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

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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 C6 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 tumoricidal 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. Barsh (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).

**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, Maryland) and stored at room temperature. For i.p. injection, O6-BG was dissolved into 10-mg discs (3.0-mm diameter, 1.0-mm height) using a stainless steel frame, and 100,000 F98 glioma cells were injected over 3 min via a 26-gauge needle lateral to the sagittal suture. The animals were then placed in a stereotactic frame, and the animals were returned to their cages.

**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 (20). 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 20:80 molar ratio was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD). Polymer discs containing 0% and 3.8% BCNU were compression-molded into 10-mg discs (3.0-mm diameter, 1.0-mm height) using a stainless steel mold.

**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.

**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 6-BG i.p. 2 h prior to 3.8% BCNU polymer implantation (Fig. 1). The modified bases were eluted on a C18 reverse phase column with 20:80 molar ratio was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD). Polymer discs containing 0% and 3.8% BCNU were compression-molded into 10-mg discs (3.0-mm diameter, 1.0-mm height) using a stainless steel mold.

**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.

**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>Drag vehicle QOD × 3, 24 h prior to empty polymer (n = 10)</td>
<td>28.5</td>
<td>22–32</td>
<td></td>
</tr>
<tr>
<td>Drag 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.
Our results demonstrate that pretreatment with systemic O\textsubscript{6}-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 O\textsubscript{6}-BG, and animal survival is improved significantly.

The success of this treatment strategy depends on several important factors. First, the systemically delivered O\textsubscript{6}-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 O\textsubscript{6}-BG administration, both O\textsubscript{6}-BG and, to an equal extent, its active metabolite O\textsubscript{6}-benzyl-8-oxoguanine, cross the blood-brain barrier and penetrate the cerebrospinal fluid in Rhesus monkeys (21). Moreover, after i.p. injection of radiolabeled O\textsubscript{6}-BG, brain tissue extracts were shown to have radioactivity (23). In experiments examining the combination of systemic O\textsubscript{6}-BG with systemic BCNU, animals bearing intracranial brain tumor xenografts had significantly improved response to the nitrosourea when first pretreated with the O\textsubscript{6}-BG. This suggests that the O\textsubscript{6}-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. O\textsubscript{6}-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 O\textsubscript{6}-BG administration.

A second important factor appears to be the timing of the O\textsubscript{6}-BG pretreatment. BCNU causes DNA damage by chloroethylation at the O\textsubscript{6} 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). O\textsubscript{6}-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 O\textsubscript{6}-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 O\textsubscript{6}-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). When we administered the O\textsubscript{6}-BG 24 h before polymer placement, there was no effect on BCNU sensitivity. This is likely the result of resynthesis of cytoprotective levels of AGT prior to BCNU administration.

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, O\textsubscript{6}-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 O\textsubscript{6}-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 O\textsubscript{6}-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 O6-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 O6-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 O6-BG are in progress, and trials to study the combination of systemic O6-BG and BCNU polymers are being initiated.

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References

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