Role of $O^6$-Alkylguanine-DNA Alkyltransferase in Protecting against 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)-Induced Long-Term Toxicities

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ABSTRACT

$O^6$-alkylguanine-DNA alkyltransferase (AGT) protects from the mutagenic and toxic lesions induced by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and in many tumors, AGT overexpression provides a means of resistance. To circumvent this, $O^6$-benzylguanine, an inactivator of AGT, has been developed and is currently in clinical development with BCNU; however, the potential long-term toxicities associated with this treatment are unknown. With the inactivation of AGT by $O^6$-benzylguanine, a higher number of toxic and mutagenic $O^5$-alkylguanine lesions introduced by methylating or chloroethylating agents would be expected. In this study, cohorts of mice were treated with $O^5$-benzylguanine (30 mg/kg), BCNU alone (low dose of 15 mg/kg or high dose of 50 mg/kg), or $O^6$-benzylguanine (30 mg/kg) plus BCNU (15 mg/kg) and followed for 12 months post-treatment. Mice treated with $O^6$-benzylguanine plus BCNU or high-dose BCNU died significantly earlier ($p < 0.0001$) than mice in the other three cohorts with a median survival of 8.3 ($O^5$-benzylguanine plus BCNU) and 7.9 months (high-dose BCNU). Histopathologic sections of tissues revealed that the most common morphological diagnosis in animals treated with $O^5$-benzylguanine plus BCNU (15 mg/kg) or BCNU (50 mg/kg) was cytomegaly in the lung with greater severity observed in mice receiving the combination $O^5$-benzylguanine plus BCNU. Four of five mice analyzed in this cohort had alveolar histiocytosis, with one also having alveolar edema. In contrast, liver and kidney toxicity was only observed in mice treated with BCNU (50 mg/kg). These results suggest that $O^6$-benzylguanine enhances long-term pulmonary toxicity associated with BCNU in mice.

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ABBREVIATIONS: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine); AGT, $O^6$-alkylguanine-DNA alkyltransferase; MNU, N-methyl-N-nitrosourea; DLCO, lung diffusing capacity for carbon monoxide measures.
promoter methylation, which has been shown to correlate with no AGT activity (Costello et al., 1994), has been associated with better response of gliomas to BCNU and temozolomide treatment (Esteller et al., 2000; Hegi et al., 2005).

The importance of AGT in protecting from toxicities associated with agents that alkylate DNA at the O6-position of guanine has been demonstrated by hypersensitivity of AGT-deficient mice to these agents (Tsuzuki et al., 1996; Glassner et al., 1999). After treatment of AGT-deficient mice with alkylating agents, the bone marrow was found to be hypocellular and, in some studies, completely ablated, with a decrease in leukocytes (Tsuzuki et al., 1996; Glassner et al., 1999). Reese et al. (2001) demonstrated that AGT wild-type mice that underwent bone marrow reconstitution with AGT−/− bone marrow show increased sensitivity to N-methyl-N-nitrosourea (MNU). Conversely, AGT−/− mice reconstituted with AGT+/− bone marrow were equally sensitive to MNU as wild-type mice. Together, these experiments demonstrate that AGT protects mice from acute death associated with alkylating agent-induced bone marrow suppression.

In attempts to increase the sensitivity of tumor cells to the cytotoxic effects of O6-alkylating agents, potent irreversible inactivators of AGT have been developed, including O6-benzylguanine (Dolan et al., 1990a). At micromolar concentrations, O6-benzylguanine rapidly depletes AGT activity to undetectable levels and increases the sensitivity of human tumor cells and xenografts to the cytotoxic effects of O6-alkylating agents (Dolan et al., 1990b, 1991; Gerson, 1993; Pegg et al., 1995). Clinical trials revealed that O6-benzylguanine alone is nontoxic; however, in combination with BCNU or temozolomide, the dose-limiting toxicity is bone marrow suppression (Spiro et al., 1999; Friedman et al., 2000). In addition, two of 23 patients on one trial displayed reduced DLCO levels, a surrogate marker of pulmonary toxicity (Friedman et al., 2000). In a recent phase II trial of O6-benzylguanine (120 mg/m²) i.v. over 1 h followed by BCNU (40 mg/m²), five of 42 patients had decreased DLCO with one grade 3 requiring discontinuation of therapy (Gajewski et al., 2005). BCNU alone has been associated with pulmonary toxicity and even fatal pulmonary fibrosis (Holoye et al., 2005). BCNU in 15% ethanol was prepared fresh by dissolving in 100% ethanol and adding filtered saline, such that the final concentrations were between 1.25 and 7.5 mg/ml (final dosage: 12.5–75 mg/kg). All injection volumes were 200 μl/20 g body weight. Mice were treated via intraperitoneal injections three weeks apart, beginning at 8 to 12 weeks of age. Mice were randomly assigned to one of five treatment groups consisting of: 1) vehicle (40% polyethylene glycol 400/60% normal saline 1 h before 15% ethanol in saline); 2) O6-benzylguanine (30 mg/kg) given 1 h before 15% ethanol in saline; 3) 40% polyethylene glycol 400/60% saline followed 1 h later with 15 mg/kg BCNU; 4) O6-benzylguanine (30 mg/kg) given 1 h before BCNU (15 mg/kg); and 5) 40% polyethylene glycol 400/60% saline followed 1 h later with 50 mg/kg BCNU.

**AGT Activity Assay.** Four female mice were sacrificed at each time point (0, 1, 6, 13, and 24 h after vehicle or O6-benzylguanine or vehicle administration) after one treatment with vehicle, O6-benzylguanine (30 mg/kg) plus BCNU (15 mg/kg), or BCNU (50 mg/kg). Solutions were made as described above. Liver, kidney, and lung tissues were harvested and immediately flash-frozen for storage at −80°C until AGT activity determination. The assay for AGT activity was performed as described previously (Dolan et al., 1990a). In brief, AGT activity was measured as the removal of O6-[3H]methylguanine from a 6-benzylguanine or vehicle as described above. Liver, kidney, and lung tissues were harvested and immediately flash-frozen for storage at −80°C until AGT activity determination. The assay for AGT activity was performed as described previously (Dolan et al., 1990a). In brief, AGT activity was measured as the removal of O6-[3H]methylguanine from a 6-benzylguanine DNA substrate (2.93 Ci/mmol) after incubation with tissue extract at 37°C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (final concentration 0.25 N) and hydrolyzed by the addition of 0.1 N HCl at 70°C for 30 min. After adjusting pH and filtering samples using a microfilter apparatus, the modified bases were separated by Beckman Ultrasphere C18 (Beckman Coulter, Fullerton, CA) reverse-phase high-performance liquid chromatography with 0.05 M ammonium formate (pH 4.5) containing 10% methanol. Protein was determined by the method of Bradford, and the amount of O6-methylguanine released from the DNA substrate per milligram of protein was calculated.

**Evaluation of Toxicity.** All mice were followed for up to 12 months post-treatment and sacrificed at the end of the observation period. During this time, weights and complete blood counts were obtained routinely on healthy mice and more often on mice that appeared ill. Complete blood counts were performed on blood collected via tail vein in EDTA-coated microtainer tubes (BD Biosciences, Franklin Lakes, NJ) using a MASCOT 850 (Drew Scientific, Oxford, CT). If at any time a mouse had an abnormal complete blood count (total leukocyte count >25,000 K/μl), a blood smear examination was done and a complete blood count was repeated 2 weeks later. If the repeat complete blood count was persistently or progressively abnormal, the mouse was sacrificed and a necropsy was performed.

**Necropsy and Tissue Collection.** At necropsy, whole-body weight was measured and gross examination was conducted on all tissues. Blood and femoral bone marrow smears were made. Spleens were isolated and weighed, condition was noted, and they were cut longitudinally to obtain touch preps. Approximately 1/3 of the spleen and a portion of the liver, after noting weight and condition, were flash-frozen in liquid nitrogen. The remaining femoral bone marrow and 1/3 of the spleen were made into single cell suspensions for cryopreservation and cytopsins. Remaining tissues were placed in Accustain formalin solution (10%) (Sigma-Aldrich, St. Louis, MO) for histologic analysis. If a tumor was detected, it was weighed, a touch prep was obtained, and portions were made into a single cell suspension, flash-frozen, or placed in formalin. Cytopsin preparations were made from 150,000 cells in fluorescence-activated cell sorter.

**Materials and Methods**

**Drugs.** O6-Benzylguanine was provided by Dr. Robert C. Moschel (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). BCNU (NSC 409962) was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).

**Animal Care.** All of the animal procedures were approved by our Institutional Animal Care and Use Committee. F1 (C57BL/6 × 129/Sv) N71−/− mice were used because of our interest in the role of AGT in protecting from alkylating agent-induced leukemia. Therapy-related leukemia after treatment with cyclophosphamide has been shown in these mice (Mahgoub et al., 1999). F1 mice were housed in an isolation facility with water and food provided ad libitum.

**Animal Treatment.** O6-Benzylguanine was administered to mice to ascertain the long-term toxicities associated with BCNU treatment (Esteller et al., 2000; Hegi et al., 2005). BCNU (NSC 409962) was kindly provided by the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). BCNU (NSC 409962) was kindly provided by the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). BCNU (NSC 409962) was kindly provided by the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD).

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buffer (Cellgro calcium/magnesium-free phenol red-free Hank's balanced salt solution (Mediatech Inc., Herdon, VA), 2% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2.5% cell dissociation buffer (Invitrogen, Carlsbad, CA) spun at 400 rpm for 8 min at medium acceleration using a Cytopep 7629 cryocentrifuge (Wescor, Logan, UT). Cells were cryopreserved at 2 × 10⁶ cells ml⁻¹ in 70% Hank's balanced salt solution/20% fetal bovine serum/10% dimethyl sulfoxide (Sigma-Aldrich). Smares, touch preps, and cytopsins were stained with Wright-Giemsa (Fisher Diagnostics, Middletown, VA).

**Histopathology.** Five ill mice (mixture of male and female) were selected for analysis from each of the cohorts treated with BCNU at 50 mg/kg or O₆-benzylguanine plus BCNU (15 mg/kg). Age-matched controls (1F/1M) from each of the remaining groups (vehicle, O₆-benzylguanine alone, and BCNU at 15 mg/kg) were sacrificed. Hematoxylin and eosin (H&E)-stained sections of formalin-fixed paraffin-embedded tissues of the heart, thymus, lungs, sternum (for bone marrow), spleen, liver, vertebrae, stomach, and intestines were made at the University of Chicago Animal Research Center. Histopathologic examination was performed by Pathology Associates (Chicago, IL).

**Statistical Analysis.** The event-free survival defined as the time to death or sacrifice due to illness was estimated for each treatment group using the Kaplan-Meier estimate and compared using the log-rank test. Various graphical displays were used to examine hematologic toxicity measures made over time, including a summary plot of means ± S.E. by treatment cohort for every time point. Formal inference included the following: 1) For each measure and at each measurement period, an analysis of variance was performed to test the overall difference among cohorts followed by post hoc Tukey range test for pairwise comparisons that controls the type I error rate. 2) A linear random effects model for longitudinal data, where the intercept and slope of the linear model are allowed to vary among rate. 2) A linear random effects model for longitudinal data, where the intercept and slope of the linear model are allowed to vary among rate.

**Results**

**Dose Finding.** Initial studies were conducted to determine the maximum tolerated dose of BCNU alone (Table 1). Mice were treated with 50 or 75 mg/kg BCNU at different time intervals, and weights were obtained up to 14 days post-treatment. Complete blood counts were performed up to 7 days; the nadir for the total white blood count was on day 4 regardless of dose. Weight loss and animal death were primarily used for dose determination. Because six of 12 mice became moribund after treatment with 75 mg/kg, we determined the maximum tolerated dose for BCNU alone to be 50 mg/kg. To parallel clinical BCNU dosing in humans, we tested multiple cycles of BCNU (50 mg/kg) with a single additional injection at 2, 3, 4, or 5.5 weeks after the first dose.

Based on severe weight loss in animals injected 2 weeks apart (24%), we chose to inject 50 mg/kg 3 weeks apart. An equitoxic dose of the combination of O₆-benzylguanine plus BCNU was determined by treating with O₆-benzylguanine (30 mg/kg) 1 h before 12.5, 15, or 18 mg/kg BCNU. O₆-Benzylguanine plus BCNU (15 mg/kg) caused weight loss and neutropenia similar to mice treated with 50 mg/kg BCNU alone with no deaths. Therefore, 30 mg/kg O₆-benzylguanine plus 15 mg/kg BCNU was chosen for further studies.

Figure 1 illustrates animal weight gain or loss over time for animals in the five treatment groups. Although the mice treated with vehicle, O₆-benzylguanine alone, or BCNU (15 mg/kg) gained weight over the 12-month time period, mice treated with O₆-benzylguanine plus BCNU (15 mg/kg) or BCNU alone (50 mg/kg) initially lost weight before regaining weight to baseline.

**AGT Inactivation.** To evaluate the effect of the maximum tolerated dose of drugs on AGT activity in mouse tissues, activity was measured in the liver, kidney, and lung of mice after treatment with vehicle, O₆-benzylguanine (30 mg/kg) plus BCNU (15 mg/kg), or BCNU (50 mg/kg) (Fig. 2). AGT activity was reduced to >97% in all tissues 1 h after treatment with O₆-benzylguanine, at which time mice were treated with BCNU. For this cohort, AGT levels remained low until 24 h with recovery to 63, 19, and 18% basal levels in liver, kidney, and lung of mice. In mice treated with BCNU.

**TABLE 1**

<table>
<thead>
<tr>
<th>O₆-Benzylguanine Dose</th>
<th>BCNU Dose</th>
<th>No. of Injections</th>
<th>Weeks between Treatments</th>
<th>Total No. of Mice</th>
<th>Weight Loss Nadir</th>
<th>% Initial WT</th>
<th>Weeks after First Injection</th>
<th>No. of Deaths within 30 Days</th>
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<tbody>
<tr>
<td>mg/kg</td>
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<td></td>
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<td>2</td>
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<td>6</td>
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</tr>
<tr>
<td></td>
<td>0</td>
<td>50</td>
<td>2</td>
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<td>15.2 (2.0)</td>
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<tr>
<td></td>
<td>0</td>
<td>50</td>
<td>2</td>
<td>1</td>
<td>38</td>
<td>12.7 (1.4)</td>
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<tr>
<td></td>
<td>0</td>
<td>50</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>8.1 (5.0)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data are from mice in O₆-benzylguanine plus BCNU cohort.
alone, AGT activity at 6 to 24 h dropped to 70% basal levels for liver and 20% for lung, respectively. This same dose of BCNU resulted in a 90% depletion of AGT activity at 6 and 13 h post-treatment in kidney with recovery to 43% baseline at 24 h. O6-Benzylationuine has been shown in previous studies to inactivate AGT in mouse tissues (Dolan et al., 1990b).

**Event-Free Survival.** Mice treated with vehicle, O6-benzylationuine alone, or BCNU (15 mg/kg) had few deaths (4/41, 1/38, and 0/40, respectively), whereas 41 of 42 mice treated with BCNU (50 mg/kg) and 40 of 45 mice treated with O6-benzylationuine plus BCNU (15 mg/kg) died or were sacrificed because of illness. The median survival of mice treated with BCNU (50 mg/kg) or O6-benzylationuine plus BCNU (15 mg/kg) was 7.9 and 8.3 months, respectively. Illness was determined either from two consecutive abnormal complete blood counts or a mouse with characteristics such as rapid breathing, shivering, hunched, or lethargy when returned to the cage after physical examination. There was a significant difference, as determined by a log rank test, in the event-free survival between the treatment groups (Fig. 3). At 6 months, event-free survival was significantly different between cohorts treated with high-dose BCNU or O6-benzylationuine plus BCNU (79 and 82% survival, respectively) and the other three cohorts (all at 100% survival, \( p < 0.007 \) when comparing vehicle, O6-benzylationuine alone, or BCNU (15 mg/kg) curves against either O6-benzylationuine plus BCNU (15 mg/kg) or BCNU (50 mg/kg)). The difference in event-free survival continued to be significant at 12 months postenrollment between these same cohorts (\( p < 0.0001 \)). Event-free survival at 12 months was 90, 97, 100, 11, and 2% for cohorts treated with vehicle, O6-benzylationuine alone, low-dose BCNU, O6-benzylationuine plus BCNU, and high-dose BCNU, respectively. Beginning at 10 months, high-dose BCNU is significantly different from O6-benzylationuine plus BCNU (\( p < 0.02 \)).

**Effect of Treatment on Complete Blood Counts.** To ascertain hematopoietic toxicities associated with treatment, mice were followed for 12 months postenrollment with serial complete blood counts. Our baseline values for white blood counts in F1 mice were \( 16.4 \times 10^3/\mu l \pm 0.5 \) (mean ± S.E.M.) total leukocytes, \( 3.8 \times 10^3/\mu l \pm 0.2 \) neutrophils, and \( 11.8 \times 10^3/\mu l \pm 0.4 \) lymphocytes. As shown in Fig. 4, mice treated with high-dose BCNU had approximately double the absolute counts (total leukocytes, neutrophils, and lymphocytes) at 3 to 4 months compared with the other cohorts and showed a decreasing trend from 3 to 10 months. The rate of decrease per month for the BCNU (50 mg/kg) cohort was significantly different from all other cohorts for all three white blood counts (\( p \) value ranges from \( p = 0.02 \) to \( p < 0.0001 \)). The absolute monocyte, eosinophil, and basophil counts were not significantly altered (data not shown).

With the exception of mice treated with high-dose BCNU, the observed ranges for hemoglobin and hematocrit were 15 to 17 g/dL and 49 to 57%, respectively, from 3 to 8 months (Fig. 5, A and B). Hemoglobin and hematocrit values obtained from mice treated with 50 mg/kg BCNU were approximately 17% lower than mice treated with vehicle, O6-benzylationuine alone, or BCNU (15 mg/kg). These differences were highly significant (controlling overall type I error at 5%)

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**Fig. 2.** AGT activity in the liver, kidney, and lung from mice treated with vehicle or O6-benzylationuine ± BCNU. AGT activity (fmol/mg) in the liver (A), kidney (B) and lung (C) 1, 6, 13, and 24 h following treatment with vehicle (open circle), O6-benzylationuine plus BCNU (15 mg/kg) (square), or BCNU (50 mg/kg) (inverted triangle). Baseline AGT activity in these tissues were 69, 88, and 33 fmol/mg protein in the liver, kidney, and lung, respectively. The arrows refer to time of O6-benzylationuine (first arrow) and BCNU (second arrow) injection. Data represents mean ± S.D. each time point of three to five replicates. O6-Benzylationuine is shown as BG.

**Fig. 3.** Event-free survival of mice treated with vehicle or O6-benzylationuine ± BCNU. Kaplan-Meyer event-free survival curve of mice treated with vehicle (n = 41), O6-benzylationuine alone (n = 38), BCNU (15 mg/kg) (n = 40), O6-benzylationuine plus BCNU (15 mg/kg) (n = 45), or BCNU (50 mg/kg) (n = 42). An event was defined as either animal death or sacrifice because of illness. Arrows indicate time of treatments. **+ + + + **, \( p < 0.0001 \) compared with vehicle, O6-benzylationuine alone, or BCNU at 15 mg/kg at 12 months. †, \( p < 0.02 \) compared with BCNU (50 mg/kg) beginning at 10 months. O6-Benzylationuine is shown as BG.
Fig. 4. Effect of treatment with vehicle or \(O^6\)-benzylguanine ± BCNU on white blood counts. Average total leukocyte (A), absolute neutrophil (B), and absolute lymphocyte (C) counts calculated from white blood counts conducted on tail vein blood collected post-treatment. Data represent mean ± S.E.M. Total leukocyte count was analyzed as the rate of decrease per month, which was 2.82, 1.09, and 0.34 for BCNU (50 mg/kg), \(O^6\)-benzylguanine plus BCNU (15 mg/kg), and BCNU (15 mg/kg), respectively. Pairwise comparisons among these rates are all highly significant (\(p < 0.0001\)). Absolute neutrophil count included the decreasing rate of 0.31 for BCNU (50 mg/kg), which was significantly different from the decreasing rate of 0.06 (\(p = 0.02\)) for BCNU (15 mg/kg) and from the increasing rate of 0.08 (\(p < 0.0001\)) for \(O^6\)-benzylguanine plus BCNU (15 mg/kg). The rates between \(O^6\)-benzylguanine plus BCNU (15 mg/kg) and BCNU (15 mg/kg) were also significantly different (\(p = 0.05\)). Absolute lymphocyte count included the rate of decrease for mice treated with BCNU (50 mg/kg) of 2.47 compared with 1.10 for \(O^6\)-benzylguanine plus BCNU (15 mg/kg) and 0.27 for BCNU (15 mg/kg). Pairwise comparisons for these rates are all highly significant (\(p < 0.0001\)). \(O^6\)-Benzylguanine is shown as BG.

Fig. 5. Effect of treatments on hemoglobin, hematocrit, and platelet counts. Hemoglobin (in gram/deciliter) (A), hematocrit (in percentage) (B), and platelet counts (\(\times 1000/\mu\text{mL}\)) (C) were determined on blood collected at various times post-treatment. Data represent mean ± S.E.M. Overall difference at each period was based on analysis of variance followed by post hoc test for pairwise comparisons. There was a significant overall difference among cohorts for hemoglobin, hematocrit, and platelet counts at each of the periods: months 3 to 4, 5 to 6, and 7 to 8 (all \(p < 0.0001\)). However, at the last measurement period (month 9–10), none of the three blood counts showed overall significant difference among the five treatment groups at 5% level. Pairwise comparisons showed that hemoglobin and hematocrit measurements in the BCNU (50 mg/kg) cohort were significantly lower (controlling overall type I error at 5%) as compared with the other four cohorts at 3 to 4, 5 to 6, and 7 to 8 months postenrollment. There was no significant pairwise difference for hemoglobin and hematocrit among the other four cohorts. For platelet counts, post hoc comparisons showed that the BCNU (50 mg/kg) cohort was significantly lower than \(O^6\)-benzylguanine alone and vehicle but significantly higher than \(O^6\)-benzylguanine plus BCNU (15 mg/kg) at measurements conducted between 3 and 4 months. \(O^6\)-Benzylguanine is shown as BG.
as compared with the other four cohorts from 3 to 8 months. In contrast, although a time-dependent decrease was observed in platelet counts for all cohorts, actual values for mice treated with BCNU, either alone or in combination with O6-benzylguanine, were lower than O6-benzylguanine alone and vehicle-treated mice (Fig. 5C). This was significant for samples analyzed between 3 and 4 months. With the exception of platelets, O6-benzylguanine did not enhance the peripheral blood effects of low dose BCNU (15 mg/kg) to the extent observed in mice exposed to high-dose BCNU, with similar values in mice treated with O6-benzylguanine plus BCNU (15 mg/kg), BCNU (15 mg/kg) alone, and controls.

**Effect of Treatment on Spleen Weight.** Spleen weights were measured at time of necropsy. There was no significant difference in spleen weight when normalized to total body weight (data not shown). In addition, no histopathological differences were observed between different treatment groups.

**Histopathology.** To ascertain cause of morbidity, microscopic analysis was performed on tissues from animals treated in each cohort. Distinctive patterns of target tissue changes were noted in the O6-benzylguanine plus BCNU and BCNU (50 mg/kg)-treated mice that probably contributed to the morbidity and mortality (Table 2). The most significant finding was cytomegaly in the lung (Fig. 6). Although all of the mice evaluated treated with BCNU (15 or 50 mg/kg alone) or with O6-benzylguanine plus BCNU (15 mg/kg) exhibited cytomegaly, morphologic changes in the lung were most severe in mice treated with O6-benzylguanine plus BCNU; this included four mice with alveolar histiocytosis with one also having alveolar edema. Alveolar edema and histiocytosis probably contributed to the death in some animals and explained the tachypnea observed in some of the mice. Hepatocyte cytomegaly (Fig. 7) and moderate tubular atrophy in the kidney (Fig. 8) were seen in all five mice analyzed that were treated with BCNU (50 mg/kg) and probably contributed to animal death in this cohort. Interestingly, animals treated with O6-benzylguanine plus BCNU did not show signs of liver or kidney toxicity.

<table>
<thead>
<tr>
<th>Histopathological Finding</th>
<th>Vehicle</th>
<th>BCNU</th>
<th>O6-Benzylguanine (30 mg/kg)</th>
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<tr>
<td></td>
<td></td>
<td>15 mg/kg</td>
<td>50 mg/kg</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Cytomegaly</td>
<td>0</td>
<td>1</td>
<td>5</td>
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<tr>
<td>Alveolar edema w/alveolar histiocytosis</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Alveolar histiocytosis alone</td>
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</tr>
<tr>
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**Fig. 6.** Micrographs of lung specimens from mice treated with vehicle or O6-benzylguanine ± BCNU. Hematoxylin and eosin-stained sections of formalin-fixed lungs from mice treated with vehicle, O6-benzylguanine alone, BCNU (15 mg/kg), O6-benzylguanine plus BCNU (15 mg/kg), or BCNU (50 mg/kg). Note lung cytomegaly, being most severe in mice treated with O6-benzylguanine plus BCNU (15 mg/kg). Cytomegaly was also seen in other mice as indicated in Table 2. Micrographs were taken with 40× objective lens; 400× total magnification. O6-Benzylguanine is shown as BG.
Although acute toxicities associated with the treatment of O6-benzylguanine plus BCNU have been described previously (Bibby et al., 1999; Reese et al., 2001), the role of AGT in protecting from long-term toxicities of BCNU have not been described. In this study, we confirm that a 70% dose reduction of BCNU is required when BCNU is combined with 30 mg/kg O6-benzylguanine, an effective AGT inactivator. The median survival of mice treated with BCNU (50 mg/kg)
and $O^6$-benzylguanine plus BCNU (15 mg/kg) is not significantly different but dramatically different from BCNU alone at 15 mg/kg. Long-term toxicity (6–9 months) concomitant with animal weight loss was likely a result of toxicities in lung, liver, and kidney. Histopathological analysis revealed that organ toxicity contributed to death in these mice. Although it is difficult to assign a definite cause of death, there is clear evidence that alveolar edema in the lung contributed significantly to death in animals treated with high-dose BCNU and those treated with $O^6$-benzylguanine plus BCNU. These results suggest that AGT protects from long-term toxicities associated with BCNU, in particular pulmonary toxicity.

Most studies have focused on the acute toxicities associated with $O^6$-benzylguanine plus BCNU and demonstrate that myelosuppression probably leads to animal death with additional toxicities observed in the intestine and liver (Bibby et al., 1999; Reese et al., 2001; Westerhof et al., 2001). Our studies are consistent in that we observed pancytopenia immediately after treatment; however, treatment of mice with high-dose BCNU resulted in lymphocytosis and low hemoglobin and hematocrit between 3 and 6 months after treatment. This was not observed in mice treated with $O^6$-benzylguanine plus BCNU (15 mg/kg). Further analysis of the lymphocyte subsets would be required to determine whether lymphocytosis could be related to animal death observed in mice treated with high-dose BCNU. We did not see evidence for intestinal toxicity in long-term follow-up, consistent with Reese et al. (2001) who observed tissue regeneration in mice sacrificed more than 30 days post-treatment (Bibby et al., 1999). No necropsies were performed early in the study. In addition to lung toxicity, hepatocyte cytomegaly and tubular atrophy in kidney specimens were observed in all five mice selected for histopathology that had been treated with 50 mg/kg BCNU. These morphologic changes were not observed in any other cohort, indicating that inactivation of AGT by $O^6$-benzylguanine does not impact BCNU-induced liver or kidney toxicity. There are several explanations for toxicity in the liver and kidney specific only in the BCNU (50 mg/kg) cohort. 1) The basal AGT levels in the liver and kidney are greater than twice that in the lung, consistent with previous reports (Gerson et al., 1985; Citron et al., 1992). 2) There is more rapid AGT resynthesis in liver, with high-dose BCNU resulting in lymphocytosis and low hemoglobin and hematocrit between 3 and 6 months after treatment. This was not observed in mice treated with $O^6$-benzylguanine plus BCNU. 3) Toxicity was due to factors unrelated to $O^6$-guanine-DNA alkylation in liver and kidney. Perhaps resistance mechanisms other than AGT play a role in protecting liver and kidney from toxicities associated with BCNU.

BCNU at higher doses in humans has been known to lead to long-term hepatic and pulmonary toxicity and, in some cases, death as a result of pulmonary fibrosis (Holoye et al., 1976; Bailey et al., 1978; Tong et al., 1982; Brandes et al., 2004). In a recent phase II study, patients with no prior chemotherapy underwent treatment with 80 mg/m$^2$ BCNU for recurrent glioblastoma (Brandes et al., 2004). Two of 40 patients developed grade 4 pulmonary toxicity, as measured by DLCO, after four cycles (8 weeks between cycles). Hepatic toxicity grade 3 was observed in two patients at the third cycle and grade 2 in two patients at cycles 2 and 3, as measured by serum bilirubin. BCNU treatment was discontinued in four patients because of pulmonary and hepatic toxicity, grade 2 or 3. Pulmonary toxicities were irreversible and possibly under-reported with only 10 of 40 patients completing four or more cycles (Brandes et al., 2004). Previous studies in AGT-deficient mice treated with methylating and chloroethylating agents did not report pulmonary toxicity, probably because the doses administered resulted in acute hematopoietic toxicity causing animal death within 30 days (Tsuzuki et al., 1996; Glassner et al., 1999). Our results show that animals exposed to BCNU after $O^6$-benzylguanine treatment are more susceptible to lung toxicities, suggesting that AGT plays a role in protecting from BCNU-induced pulmonary toxicities observed clinically.

The role of glutathione as a means to protect against BCNU-induced lung toxicity is not well understood. In mice and rats, numerous studies reported that BCNU administration (up to 100 mg/kg) has no effect on glutathione levels in the lung, whereas glutathione reductase activity is reduced or completely inhibited (McConnell et al., 1979; Kehrer and Paraidathathu, 1984; Smith and Boyd, 1984). In conjunction with reduced glutathione reductase activity, Smith and Boyd (1984) observed delayed onset of striking pulmonary damage in rats, with increasing severity during and continuing after exposure to multiple doses of BCNU (5 mg/kg/week for 6 weeks). Although mechanisms of resistance in cancer cells may be different from normal cells, Eghahzii et al. (1997) showed that the glutathione pathway, as well as AGT, contributes to resistance to BCNU in a human nonsmall cell lung cancer cell line. In contrast, Chinese hamster fibroblasts exposed to buthionine sulfoxime, a specific inhibitor of the glutathione pathway, before BCNU treatment did not result in enhanced BCNU toxicity (Lunel-Orsini et al., 1995). These data suggest that, in some settings, the glutathione pathway, in addition to AGT, can contribute to protection from BCNU-induced toxicities.

There is strong evidence that AGT is the predominant mechanism protecting cells from toxic and mutagenic effects associated with BCNU (Pegg, 1990, 1995; Gerson, 2004). Because of this, we were interested in the role of AGT in protecting against therapy-related leukemia caused by BCNU and therefore used the Nf1 mouse model for alkylating agent-induced leukemia (Mahgoub et al., 1999). Mice heterozygous for the Nf1 gene have been shown to be more susceptible to cyclophosphamide-induced leukemia. Although we did not observe leukemia, either by serial complete blood count analysis or histologically, three of five mice treated with $O^6$-benzylguanine plus BCNU examined histologically showed evidence of hematopoietic abnormalities (one with moderate atypical hyperplasia in the thymus, including loss of normal cortex/medulla arrangement, and two with minimal extramedullary hematopoiesis and relative myeloid hyperplasia). The lack of leukemia observed in these mice may be because of: 1) the lack of susceptibility of F1 (C57BL/6 × 129/Sv) mice to BCNU-induced mutations; 2) the fact that the dosage regimen used in this study caused death before the onset of leukemia; and 3) the fact that BCNU may not be leukemogenic at this dose in mice.

In conclusion, our data demonstrate that AGT plays a role in protecting against BCNU-induced long-term toxicities, particularly pulmonary toxicity. Although myelosuppression is associated with acute toxicity, our results are the first to suggest that AGT protects in vivo from BCNU-induced lung toxicity that leads to animal death. (We note that our data do not rule out a role of the glutathione pathway.) In addition,
AGT inactivation is not associated with organ toxicities seen at the maximum tolerated dose of BCNU, suggesting that O6-benzylguanine plus low-dose BCNU might have less long-term toxicity in peripheral blood, liver, and kidney than high-dose BCNU when used in humans. As current clinical trial protocols use the combination of O6-benzylguanine plus BCNU, long-term lung toxicity may be observed in these patients.

References

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