O6-Benzylguanine-mediated Enhancement of Chemotherapy

Henry S. Friedman,2 Stephen Keir, Anthony E. Pegg, Peter J. Houghton, O. Michael Colvin, Robert C. Moschel, Darell D. Bigner, and M. Eileen Dolan

Departments of Surgery [H. S. F., S. K., D. D. B.], Pathology [H. S. F., D. D. B.], and Medicine [H. S. F., O. M. C., D. D. B.], Duke University Medical Center, Durham, North Carolina 27710; Department of Cellular and Molecular Physiology, The Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033 [A. E. P.]; Department of Molecular Pharmacology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101 [P. J. H.]; Laboratory of Comparative Carcinogenesis, National Cancer Institute of Frederick, Frederick, Maryland 21702 [R. C. M.]; and Department of Medicine, University of Chicago, Chicago, Illinois 60637 [M. E. D.]

Abstract
We have previously demonstrated (A. E. Pegg, Cancer Res., 50: 6119–6129, 1990) that O6-benzylguanine (O6-BG) enhances nitrosourea, temozolomide, and cyclophosphamide activity in malignant glioma xenografts growing in athymic nude mice. More recently, we have demonstrated (V. J. Patel et al., Clin. Cancer Res., 6: 4154–4157, 2000; P. Pourquier et al., Cancer Res., 61: 53–58, 2001) that the combination of temozolomide plus irinotecan (CPT-11) displays a schedule-dependent enhancement of antitumor activity secondary to trapping of topoisomerase I by O6-methylguanine residues in DNA. These studies suggested that there might be favorable therapeutic interactions between O6-BG and combinations of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) plus cyclophosphamide or temozolomide plus CPT-11, respectively. Our present results indicate that the combination of cyclophosphamide plus BCNU plus O6-BG produces growth delays modestly-to-markedly superior to combinations of cyclophosphamide with BCNU. Although the combination of temozolomide and CPT-11 reveals a marked increase in activity compared with either agent used alone, the addition of O6-BG to this combination dramatically increased the growth delay of the O6-alkylguanine-DNA alkytransferase (AGT)-positive malignant glioma D-456 MG. These results suggest that a Phase I trial of CPT-11 plus temozolomide plus O6-BG in AGT-positive tumors may be an important intervention to maximize the therapeutic benefits of the combination of CPT-11 and temozolomide.

Introduction
Several preclinical and clinical studies have demonstrated that the DNA repair protein AGT3 is responsible for resistance to chloroethylation or methylation damage at the O6 position of guanine in DNA (1–15), and several studies have shown that this enhances BCNU, temozolomide, and cyclophosphamide resistance. O6-BG inactivates AGT activity both in vitro and in vivo (16–27). More recently, we have demonstrated that the combination of temozolomide plus CPT-11 displays a schedule-dependent enhancement of antitumor activity secondary to the trapping of topoisomerase I by O6-methylguanine residues in DNA (28, 29). These studies suggested that there might be favorable therapeutic interactions between O6-BG, and combinations of BCNU plus cyclophosphamide or temozolomide plus CPT-11, respectively. We now report the marked increase in antitumor activity produced by these combinations after O6-BG-mediated AGT inactivation.

Materials and Methods
Animals. Male and female athymic BALBc mice (nu nu genotype, 6 weeks of age or older) were used for all of the studies and were maintained as described previously (30). Athymic mice were housed in an isolation facility with high-efficiency particulate air-filtration. All of the mice were maintained under a controlled 12-h light/12-h dark cycle and were provided food and water ad libitum.

Xenografts. Human central nervous system tumor-derived xenografts were used for in vivo studies. The xenografts were maintained as described previously (31). The derivation and AGT status of these xenografts is detailed in Table 1 from a previous publication (25).

Drugs. Temozolomide was provided by Schering-Plough Research Institute (Kenilworth, NJ). CPT-11 was provided by Pharmacia and Upjohn Global Distribution Center (Kalama-zoo, MI). Cyclophosphamide and BCNU were purchased from Sigma (St. Louis, MO). O6-BG was synthesized as described previously (32).

s.c. Xenograft Transplantation. s.c. tumor transplantation was performed into the right flank of the animals with an inoculation volume of 50 μl using a brei prepared from xenografts (33).

Tumor Measurements. Tumors were measured twice weekly with hand-held vernier calipers (Scientific Products, McGraw, IL). Tumor volume was calculated according to the following formula: V = [(width)2 × (length)]/2.

Drug Toxicity. Mice were weighed twice weekly to assess weight loss and were checked daily for survival.

1 This work was supported by NIH Grants NS30245 (to H. S. F.), NS20023 (to H. S. F.), CA57725 (to A. E. P., H. S. F., and M. E. D.), and CA81485 (to M. E. D.). Drs. Pegg, Moschel, and Dolan have a financial relationship with Access Oncology, the company that is presently licensing CPT-11 (to H. S. F.), CA57725 (to A. E. P., H. S. F., and M. E. D.), and CA81485 (to M. E. D.).

2 To whom requests for reprints should be addressed, at Duke University Medical Center, Room 047, Baker House, Trent Drive, Durham, North Carolina 27710.

Received 5/6/02; revised 7/23/02; accepted 7/31/02.
O6-BG-mediated Enhancement Chemotherapy

via i.p. injection at a single dose of 105 mg/m² (35 mg/kg) in 240 mg/m² (80 mg/kg) in polyethylene glycol-400/0.9% saline mice were treated with the combination of temozolomide at or with O6-BG. Similar studies with five non-tumor-bearing mice/group using temozolomide at 50% of the LD10 plus CPT-11 (0.25 LD10) produced no toxic deaths. The combination of temozolomide (0.50 LD10) plus CPT-11 (0.5 LD10) produced two of five deaths with O6-BG and two of five deaths without O6-BG.

### Activity

**Cyclophosphamide/BCNU/O6-BG.** The combination of cyclophosphamide plus O6-BG produced increased antitumor activity in five AGT-positive tumors, compared with cyclophosphamide alone (Table 2). Cyclophosphamide produced growth delays of 8.0, 12.0, 4.6, 15.3, and 16.3 days, whereas cyclophosphamide plus O6-BG produced growth delays of 9.5, 21.9, 13.1, 18, and 17.7 days against d-245 MG, d-341 MED, d-456 MG, d-528 EP, and d-612 EP, respectively. The addition of O6-BG to BCNU increased growth delays of 11.7, 1.0, 2.0, 6.5, 9.2, and 2.3 days with BCNU alone to 24.7, 20.2, 14.4, 42.5, and 45.6 days with BCNU plus O6-BG. The combination of BCNU plus cyclophosphamide produced growth delays of 23.9, 13.3, 12.0, 10.8, and 32.7 days. The use of BCNU, cyclophosphamide, and O6-BG together produced the longest growth delays of 46.6, 60.1, 70.4, 49.0, and 55.4 days.

### Discussion

The use of O6-BG-mediated AGT depletion to augment the antineoplastic activity of chemotherapeutic agents remains an exciting possibility. The combination of BCNU plus O6-BG, a very effective strategy in vitro and in rodents (16–24, 26, 27) has not yet produced similar results in humans, presumably because of the marked reduction of BCNU dose required to deliver this regimen safely. The dose of BCNU when given with O6-BG must be reduced by 80%, from 200 to 40 mg/m², because of dose-limiting myelosuppression (35). The use of O6-BG plus this reduced dose of BCNU was not effective against BCNU-resistant glioblastoma multiforme (36). Nevertheless, AGT depletion is a very enticing strategy if rational and safe regimens can be designed.

The current laboratory studies focused on two different strategies to exploit O6-BG-mediated AGT depletion. BCNU and cyclophosphamide are frequently administered in combination, particularly in high-dose chemotherapy regimens...
using stem cell support. Our results confirm previous work in our laboratory that demonstrated modestly enhanced activity of cyclophosphamide and substantially enhanced activity of BCNU produced by O6-BG (21, 37). The concomitant use of three agents produced markedly enhanced antitumor activity, even compared with the combination of cyclophosphamide and BCNU. A precise molecular explanation for this interaction remains somewhat speculative. The interaction between BCNU and O6-BG is clearly a result of reduced removal of BCNU-induced mono-adducts with higher formation of the lethal DNA interstrand cross-link (11). The interaction between cyclophosphamide and O6-BG is more complex.

Evidence has emerged recently that suggests a role for AGT in the repair of certain cyclophosphamide-induced lesions. Cyclophosphamide is metabolized to acrolein and phosphoramidate mustard. The antitumor effect is thought to be mainly mediated by interstrand cross-links formed from the reaction of phosphoramidate mustard and DNA (38). We demonstrated that AGT-expressing CHO cells were significantly less sensitive to the toxic and mutagenic effects of both 4-HC (activated form of cyclophosphamide) and 4-hydroperoxycyclophosphamide (4-HDC; a generator of acrolein and a nonalkylating form of phosphoramidate mustard) than CHO cells without detectable AGT (37, 39). However, neither the toxic nor the mutagenic effects of phosphoramidate mustard were altered in the presence or absence of AGT. These results suggest that inactivation of AGT by O6-BG in AGT-expressing CHO cells was significant. Interestingly, we observe a more dramatic tumor growth inhibition when O6-BG is combined with BCNU and cyclophosphamide. In D-245 MG, D-341 MED, and D-456 MG, the

<table>
<thead>
<tr>
<th>Drug</th>
<th>Xenograft</th>
<th>T − C</th>
<th>P</th>
<th>Regressions</th>
<th>P</th>
<th>Toxic deaths</th>
<th>Mean nadir weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclo (0.22)</td>
<td>D-245 MG</td>
<td>8.0</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>1.6</td>
</tr>
<tr>
<td>BCNU (0.24)</td>
<td>D-245 MG</td>
<td>11.7</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0.1</td>
</tr>
<tr>
<td>O6-BG</td>
<td>D-245 MG</td>
<td>-0.3</td>
<td>NS</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0.0</td>
</tr>
<tr>
<td>Cyclo (0.22) + O6-BG</td>
<td>D-245 MG</td>
<td>9.5</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>1.1</td>
</tr>
<tr>
<td>BCNU (0.24) + O6-BG</td>
<td>D-245 MG</td>
<td>24.7</td>
<td>&lt;0.001</td>
<td>10/10</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>7.7</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24)</td>
<td>D-245 MG</td>
<td>23.9</td>
<td>&lt;0.001</td>
<td>7/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>0.15</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24) + O6-BG</td>
<td>D-245 MG</td>
<td>46.6</td>
<td>&lt;0.001</td>
<td>10/10</td>
<td>&lt;0.001</td>
<td>6/10</td>
<td>19.0</td>
</tr>
<tr>
<td>Cyclo (0.22)</td>
<td>D-341 MED</td>
<td>12.0</td>
<td>&lt;0.001</td>
<td>6/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>3.7</td>
</tr>
<tr>
<td>BCNU (0.24)</td>
<td>D-341 MED</td>
<td>1.0</td>
<td>NS</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>1.5</td>
</tr>
<tr>
<td>O6-BG</td>
<td>D-341 MED</td>
<td>0</td>
<td>NS</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Cyclo (0.24) + O6-BG</td>
<td>D-341 MED</td>
<td>21.9</td>
<td>&lt;0.001</td>
<td>9/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>7.1</td>
</tr>
<tr>
<td>BCNU (0.24) + O6-BG</td>
<td>D-341 MED</td>
<td>20.2</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>5.5</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24)</td>
<td>D-341 MED</td>
<td>13.3</td>
<td>&lt;0.001</td>
<td>8/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>3.6</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24) + O6-BG</td>
<td>D-341 MED</td>
<td>60.1</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>15.4</td>
</tr>
<tr>
<td>Cyclo (0.22)</td>
<td>D-456 MG</td>
<td>4.6</td>
<td>0.01</td>
<td>3/10</td>
<td>NS</td>
<td>0/10</td>
<td>4.5</td>
</tr>
<tr>
<td>BCNU (0.24)</td>
<td>D-456 MG</td>
<td>2.3</td>
<td>0.03</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0.1</td>
</tr>
<tr>
<td>O6-BG</td>
<td>D-456 MG</td>
<td>0.0</td>
<td>NS</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Cyclo (0.24) + O6-BG</td>
<td>D-456 MG</td>
<td>13.1</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>3.7</td>
</tr>
<tr>
<td>BCNU (0.24) + O6-BG</td>
<td>D-456 MG</td>
<td>14.4</td>
<td>&lt;0.001</td>
<td>9/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>6.4</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24)</td>
<td>D-456 MG</td>
<td>12.0</td>
<td>&lt;0.001</td>
<td>9/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>4.5</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24) + O6-BG</td>
<td>D-456 MG</td>
<td>70.4</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>16.3</td>
</tr>
<tr>
<td>Cyclo (0.22)</td>
<td>D-528 EP</td>
<td>13.5</td>
<td>&lt;0.001</td>
<td>4/10</td>
<td>0.01</td>
<td>0/10</td>
<td>0.1</td>
</tr>
<tr>
<td>BCNU (0.24)</td>
<td>D-528 EP</td>
<td>6.5</td>
<td>0.003</td>
<td>6/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>1.5</td>
</tr>
<tr>
<td>O6-BG</td>
<td>D-528 EP</td>
<td>0</td>
<td>NS</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Cyclo (0.22) + O6-BG</td>
<td>D-528 EP</td>
<td>18.1</td>
<td>&lt;0.001</td>
<td>8/8</td>
<td>&lt;0.001</td>
<td>1/9</td>
<td>4.7</td>
</tr>
<tr>
<td>BCNU (0.24) + O6-BG</td>
<td>D-528 EP</td>
<td>42.5</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>0/9</td>
<td>9.2</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24)</td>
<td>D-528 EP</td>
<td>10.8</td>
<td>0.04</td>
<td>6/9</td>
<td>&lt;0.001</td>
<td>0/0</td>
<td>7.2</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24) + O6-BG</td>
<td>D-528 EP</td>
<td>49.0</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>12.0</td>
</tr>
<tr>
<td>Cyclo (0.22)</td>
<td>D-612 EP</td>
<td>16.3</td>
<td>&lt;0.001</td>
<td>7/10</td>
<td>&lt;0.001</td>
<td>0/10</td>
<td>1.3</td>
</tr>
<tr>
<td>BCNU (0.24)</td>
<td>D-612 EP</td>
<td>9.2</td>
<td>&lt;0.001</td>
<td>2/10</td>
<td>NS</td>
<td>0/9</td>
<td>1.0</td>
</tr>
<tr>
<td>O6-BG</td>
<td>D-612 EP</td>
<td>0</td>
<td>NS</td>
<td>0/10</td>
<td>NS</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Cyclo (0.22) + O6-BG</td>
<td>D-612 EP</td>
<td>17.7</td>
<td>&lt;0.001</td>
<td>8/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>4.2</td>
</tr>
<tr>
<td>BCNU (0.24) + O6-BG</td>
<td>D-612 EP</td>
<td>45.6</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>11.4</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24)</td>
<td>D-612 EP</td>
<td>32.7</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>0/9</td>
<td>6.2</td>
</tr>
<tr>
<td>Cyclo (0.22) + BCNU (0.24) + O6-BG</td>
<td>D-612 EP</td>
<td>55.4</td>
<td>&lt;0.001</td>
<td>9/9</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>18.9</td>
</tr>
</tbody>
</table>

* F, percent of LD10; Cyclo, cyclophosphamide; NS, not significant.

| T − C, the difference in days between the median time required in treated (T) and control (C) animals to reach a volume five times greater than that measured at the start of treatment.
growth delay with the three-drug regimen was more than the
additive effect of O6-BG with cyclophosphamide or O6-BG
with BCNU. The critical toxic lesion formed in DNA by BCNU
is the 1→3-cytosine-2→1-guanineethane interstrand cross-
link after chloroethylation at the O6 position of guanine (40).
Cyclophosphamide produces interstrand N7-N7 cross-links
involving the two guanines in GNC→GNC (5→3′→3′→3′)
sequences (41). We have observed that, in some cell lines
deficient of AGT, O6-BG enhances the toxicity of 4-HC (42).
The mechanism of enhancement is unrelated to AGT inacti-
vation because the known toxic DNA lesions associated with
nitrogen mustards occur at nucleophilic nitrogens of gua-
mine, not the O6 position of guanine, and there is no evidence
to suggest that AGT can repair these lesions. In addition,
enhancement is observed in cell lines such as CHO and
SQ20b (squamous cell carcinoma) that are apparently de-
void of the AGT protein.4 Preliminary results from our labo-
ratory indicate that the formation or processing of intrastrand
and/or interstrand DNA cross-links is critical for the interac-
tion of O6-BG with cyclophosphamide (43). The dramatic
growth delay when O6-BG is combined with cyclophospha-
mide and BCNU could be attributable to the abundance of
structurally different cross-links on DNA. O6-BG might act by
two mechanisms: (a) inactivation of AGT, thus producing
more BCNU cross-links and (b) a more elusive mechanism
involving less repair of cyclophosphamide cross-links. Both
of the interstrand cross-links formed on DNA may act in
concert, resulting in greater tumor growth inhibition. Nev-
evertheless, the concomitant use of the three agents produced
marked increases in antitumor activity, which suggests that
translation to the clinic, presumably in a program using stem
cell support to offset dose-limiting myelosuppression, may
be warranted.

The striking increases in antitumor activity seen with the
combination of temozolomide, CPT-11, and O6-BG are bet-
derstood and may provide a rational combination for the
clinic. Temozolomide resistance is produced, at least in
part, by tumor AGT (44). However, it is also possible that
altered drug pharmacokinetics may be contributing to the
increase in antitumor activity. A current Phase I trial of
temozolomide plus O6-BG will ultimately culminate in a Phase
II trial of this combination in patients with temozolomide-
resistant malignant glioma. Nevertheless, it is possible that
dose-limiting toxicity will, similar to the BCNU plus O6-BG
trial (36), minimize the dose and potentially limit the benefit of
temozolomide when given in combination with O6-BG. Ac-
cordingly, we are also conducting a Phase I trial of CPT-11
plus temozolomide designed to use methylation of the O6
position of guanine produced by temozolomide to trap topo-
isozeromerase I and enhance CPT-11 activity. Unfortunately,
AGT removal of the methyl adduct may minimize any ther-
apeutic gain if it occurs before CPT-11-induced cytotoxicity.

Therefore, although a Phase II trial of CPT-11 plus temo-
zolomide will be warranted, a design of strategies to reduce
the impact of AGT-mediated methyl adducts before CPT-11
cytotoxicity may be necessary. Our current results suggest
that the use of temozolomide (to methylate tumor cell DNA),
O6-BG (to prevent AGT-mediated adduct removal), and
CPT-11 (to target trapped topoisomerase I) may be a highly
effective clinical intervention. A Phase I trial of this three-drug
regimen is currently being designed that will reflect the
schedule-dependent interaction between CPT-11 and temo-
zolomide (28) and the need to deplete AGT before temozol-
omide-induced methylation.

References
1. Zlotogorski, C., and Erickson, L. C. Pretreatment of human colon
tumor cells with DNA methylating agents inhibits their activity to repair


O6-Benzylguanine-mediated Enhancement of Chemotherapy

This work was supported by NIH Grants NS30245 (to H. S. F.), NS20023 (to H. S. F.), CA57725 (to A. E. P., H. S. F., and M. E. D.), and CA81485 (to M. E. D.). Drs. Pegg, Moschel, and Dolan have a financial relationship with Access Oncology, the company that is presently licensing O6-benzylguanine. Dr. Friedman is a paid consultant for Access Oncology.

Henry S. Friedman, Stephen Keir, Anthony E. Pegg, et al.

Mol Cancer Ther 2002;1:943-948.