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 intraocular 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 toxicity 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 tolerable 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
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Oβ-BENZYLGUANINE (Oβ-BG) is a DNA alkylation agent that has been shown to increase the sensitivity of glioma cells to interstitial BCNU. In a recent study, the effects of Oβ-BG on the efficacy of intracranial BCNU polymers and systemically administered Oβ-BG against intracranial gliomas were investigated.

### Methods

#### Chemicals and Drugs

BCNU (Bristol-Myers-Squibb, Princeton, NJ) was used for the BCNU polymer. Oβ-BG (National Cancer Institute, Frederick, MD) was dissolved in 33.3% polyethylene glycol (PEG) in phosphate-buffered saline and administered at a dose of 50 mg/kg i.p. 2 h prior to polymer placement.

#### Tumor Cell Lines

Samples of several human and rat brain tumor cell lines were analyzed for AGT activity using a previously described technique. These cell lines were selected as experimental models in which to test the effects of Oβ-BG-mediated AGT inhibition on the efficacy of locally delivered BCNU chemotherapy.

### Results

The results of the first experiment, involving 30 rats challenged with intracranial F98 glioma, showed that the combination of Oβ-BG and interstitial BCNU resulted in a significant increase in survival compared to controls. The second experiment, involving 55 rats, confirmed the efficacy of Oβ-BG in conjunction with BCNU polymers, with a significant improvement in survival rates observed in animals treated with Oβ-BG prior to polymer implantation.

### Discussion

The study demonstrates the potential of Oβ-BG as an adjuvant to BCNU for the treatment of intracranial gliomas. The results suggest that Oβ-BG-mediated AGT inhibition could enhance the therapeutic efficacy of BCNU polymers by increasing the sensitivity of glioma cells to the drug. Further studies are needed to optimize the dosing regimens and to evaluate the long-term effects on tumor control and patient outcomes.

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### References

1. Guilford Pharmaceuticals Corp. (Baltimore, MD). Polymer discs containing BCNU/polymer mixtures of 0 and 3.8% BCNU by weight were used for the statistical analyses.

2. Samples of several human and rat brain tumor cell lines 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).

3. The second experiment was conducted in a similar fashion. On the 5th day after intracranial tumor challenge, 30 rats were randomized into four treatment groups and received either i.p. drug vehicle 2 h prior to empty polymer implantation, or i.p. drug vehicle 2 h prior to 3.8% BCNU polymer implantation, or 50 mg/kg Oβ-BG i.p. 2 h prior to 3.8% BCNU polymer implantation (Fig. 1).

4. In both experiments, animals were evaluated daily for physical or behavioral evidence of toxicity, such as decreased alertness, impaired grooming, lacrimal debris around the eyes, focal motor deficits, or gait disturbances, and survival was recorded.

5. The AGT activity of human and rat brain tumor cell lines was assayed by the method of Bradford (21). The results were expressed as fmol/mg protein.

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### Table 1

<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>Dasy</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.

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### Table 2

<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>0.959</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></td>
</tr>
<tr>
<td>Oβ-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>Oβ-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>Oβ-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>Oβ-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.

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### Additional Notes

- The AGT activity of human and rat brain tumor cell lines was determined by using the 3H-methylated DNA substrate for AGT activity.
- The statistical analyses were performed using the log-rank (Mantel-Cox) test in Kaplan-Meier nonparametric analysis of survival.
Our results demonstrate that pretreatment with systemic O\textsuperscript{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\textsuperscript{6}-BG, and animal survival is improved significantly.

The success of this treatment strategy depends on several important factors. First, the systemically delivered O\textsuperscript{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\textsuperscript{6}-BG administration, both O\textsuperscript{6}-BG and, to an equal extent, its active metabolite O\textsuperscript{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\textsuperscript{6}-BG, brain tissue extracts were shown to have radioactivity (23). In experiments examining the combination of systemic O\textsuperscript{6}-BG with systemic BCNU, animals bearing intracranial brain tumor xenografts had significantly improved response to the nitrosourea when first pretreated with the O\textsuperscript{6}-BG. This suggests that the O\textsuperscript{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\textsuperscript{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\textsuperscript{6}-BG administration.

A second important factor appears to be the timing of the O\textsuperscript{6}-BG pretreatment. BCNU causes DNA damage by chloroethylation at the O\textsuperscript{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\textsuperscript{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\textsuperscript{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 these protective levels of AGT are restored by de novo protein synthesis.

In our experiments, treatment with O\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{6}-BG...
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.

Acknowledgments

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References