O6-Benzylguanine Potentiates the Antitumor Effect of Locally Delivered Carmustine against an Intracranial Rat Glioma

Laurence D. Rhines, Prakash Sampath, M. Eileen Dolan, Betty M. Tyler, Henry Brem, and Jon Weingart

Department of Neurological Surgery, Johns Hopkins School of Medicine, Baltimore, Maryland 21205 [L. D. R., P. S., B. M. T., H. B., J. W.], and Section of Hematology-Oncology, Department of Medicine, University of Chicago, Chicago, Illinois 60637 [M. E. D.]

Abstract

Local delivery of carmustine (BCNU) via biodegradable polymers prolongs survival against experimental brain tumors and in human clinical trials. O6-Benzylguanine (O6-BG), a potent inhibitor of the DNA repair protein, O6-alkylguanine-DNA alkyltransferase (AGT), has been shown to reduce nitrosourea resistance and, thus, enhance the efficacy of systemic BCNU therapy in a variety of tumor models. In this report, we demonstrate that O6-BG can potentiate the activity of BCNU delivered intracranially via polymers in rats challenged with a lethal brain tumor. Fischer 344 rats received a lethal intracranial challenge of 100,000 F98 glioma cells (F98 cells have significant AGT activity, 328 fmol/mg protein). Five days later, animals receiving an i.p. injection of O6-BG (50 mg/kg) 2 h prior to BCNU polymer (3.8% BCNU by weight) implantation had significantly improved survival (n = 7; median survival, 34 days) over animals receiving either O6-BG alone (n = 7; median survival, 22 days; P = 0.0002) or BCNU polymer alone (n = 8; median survival, 25 days; P = 0.0001). Median survival for the control group (n = 8) was 23.5 days. Moreover, there was no physical, behavioral, or pathological evidence of treatment-related toxicity. These findings suggest that O6-BG can potentiate the effects of interstitially delivered BCNU and, for tumors expressing significant AGT, may be necessary for the BCNU to provide a meaningful therapeutic benefit. Given the clinical use of BCNU polymers against malignant gliomas, concurrent treatment with O6-BG may provide an important addition to our therapeutic armamentarium.

Introduction

Despite advances in neuroimaging, surgical technique, radiation therapy, and chemotherapy, primary malignant brain tumors remain a difficult therapeutic challenge. Historically, systemic BCNU has been the most commonly used and most effective chemotherapeutic agent for brain tumors; however, it has provided only modest improvements in patient survival, and its efficacy has been limited by side effects including myelosuppression, hepatic toxicity, and pulmonary fibrosis (1). In an effort to improve the effectiveness of nitrosoureas against malignant gliomas, BCNU has been incorporated into biodegradable polymer wafers that can be placed directly into the brain at the site of a tumor. These polymers are able to release BCNU in a sustained fashion, generating high local drug concentrations at the tumor site while minimizing the problems of systemic toxicity. Use of the BCNU polymers has been both safe and effective in prolonging survival in experimental brain tumor models (2, 3), as well as in human clinical trials (4–6).

One limitation of this therapy is that many brain tumors are resistant to BCNU and other alkylating agents. This resistance may be attributable, in part, to AGT, a DNA-repair protein found in a majority of human brain tumors (7, 8). BCNU exerts its toxic effects via chloroethylation of DNA at the O6 position of guanine. AGT is able to protect tumor cells from this damage by removing DNA adducts at this position before cytotoxic interstrand cross-linking can occur (9). AGT can be irreversibly inactivated by reaction with the substrate analogue O6-BG, which transfers a benzyl group to a cysteine residue at the active site of the repair protein (10, 11). This O6-BG-mediated AGT inhibition has been shown to enhance sensitivity to BCNU and, thus, improve its efficacy in both in vitro and in vivo tumor models (12–16).

Although systemic administration of O6-BG alone is safe in animals and humans, it can cause a significant increase in the toxicity of BCNU when given as a pretreatment prior to systemic BCNU therapy. Preclinical toxicology studies in animals have shown O6-BG alone to be nontoxic. However, when combined with BCNU, bone marrow toxicity is dose-limiting, and the maximal tolerated dose of BCNU is 2- to 3-fold lower in mice and 6-fold lower in dogs than in the absence of O6-BG (16, 17). Similarly, in humans, O6-BG has proven to be nontoxic; however, the dose of systemic BCNU has to be reduced when administered after O6-BG to avoid unwanted toxicity (18, 19).

In an attempt to take advantage of the potentiating effects of O6-BG on BCNU therapy and to avoid systemic BCNU toxicity, the present study investigates whether pretreatment with systemic O6-BG can be used successfully in combination with BCNU delivered locally via biodegradable polymers against an intracranial rat glioma. We hypothesized that O6-BG-mediated AGT suppression would increase the efficacy of the interstitial BCNU, and that systemic complications would be avoided because so little of the locally delivered BCNU leaves the brain. Given the clinical use of BCNU-impregnated polymers in the treatment of patients with malignant gliomas, such a finding may have important therapeutic implications.

Materials and Methods

Animals. Male Fischer 344 rats weighing 200–250 g were obtained from Harlan Sprague Dawley (Indianapolis, IN). They were housed in standard facilities and given free access to water and rodent chow. All of the rats were treated in accordance with the policies and principles of laboratory animal care of the Johns Hopkins University School of Medicine Animal Care and Use Committee.

Tumor Lines. The F98 glioma was obtained from Dr. R. Barth (Ohio State University, Columbus, OH). Tumor cells were maintained in RPMI culture medium (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum in humidified incubators. The 9L gliosarcoma was obtained from Dr. M. Barker at the University of California-San Francisco Brain Tumor Research Center (San Francisco, CA). The C6 glioma, the U87 glioma, and the...
Daoy medulloblastoma were obtained from the American Type Culture Collection (Manassas, VA). The U251 glioma was obtained from Duke University (Durham, NC). The AGT activities of these tumor lines were assayed by the technique described below (Table 1).

### Table 1: AGT activity of human and rat brain tumor cell lines

<table>
<thead>
<tr>
<th>Species</th>
<th>Tumor type</th>
<th>AGT activity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Daoy</td>
<td>333</td>
</tr>
<tr>
<td></td>
<td>U251</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>U87</td>
<td>ND</td>
</tr>
<tr>
<td>Rat</td>
<td>9L</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C6</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>F98</td>
<td>328</td>
</tr>
</tbody>
</table>

*ND, none detected using the 3H-methylated DNA substrate for AGT activity.

#### Chemicals and Drugs.
BCNU (Bristol-Myers-Squibb, Princeton, NJ) was purchased commercially and stored at 4°C prior to polymer preparation (see below). O6-BG was obtained from the National Cancer Institute (Frederick, MD) and stored at room temperature. For i.p. injection, O6-BG was dissolved in a vehicle of 33.3% PEG in PBS and administered at a dose of 50 mg/kg.

### BCNU Polymer Preparation.
Briefly, BCNU/polymer mixtures of 0 and 3.8% BCNU by weight were prepared as described previously (20). The poly[1,3-bis(carboxyphenoxy) propane: sebacic acid polyanhydride polymer with a 20:80 molar ratio was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD). Polymer discs containing 0% and 3.8% BCNU by weight were prepared as described previously (20).

#### Assay for AGT Activity.
Extracts were prepared from cells or tumor by homogenization in 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM DTT. AGT activity was determined as described previously (10). Briefly, cell extracts were incubated with 3H-methylated DNA substrate (5.8 Ci/mmol) for 30 min at 37°C. The DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 N and hydrolyzed in 0.1 N hydrochloric acid at 70°C for 30 min. The modified bases were eluted on a C18 reverse phase column with 10% methanol/0.05 M ammonium formate. Protein concentration was determined by the method of Bradford (21). The results were expressed as fmol of O6-methylguanine released from DNA per mg of protein.

### Intracranial Tumor Implantation.
Eighty-five rats were anesthetized with an i.p. injection of 2–4 ml/kg of a stock solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethanol in normal saline. The heads were shaved and disinfected with 70% ethanol and povidone-iodine solution. After a midline scalp incision, the galea overlying the left cranium was swept laterally. A 3-mm burr hole was made over the left parietal region with its center 2–3 mm posterior to the coronal suture and 3–4 mm lateral to the sagittal suture. The animals were then placed in a stereotactic frame, and 100,000 F98 glioma cells were injected over 3 min via a 26-gauge needle inserted to a depth of 4 mm at the center of the burr hole. After tumor cell inoculation, the needle was removed, the site was irrigated with normal saline, and the incision was closed with surgical staples.

### Treatment of Intracranial Tumors.
Two separate treatment experiments were conducted. The first examined the relative efficacy of intracranially implanted BCNU polymers in conjunction with three different i.p. O6-BG dosing regimens. The second experiment confirmed the results of the most effective treatment paradigm used in the initial experiment.

#### Table 2: Efficacy of intracranial BCNU polymers and systemically administered O6-BG against intracranial F98 glioma

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Median survival (days)</th>
<th>Range (days)</th>
<th>P* (vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug vehicle QOD × 3, 24 h prior to empty polymer (n = 10)</td>
<td>28.5</td>
<td>22–32</td>
<td>0.810</td>
</tr>
<tr>
<td>Drug vehicle QOD × 3, 24 h prior to BCNU polymer (n = 8)</td>
<td>26.0</td>
<td>22–32</td>
<td>0.959</td>
</tr>
<tr>
<td>O6-BG QOD × 3, 24 h prior to empty polymer (n = 9)</td>
<td>28.0</td>
<td>21–32</td>
<td>0.810</td>
</tr>
<tr>
<td>O6-BG QOD × 3, 24 h prior to BCNU polymer (n = 9)</td>
<td>32.0</td>
<td>27–38</td>
<td>0.004</td>
</tr>
<tr>
<td>O6-BG × 1, 24 h prior to BCNU polymer (n = 10)</td>
<td>30.5</td>
<td>24–38</td>
<td>0.119</td>
</tr>
<tr>
<td>O6-BG × 1, 2 h prior to BCNU polymer (n = 9)</td>
<td>42.0</td>
<td>32–53</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Using log-rank (Mantel-Cox) test in Kaplan-Meier nonparametric analysis of survival.

#### Results.
Several rat and human brain tumors were evaluated for AGT activity to determine an optimal model for the study of O6-BG-mediated AGT modulation in conjunction with interstitial BCNU therapy (Table 1). We found that the 9L rat gliosarcoma and the U251 and U87 human gliomas had undetectable AGT activity, which made them unsuitable models in which to examine this issue. The F98 rat glioma, on the other hand, had moderate-to-high alkyltransferase activity (328 fmol/mg protein). Thus, we would expect this tumor to be relatively resistant to local BCNU therapy. Moreover, when stereotactically injected into the left parietal lobes of Fischer 344 rats, F98 glioma cells formed intracranial tumors that were uniformly fatal in untreated control animals in 22–32 days. The F98 rat glioma was, therefore, selected as our experimental brain tumor model.

In the initial experiment, animals treated with an i.p. injection of 50 mg/kg O6-BG 2 h prior to polymer placement (× 1, 24 h prior to polymer)
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mg/kg O6-BG 2 h prior to intracranial implantation of a 3.8% BCNU polymer (median survival, 42 days) had a significant improvement in survival not only compared with control animals receiving drug vehicle and empty polymer (median survival, 28.5 days; P < 0.0001) but also compared with animals receiving drug vehicle and BCNU polymer (median survival, 26.0 days; P < 0.0001). The resistance of the F98 glioma to local BCNU alone is noteworthy. This combination of O6-BG 2 h prior to BCNU polymer placement also statistically outperformed the other O6-BG/BCNU regimens [P = 0.0026 versus O6-BG every other day for three doses beginning 24 h prior to BCNU polymer placement (median survival, 32 days); P = 0.0004 versus O6-BG single dose 24 h prior to BCNU polymer placement (median survival, 30.5 days); Table 2].

Given these findings, a second experiment, focused on this optimal treatment group, was undertaken to reproduce these results. In this experiment, animals that received an i.p. injection of 50 mg/kg O6-BG 2 h prior to 3.8% BCNU polymer implantation had significantly improved survival over control animals receiving drug vehicle and empty polymer, animals receiving O6-BG following by empty polymer, or those receiving drug vehicle and BCNU polymer (Fig. 1). Control animals had a median survival of 23.5 days (n = 8). Animals receiving O6-BG 2 h prior to empty polymer had a median survival of 22 days (n = 7; P = 0.4726 versus control). Animals receiving drug vehicle followed by BCNU polymer had a median survival of 25 days (n = 8; P = 0.0352 versus control). Animals receiving the combination of O6-BG pretreatment and BCNU polymer implantation had a median survival of 34 days (n = 7; P = 0.0002 versus control; P = 0.0002 versus O6-BG/empty polymer; P = 0.0001 versus drug vehicle/BCNU polymer).

Rats treated with 50 mg/kg i.p. O6-BG 2 h prior to intracranial implantation of a 3.8% BCNU polymer wafer showed no signs of toxicity. Complete necropsies with organ histopathology similarly showed no evidence of systemic toxicity, and the brains showed only the mild reactive gliosis that is typical after polymer placement (2, 20).

Discussion

Our results demonstrate that pretreatment with systemic O6-BG prior to intracranial implantation of BCNU polymer is a safe and effective means of increasing the sensitivity of brain tumors to alkylating agents, thereby enhancing the efficacy of the locally delivered BCNU. The intracranial F98 glioma, which is largely resistant to the action of BCNU alone, is made sensitive by O6-BG, and animal survival is improved significantly.

The success of this treatment strategy depends on several important factors. First, the systemically delivered O6-BG must be able to inhibit AGT in the brain at the tumor site where the locally delivered BCNU is present in high concentration. Previous studies have shown that after systemic O6-BG administration, both O6-BG and, to an equal extent, its active metabolite O6-benzyl-8-oxoguanine, cross the blood-brain barrier and penetrate the cerebrospinal fluid in Rhesus monkeys (21). Moreover, after i.p. injection of radiolabeled O6-BG, brain tissue extracts were shown to have radioactivity (23). In experiments examining the combination of systemic O6-BG with systemic BCNU, animals bearing intracranial brain tumor xenografts had significantly improved response to the nitrosourea when first pretreated with the O6-BG. This suggests that the O6-BG (or some active metabolite) crosses the blood-brain barrier and inhibits tumor AGT (16). Finally, more recent studies in humans have shown that when patients were treated preoperatively with i.v. O6-BG, resected brain tumors had markedly depleted AGT activity (18). Our results using local BCNU therapy corroborate these findings that intracranial AGT is inhibited after systemic O6-BG administration.

A second important factor appears to be the timing of the O6-BG pretreatment. BCNU causes DNA damage by chloroethylation at the O6 position of guanine. This process occurs rapidly and leads to the subsequent formation of cytotoxic DNA interstrand cross-links. AGT protects cells by removing DNA adducts at this position before these cross-links can form. The reaction is stoichiometric and results in transfer of the adduct to a cysteine residue within the protein, leaving the normal guanine within the substrate DNA. While the AGT performs this transfer rapidly and with high affinity, the protein is permanently inactivated once bound at its acceptor site, and de novo synthesis is required to replenish the AGT supply (9, 24). O6-BG acts as a substrate analogue for the alkyltransferase. Its benzyl group binds the AGT at its active site causing irreversible inactivation (11). Because of the high efficiency with which AGT corrects BCNU-induced DNA damage, depletion of this repair protein must be virtually complete in order for cytotoxic interstrand cross-link formation to proceed (14). Thus, the optimal timing for O6-BG pretreatment must satisfy two criteria. It must effectively deplete AGT prior to BCNU administration and maintain these low AGT levels until a sufficient number of DNA cross-links have formed to result in cell death. On the other hand, it must not be given so far in advance of the BCNU that protective levels of AGT are restored by de novo protein synthesis.

In our experiments, treatment with O6-BG 2 h prior to implantation of BCNU polymer appeared to satisfy these criteria and markedly enhanced the efficacy of the nitrosourea. This time course for AGT inhibition is consistent with that observed by other authors (15, 16). Moreover, after i.p. injection of radiolabeled O6-BG, brain tissue extracts were shown to have radioactivity (23). In experiments examining the combination of systemic O6-BG with systemic BCNU, animals bearing intracranial brain tumor xenografts had significantly improved response to the nitrosourea when first pretreated with the O6-BG. This suggests that the O6-BG (or some active metabolite) crosses the blood-brain barrier and inhibits tumor AGT (16). Finally, more recent studies in humans have shown that when patients were treated preoperatively with i.v. O6-BG, resected brain tumors had markedly depleted AGT activity (18). Our results using local BCNU therapy corroborate these findings that intracranial AGT is inhibited after systemic O6-BG administration.

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The third factor on which the success of this treatment depends is its safety. In addition to increasing the therapeutic efficacy of systemic BCNU therapy, O6-BG pretreatment also increases its toxicity, leading to a higher incidence of BCNU-induced complications, particularly bone marrow suppression. Presumably, this is attributable to the depletion of AGT in sensitive normal tissues. In fact, in experiments using systemically delivered O6-BG and BCNU to treat intracranial and s.c. brain tumor xenografts, the maximal tolerated dose of BCNU alone had to be reduced by 50% or more when combined with O6-BG.
(15, 16). The ability to deliver BCNU via biodegradable polymers directly into the brain at the site of tumor has been a significant therapeutic advance (5). Not only are tumor cells exposed to markedly higher drug levels, but issues of systemic toxicity are minimized. In our experiments, pretreatment with systemic O\textsubscript{6}-BG prior to intracranial implantation of a BCNU polymer did not result in any new toxicity. This was expected given that this mode of delivery results in very low levels of extracranial BCNU exposure (3).

Intracranially implanted BCNU polymers are currently in use for patients with malignant gliomas, however, their efficacy may be compromised by the significant levels of AGT activity found in most human brain tumors. The findings reported here suggest that treatment with O\textsubscript{6}-BG prior to surgical resection and polymer placement may help overcome tumor resistance to the nitrosourea and make the local BCNU therapy more effective. Clinical trials assessing the safety and optimal dosing of O\textsubscript{6}-BG are in progress, and trials to study the combination of systemic O\textsubscript{6}-BG and BCNU polymers are being initiated.

Acknowledgments

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References

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