among treatment groups using the exact log-rank test. No adjustments were made for multiple comparisons in this exploratory study.

Although it is not possible to accurately predict toxicities that may become evident in phase I trials in murine models, the most likely potential toxicity was myelosuppression. Therefore, complete blood counts were done by the Pathology Core Facility of the St. Jude Animal Resource Center using standard methods. This analysis was done using blood samples collected on day 35 from all mice, just before starting the second course of treatment. Low blood counts on this day would indicate prolonged myelosuppression from the first course of treatment which might preclude the administration of a second course of therapy.

### Table 1. Immunohistochemical detection of MGMT protein expression in primary neuroblastoma specimens

<table>
<thead>
<tr>
<th>International Neuroblastoma Staging System stage</th>
<th>N</th>
<th>MGMT</th>
<th>MSH-2</th>
<th>MLH-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-19%</td>
<td>20-79%</td>
<td>≥80%</td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>II</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>III</td>
<td>13</td>
<td>0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>IV</td>
<td>24</td>
<td>0</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>IVS</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>0 (0%)</td>
<td>9 (12%)</td>
<td>65 (88%)</td>
</tr>
</tbody>
</table>

NOTE: The percentage of tumor cells expressing MGMT was identified by a neuropathologist having expertise with neuroblastoma, using a previously established assay (13). The values in the table indicate percentage of tumor cells expressing target protein.

![Fig. 1. Primary neuroblastoma cells with undifferentiated neuroblast morphology expressed MGMT in all samples analyzed. Although MGMT expression was less prevalent in occasional samples (top), expression in ≥80% of tumor cells (bottom) was seen in 88% of specimens in the tissue microarray obtained from the Children's Oncology Group. MGMT expression was also readily detectable in neuroblastoma cells having a more differentiated, ganglionic morphology (Table 1; data not shown).](image-url)
Results

MLH-1 and MSH-2 expression in primary neuroblastoma tumors. Relatively low levels of the mismatch repair proteins MSH-2 and MLH-1 have been associated with resistance to TEM in neuroblastoma cells (15). In contrast, tumors in which MSH-2 and MLH-1 are detectable in at least 60% of tumor cells by immunostaining have been reported to be more sensitive to TEM (9). Data in Table 1 show that both of these DNA repair proteins were readily detectable in 91% to 99% of the primary neuroblastoma cells analyzed, with ≥60% of the tumor cells in each sample expressing these proteins. Our results suggest that deficiencies of mismatch repair proteins are uncommon in neuroblastoma tumors, and therefore unlikely to account for the resistance to TEM.

MGMT expression in primary neuroblastoma tumors. In contrast to the mismatch repair proteins, high levels of MGMT have been shown to correlate with resistance to TEM in several different types of solid tumor cells, particularly malignant gliomas (9, 10). One established definition of high MGMT expression has been the detection of MGMT protein in at least 20% of tumor cells in a sample, which correlated inversely with response to TEM (9). Data in Table 1 show that MGMT expression was detected to this degree in all 74 interpretable primary neuroblastoma samples analyzed, regardless of disease stage. In fact, 88% of evaluable samples expressed MGMT protein by immunohistochemistry in a minimum of 80% of tumor cells (Fig. 1). Duplicate or triplicate samples were evaluable for 82% of cases, and in 67% of these cases, there was no variation at all in the reported percentage of expression. There was a variation of >40% in only 6 (8%) tumors, which could possibly be related to the heterogeneous expression of MGMT within a single tumor. There was no correlation between stage of disease and level of MGMT expression (Table 1). We conclude that, similar to published results for neuroblastoma cell lines (15), a majority of primary neuroblastoma cells express the mismatch repair proteins MLH-1, MSH-2, as well as MGMT. These results suggest that expression of MGMT may be the more common mechanism of resistance to TEM in neuroblastoma cells.

Effect of BG on in vitro activity of TEM and IRN. We then evaluated a panel of neuroblastoma cell lines to identify a model system with which to assess the ability of BG to sensitize cells to TEM, ostensibly by inactivating MGMT. The immunoblots in Fig. 2A show that all four cell lines evaluated express similar levels of MLH-1 and MSH-2, with NB-1643 cells expressing the lowest level of MGMT. Therefore, we transfected NB-1643 cells to express elevated levels of MGMT in order to compare the effect of BG on TEM sensitivity in neuroblastoma cells that express contrasting levels of this DNA repair protein. The immunoblot in Fig. 2B shows that the NB-1643pIRES.MGMT transfectants expressed ~5-fold more MGMT than NB-1643 cells transfected with control plasmid (pIRESneo).

We next assessed the effect of BG on the sensitivity of this pair of neuroblastoma cell transfectants to TEM. BG at 25 μmol/L has been previously shown to inactivate MGMT activity in cell lines (30), and this concentration was added to cell cultures in exponential growth 24 h prior to the addition of the concentrations of TEM indicated in Fig. 3B. The data show that BG sensitized both NB-1643pIRESneo cells and NB-1643pIRES.MGMT cells to TEM by 11- to 12-fold (Table 2). We concluded that the effect of BG was similar regardless of the level of expression of MGMT.

In contrast to the effects seen with TEM, IC_{50} concentrations of IRN were similar in both cell lines (1.8 μmol/L for NB1643pIRESneo cells and 2.2 μmol/L for NB-1643pIRES.MGMT cells). Furthermore, the addition of BG to IRN did not provide additional benefit, as the IC_{50} concentrations remained essentially unchanged (1.3 and 1.8 μmol/L, respectively).

Because the overall goal of this work was to evaluate the efficacy of the three-drug combination BG → TEM → IRN in neuroblastoma cells, we next exposed the two NB-1643 transfectants to the three-drug combination to assess whether adding BG and/or IRN to TEM would produce additive, greater than additive, or less than additive cytotoxicity. Based on the schedules of administration previously reported to produce synergistic (greater than additive) cytotoxicity, the schedule of drug exposure was 25 μmol/L BG × 24 h → TEM × 1 h → IRN × 4 h, without changing the tissue culture medium until IRN had been present for 4 h. For all in vitro experiments, the time of exposure to BG was a total of 29 h; the time of exposure to TEM was 5 h and the time of exposure to IRN was 4 h. As single agents, BG was nontoxic and IRN was equitoxic for NB-1643pIRESneo and NB-1643pIRES.MGMT cells (data not presented).
shown). BG did not affect the sensitivity of the cells to IRN, but did reduce the IC50 of TEM, as noted above, in both cell lines by a similar amount (~ 10-fold). The addition of IRN to the BG → TEM two-drug combination further reduced the IC50 of TEM such that an incremental decrease was seen for the IC50 of TEM when BG was added prior to TEM as well as when IRN was added after TEM. For NB-1643pIRESneo cells, the IC50 of TEM decreased from 42 μmol/L as a single agent to 3.4 μmol/L in combination with BG, to 1.3 μmol/L in the three-drug combination, for an overall increase in sensitivity to TEM of ~ 30-fold. The overall increase in sensitivity to TEM, when used as a component of the three-drug combination, was in the same range for cells expressing low versus high levels of MGMT (30- to 80-fold).

Importantly, we observed a stepwise augmentation in the anticancer effect of the TEM with the addition of each drug. Also, critical to the rationale on which this proposal is based, the combination index of the three drugs for each transfectant was 0.27 to 0.30, indicative of strong synergy for the combination in cells expressing either high or low levels of MGMT.

### Table 2. IC50 values in micromolar for TEM, alone and in combination with BG and/or IRN

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 of TEM concentration (μmol/L)</th>
<th>Combination index*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single agent</td>
<td>with IRN</td>
</tr>
<tr>
<td>NB-1643pIRESneo</td>
<td>41</td>
<td>9.1 †</td>
</tr>
<tr>
<td>NB-1643pIRES.MGMT</td>
<td>602</td>
<td>59.0 †</td>
</tr>
</tbody>
</table>

**NOTE:** Combination indices for three-drug combination. Combination index: 0.1, very strong synergy; 0.1 to 0.3, strong synergy; 0.3 to 0.7, synergy; 0.7 to 0.85, moderate synergy; 0.9 to 1.45, ranges from slight synergy to nearly additive to moderate antagonism. † Differs significantly from IC50 of TEM as a single agent. IC50s were determined in duplicate experiments with three replicate wells at each drug concentration in each experiment, using a fixed ratio of TEM/IRN of 10:1. Significance was evaluated using a two-tailed t test and a minimum P < 0.05.
controls, 47d; TEM

Survival distributions for the 29 mice assigned to one of three treatment groups are shown in Fig. 4. The median survival of each of the three groups was: untreated controls, 47 d; TEM → IRN, 72 d (P = 0.047 versus control); BG → TEM → IRN, 111 d (P < 0.001 versus control; P = 0.081 versus TEM → IRN).

Activity in the mouse model. These in vitro data predicted that BG + TEM + IRN should be more effective than single or two-drug combinations of these drugs, regardless of the level of MGMT expression. We therefore selected the neuroblastoma cell line that we had determined to have one of the highest levels of expression of MGMT (NB-1691 cells), which would be predicted to be relatively resistant to TEM. Key features of the experimental design for this proposal included: (a) i.v. injection of NB-1691 cells which has been shown to produce bone and bone marrow metastases, (b) drug sequencing that capitalizes on the mechanism of action of these agents, and (c) the use of dosages expected to be relevant for clinical trials.

The drugs were given in sequence with BG followed by TEM followed by IRN. BG was administered i.p. at 40 mg/kg, the highest nontoxic dose. One hour later, 25 mg/kg of TEM was given by oral gavage, followed 1 h later by 7.5 mg/kg of IRN as a tail vein injection. A treatment course consisted of BG on days 1 to 5, TEM on days 1 to 5, and IRN given on days 1 to 5 and 8 to 12. The first course of treatment started 14 days after i.v. injection of 1 million NB-1691 cells. A second course of treatment started 21 days after the first course (i.e., 35 days after tumor cell infusion). Each group comprised 8 to 10 animals in the following groups: no treatment, TEM + IRN, and BG + TEM + IRN. Animals were monitored daily for signs of illness or discomfort. Any mouse whose tumor caused sufficient discomfort (as judged by animal care personnel not associated with this protocol) was euthanized.

Survival distributions for the 29 mice assigned to one of three treatment groups are shown in Fig. 4. The median survival of each group was: control, 47 days; TEM + IRN, 72 days; BG + TEM + IRN, 111 days. Significant differences in survival distributions were observed between the control group and each of the treatment groups (P = 0.047 for TEM + IRN; P < 0.001 for BG + TEM + IRN). All untreated mice were dead of disease within 62 days of i.v. injection of tumor cells. Survival estimates were highest for the nine mice treated with all three drugs, with 100-day survival estimates for BG + TEM + IRN being 56 ± 12% compared with 10 ± 7% for TEM + IRN (P = 0.081; exact log-rank test). The data shows an apparent advantage in efficacy at day 100 when BG was included in the treatment regimen, but as seen with current therapies for neuroblastoma, late relapses negated any differences at day 300.

Discussion

This study is the first to systematically assess MGMT protein expression in a large and varied assortment of primary neuroblastoma tumors. As expected by the limited clinical success of TEM as a single agent in this disease, MGMT expression was seen to some degree in all tumors tested, being readily detectable in at least 20% of tumor cells in all 74 primary tumor samples. We chose immunohistochemistry to assess MGMT phenotype because protein expression correlated with MGMT biochemical activity (31), and with response to alkylator-based chemotherapy for other pediatric tumors (32). Our findings are consistent with a previous report by Gonzalez-Gomez showing that MGMT promoter hypermethylation, which is usually associated with absent or low MGMT protein expression, occurred in only 12 (27%) of 44 neuroblastic tumors (33). Similarly, our finding of frequent expression of common mismatch repair proteins in neuroblastoma is in keeping with other reports suggesting that deficiency in this system is an unlikely cause of resistance for this tumor (15, 16). Taken together, these findings support the hypothesis that MGMT expression may be a key factor determining the resistance of neuroblastoma to TEM.

The importance of MGMT as a therapeutic target is increased by the clinical availability of an agent such as BG which can effectively inactivate this resistance mechanism. As predicted by experiments with other tumor types, the in vitro activity of TEM was increased by the addition of BG in a neuroblastoma cell line with either low endogenous MGMT expression or forced high-level expression. We chose this study design to minimize other known and unknown factors that could affect the response to TEM. In contrast to its effect on TEM, pretreatment with BG did not substantially affect the response to IRN. However, we did observe the expected synergy when TEM was followed by IRN (4). This synergy could be further enhanced to produce a combination index of 0.27 by pretreatment with BG in cell lines with both high and low MGMT expression. Of particular note is that chemotherapy concentrations required to observe this synergy are within levels achievable in patients (2, 27).

To evaluate the in vivo effects of treatment, we used a mouse model of disseminated neuroblastoma instead of the traditional hind-flank xenograft model. A major advantage of this model is the reliable development of lethal metastatic tumors in clinically relevant organs including adrenal glands, lymph nodes, liver, bone, and bone marrow. The NB-1961 cells used in this model are included in the national Pediatric Preclinical Testing Program implemented by the National Cancer Institute for evaluation of new agents (34), and have biological features of refractory/resistant disease including unfavorable histology and MYCN gene amplification. In addition, NB-1961 cells express MGMT, as well as functional mismatch repair proteins MLH-1 and MSH-2. The use of this cell line and animal model of disseminated disease therefore closely recapitulates several hallmarks biological and clinical features of refractory neuroblastoma, and so might possibly be more predictive of the response of high-risk patients.

The in vivo studies were designed to assess the benefit in overall survival afforded by adding BG to the combination of TEM and
IRN in mice bearing otherwise lethal burdens of metastatic tumor. Using this model in a limited number of mice, a strong trend for improved survival with the addition of BG was observed, in that 56% (±12%) of mice receiving triple drug therapy survived 100 days after the infusion of tumor cells, compared with 10% (±7%) of mice receiving TEM and IRN without BG. The toxicity of the three-drug regimen at the doses used was negligible, with no treatment-related deaths, substantial weight loss, or significant myelosuppression observed.

Because so little toxicity was encountered, it is possible that higher and potentially more effective dosages of chemotherapy could have been administered, and this hypothesis will be explored in future experiments. However, we note that in a previous clinical trial, the addition of BG to TEM-containing regimens exacerbated the myelosuppression of TEM (28), likely due to the inhibition of MGMT in hematopoietic cells (29). This myelosuppression necessitated a reduction in the daily TEM dosage from 200 to 75 mg/m². We reflected this required dose reduction by making similar reductions in our xenograft experiments, with additional reductions also made in IRN dosing based on data from a pediatric clinical trial of TEM + IRN (5). However, our attempts to administer drug dosages that are relevant for human trials may have resulted in undertreatment. Ultimately, the tolerated dosages for humans in this complex regimen will have to be determined in a clinical trial. Nevertheless, our results are consistent in principle with previous experiments using a mouse model of glioblastoma multiforme in which BG pretreatment given before noncurative doses of TEM and IRN were administered enhanced antitumor responses (20).

It is notable that although five of nine mice in the three-drug treatment group were alive ~2 months after finishing therapy, only one mouse survived to day 300. In fact, two mice died from tumor very late (at 290 and 293 days), almost 250 days after completing the planned treatment. These results reflect the clinical experience with high-risk neuroblastoma patients, nearly half of whom relapse from the minimal residual disease setting. It is unknown whether higher drug dosages or additional courses of therapy may have been curative for these mice, or whether prolonging BG infusion time to eradicate low levels of residual MGMT may be helpful (35), although the BG dosage of 40 mg/kg is similar to dosages documented to completely inactivate MGMT in mouse xenograft tumor tissue (36). Alternatively, other mechanisms of resistance may also be affecting response to therapy, such as differences in levels of expression of apurinic/apyrimidinic endonucleases (37). Further evaluation of potential mechanisms of antitumor efficacy of this regimen in other mouse models is ongoing to better define the benefits of this approach.

In conclusion, we provide evidence that MGMT is commonly expressed in neuroblastoma and represents an attractive therapeutic target because modulation with BG may improve the in vitro and in vivo antitumor activity of combined TEM and IRN. This strategy would represent a logical extension of existing treatments, and plans are in place for a clinical trial in relapsed neuroblastoma provided that testing in additional neuroblastoma models confirms these preliminary results.

Acknowledgments

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References


Targeting Methylguanine-DNA Methyltransferase in the Treatment of Neuroblastoma


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