Biochemical Pharmacology, Vol. 45, No. 2, pp. 483–491, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SYNERGISTIC EFFICACY OF O^6-BENZYLGUANINE AND 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU) IN A HUMAN COLON CANCER XENOGRAFT COMPLETELY RESISTANT TO BCNU ALONE

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(*Received* 29 *June* 1992; *accepted* 28 *August* 1992)

Abstract—The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (alkyltransferase) repairs cytotoxic DNA damage formed by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). High levels of this repair protein cause tumor drug resistance to nitrosoureas. To investigate the ability of a direct alkyltransferase inhibitor, O^6-benzylguanine, to reverse the nitrosourea resistance of human colon cancer cells, we studied the VACO 6 cell line which has high alkyltransferase and is completely resistant to BCNU at maximal tolerated doses in the xenograft model. O^6-Benzylguanine at 0.5 μg/mL for 1 hr inactivated VACO 6 alkyltransferase by >98% and reduced the IC_{50} of BCNU by 3- to 4-fold. Further analysis indicated that these two agents act in a highly synergistic fashion. In xenograft bearing athymic mice, dose-dependent depletion of hepatic and tumor alkyltransferase was noted. To maintain alkyltransferase depletion in the xenograft for at least 24 hr, two doses of 60 mg/kg O^6-benzylguanine were given 1 hr prior and 7 hr after BCNU. Under these conditions, VACO 6 xenografts became responsive to BCNU with significant reductions ($P < 0.001$) in the tumor growth rate. The combination increased toxicity to the host, reducing the maximum tolerated dose of BCNU by approximately 50%. This study provides definitive evidence that high alkyltransferase activity is responsible for BCNU resistance in human colon cancer xenografts and that with careful drug scheduling, O^6-benzylguanine can sensitize a tumor which is completely unresponsive to BCNU alone. Further studies which optimize the therapeutic index of BCNU and O^6-benzylguanine *in vivo* will define the schedule to be used in broader preclinical studies.

One of the major cytotoxic lesions formed by chloroethylating chemotherapeutic agents is the interstrand DNA cross-link [1]. With 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)‡, the first step in the DNA cross-link reaction is formation of O^6-chloroethylguanine which then rearranges to N^1,O^6-ethanoguanine and is the direct precursor to the interstrand cross-link [2]. The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (alkyltransferase) (EC 2.1.1.63) repairs the monoadduct by direct transfer of the alkyl group to the cysteine residue in the active site of the protein, inactivating the protein and regenerating a normal DNA strand [3,4]; it also attacks at the oxygen position of the N^1,O^6-ethanoguanine adduct generating a protein–DNA cross-link [5, 6]. Both reactions prevent interstrand cross-link formation and reduce the cytotoxic effects of BCNU [3, 7, 8]. Thus, cells with increased levels of alkyltransferase are resistant to BCNU and other chloroethylating agents which attack at the O^6-position of guanine [7–10]. Numerous investigators have found a correlation between alkyltransferase activity and nitrosourea resistance in human tumor cell lines and in xenograft models of rhabdomyosarcoma and gastric carcinomas [7, 11, 12].

Two methods have been used to overcome BCNU resistance by inactivation of the alkyltransferase. Erickson and coworkers [8, 13] determined that methylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and streptozotocin form sufficient O^6-methylguanine DNA adducts to deplete alkyltransferase activity due to its "suicide" mechanism of action. Thus, the sequential administration of MNNG or streptozotocin followed by a chloroethylating agent results in increased cytotoxicity and cross-link formation over that seen with equivalent doses of the two compounds alone [8, 13].

The second method is the use of direct alkyltransferase inhibitors, such as O^6-methylguanine, to inactivate the alkyltransferase and sensitize cells to nitrosoureas [14–18]. Approximately 80–90% inactivation of the alkyltransferase can be achieved at concentrations of O^6-methylguanine of 500 μM, and a reduction in the IC_{50} of BCNU of 2- to 4-fold has been observed [14–18]. Dolan *et al.* [19] first reported that O^6-benzylguanine was a more

* Recipient of an Edward Mallinckrodt Jr. Foundation Scholar Award.

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‡ Abbreviations: BCNU, 1,3,-bis(2-chloroethyl)-1-nitrosourea; and alkyltransferase, O^6-alkylguanine-DNA alkyltransferase.

potent inhibitor of the alkyltransferase than O^6-methylguanine, capable of over 95% inactivation of the protein at concentrations of 5–25 μM. These investigators also found that O^6-benzylguanine and certain analogues potentiate BCNU cytotoxicity in the HT-29 cell line with a reduction in the IC_{50} of 4.8-fold and similar reductions with clomesome and 1-(2-chloroethyl)-3-cyclohexyl-nitrosourea. In xenograft studies they observed an improved response combining O^6-benzylguanine with the chloroethylating agent 1-(2-chloroethyl)-3-(4-methyl-cyclohexyl)nitrosourea (methylCCNU) in the SF767 human glioma cell line which has intermediate alkyltransferase activity and a moderate response to methylCCNU alone [20]. More recently, Mitchell *et al.* [21] found similar results with O^6-benzylguanine and BCNU in the SF767 xenograft and observed a partial response to the combination in the HT-29 colon cancer cell line which has much higher levels of alkyltransferase. While the specificity of O^6-benzylguanine and O^6-methylguanine is quite different (the latter inhibits both the mammalian and bacterial *ada*-derived alkyltransferase whereas the former inhibits only the activity of the mammalian protein), both inhibitors react directly with the active site of the alkyltransferase with generation of guanine, suggesting covalent transfer of the alkyl group from the inhibitor to the protein [22].

Human colon cancer is resistant to most chemotherapeutic agents, including the chloroethylnitrosoureas, although responses are seen at high doses [23]. In a recent study we found a high degree of correlation between alkyltransferase activity in seven different human colon cancer cell lines and their resistance to BCNU [24]. These colon cancer cell lines contain the spectrum of alkyltransferase activity seen in human colon cancer tumors, the majority of which contain high levels of alkyltransferase. O^6-Methylguanine inactivated the alkyltransferase in the VACO 6 human colon cancer cell lines by 80% and sensitized this line approximately 2-fold to BCNU [24]. This suggested that biochemical modulation of alkyltransferase activity might overcome BCNU resistance in human colon cancer.

To address further the nature of nitrosourea resistance in human colon cancer, we evaluated the ability of O^6-benzylguanine to reverse the BCNU resistance, both *in vitro* and in human colon cancer xenografts. The VACO 6 cell line was used because it has high alkyltransferase activity, regenerates alkyltransferase activity rapidly, and is completely resistant to BCNU in xenografts at maximally tolerated doses. We found that O^6-benzylguanine completely inactivated the alkyltransferase activity, both *in vitro* and in the xenograft tumor, could synergistically increase the cytotoxicity of BCNU in the VACO 6 cell line, and was an effective modulator of BCNU resistance in the xenograft model. This provides definitive evidence that biochemical modulation of alkyltransferase can sensitize tumors that are completely resistant to BCNU *in vivo*.

MATERIALS AND METHODS

Chemical and reagents. BCNU was obtained from the Drug Synthesis and Chemistry Branch, Drug Therapeutics Program, National Cancer Institute. BCNU was reconstituted fresh in 0.5 mL of 100% ethanol at 22° and diluted to 10 mM in phosphate-buffered saline (PBS). It was used within 20 min of dilution for both *in vitro* and *in vivo* experiments. O^6-Benzylguanine was synthesized by Dr. Robert Moschel at the Frederick Cancer Research Institute, National Cancer Institute. For *in vitro* experiments O^6-benzylguanine was prepared fresh at 1.8 mg/mL in dimethyl sulfoxide (DMSO) and diluted into tissue culture medium. For *in vivo* experiments, O^6-benzylguanine was dissolved by sonication at 4 mg/mL in PBS/10% cremophor, pH 7.4, and passed through a 0.2 μm filter prior to injection [20]. In pilot *in vivo* experiments using 5% DMSO as solvent, an identical pattern of alkyltransferase inactivation and recovery was observed. However toxicity to the mice was observed at 80 mg/kg O^6-benzylguanine and this solvent was not used in the experiments reported here.

In vitro cytotoxicity in colon cancer cell lines. The CWRU colon cancer cell line bank has been described previously [24, 25]. Cells were grown with 8% calf serum as described previously [24]. Inhibition of cell growth was measured with a cell regrowth assay as described previously [25]. Briefly, cells were treated 48 hr after replating to ensure analysis during the logarithmic growth phase. O^6-Benzylguanine was added for 1–24 hr at concentrations of 0.5 to 6 μg/mL. In cytotoxicity studies, 6 μg O^6-benzylguanine was added to the cell culture and 1 hr later increasing concentrations of BCNU were added for an additional hour. The cells were washed free of drug three times using fresh medium containing 8% calf serum. O^6-Benzylguanine (0.5 μg/mL) was added to the cells immediately after washing for an additional 5 days before counting the number of viable cells in triplicate plates. BCNU cytotoxicity was determined by measuring the IC_{50} and IC_{90} from the survival curves. Synergy between O^6-benzylguanine and BCNU was measured according to the method of Berenbaum [26]. Separate sets of concentration–response curves were performed with the VACO 6 cell line using a fixed ratio of O^6-benzylguanine: BCNU of 1:3, which is approximately the ratio of the IC_{50} of the two agents given separately. In these experiments, cells were incubated in each drug alone in the ratio of 1:3 or with the combination at the same concentrations and ratio. Drugs were not washed from the cells at 1 hr and overall cytotoxicity was somewhat greater than in the above experiments. To measure regeneration of alkyltransferase activity following removal of O^6-benzylguanine, the O^6-benzylguanine-containing medium was replaced with an equal volume of culture medium containing 10% calf serum at 37% for 10 min and this procedure was repeated two times at 10-min intervals.

Xenograft studies. BALB-C derived athymic mice (nu/nu athymic) were maintained as a breeding colony. Animals were inoculated at 6–8 weeks of age (20–25 g) with 5×10^6 cultured VACO 6 colon cancer cells in bilateral upper back subcutaneous inoculations. When tumors had reached approximately 500 mm^3, animals were treated with O^6-

benzylguanine and/or BCNU by i.p. injection. Xenograft tumor measurements were made every other day and tumor volume was calculated by the following formula: tumor volume = longest volume × [perpendicular volume]^2 × 0.5. Animals were weighed twice weekly. To measure alkyltransferase activity following drug exposure, animals were anesthetized at the indicated time using pentobarbital and tumors were dissected free of their capsule and surrounding stromal tissue, flash frozen in liquid nitrogen and stored at −80° until analyzed as we have described [27]. Liver was taken from these animals as well. BCNU dose levels ranged from 30 to 130 mg/m^2 (8–34 mg/kg) of BCNU for animals treated with this compound alone and from 30 to 100 mg/m^2 for mice with the combination of BCNU and O^6-benzylguanine.

Alkyltransferase assay. Xenograft tumors and tissues were rapidly thawed and dissociated using a Ten-Brock homogenizer in 3 vol. per weight cell extract buffer (70 mM HEPES, pH 7.8, 0.1 mM EDTA, 5% glycerol and 1 mM dithiothreitol) at 4° and then sonicated and processed as previously described. Tissue culture cells were washed twice in 50 mL PBS–1 mM EDTA at 4°, resuspended at 4×10^7 cells/mL in cell extract buffer and then processed as previously described [27, 28]. The enzyme assay measures the removal of O^6-methylguanine from [^{3}H]methyl DNA containing O^6-methylguanine using HPLC separation of the ^{3}H-labeled adducts as we have described previously [28]. One femtomole of alkyltransferase activity is defined as removal of 1 fmol of O^6-methylguanine and is expressed as fmol/μg cellular DNA to compare the activity levels in different tissues and cells [27, 28].

Statistical analysis. *In vitro* synergy was determined for the experiments using the fixed ratio of 1:3 for O^6-benzylguanine and BCNU. Graphs relating the fraction affected to concentration were obtained by linear regression. The best curve fit within the range of concentrations used for these relationships yielded a linear model for the agents alone and the Weibull distribution for the combination. The method for the assessment of synergy described by Berenbaum [26] was used to calculate the isoboles and the combination index [$CI = d_a/D_a + d_b/D_b$] where d is the concentration of drug in the combination and D is the concentration of drug when used separately which causes the identical cytotoxic effect (fraction affected). The xenograft growth rate coefficient [β] was obtained by linear regression assuming an exponential growth rate according to the equation $N(t) = N_o e^{\beta t}$. The linear regression model related the natural logarithm of tumor volume to time for each treatment group.

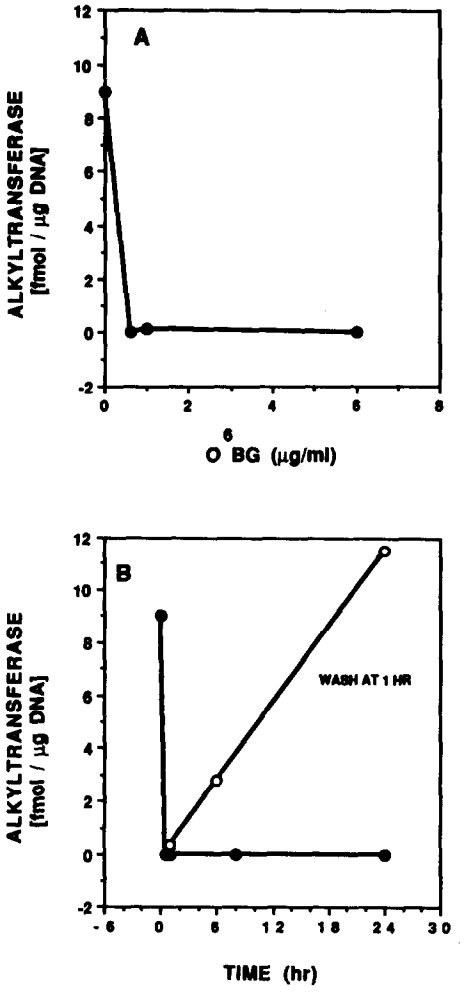


Fig. 1. O^6-Benzylguanine inhibition of VACO 6 alkyltransferase. (A) VACO 6 colon cancer cells in exponential growth phase were passaged into complete culture medium 2 days before addition of 0 to 6 μg/mL O^6-benzylguanine for 1 hr at 37°. At the end of the 1-hr incubation, cells were collected for analysis of residual alkyltransferase activity as described in the Materials and Methods. (B) VACO 6 cells were cultured in the presence of 0.5 μg/mL O^6-benzylguanine for up to 24 hr (●). In one set of cultures, the O^6-benzylguanine was removed at 1 hr by three washes in serum-containing medium (○). After 24 hr, the alkyltransferase in these cells stabilized at baseline levels (data not shown). For both experiments, results indicate the average of duplicate or triplicate determinations at each time point.

RESULTS

Inactivation of alkyltransferase by O^6-benzylguanine in colon cancer cells. We studied the inactivation kinetics of alkyltransferase by O^6-benzylguanine in the VACO 6 cell line (which we have described previously [24]) as shown in Fig. 1. As little as 0.5 μg/mL O^6-benzylguanine inactivated the alkyltransferase by over 97% within 1 hr (Fig. 1A). If the compound was left in the culture medium, this effect was persistent for at least 24 hr. When as much as 6 μg/mL O^6-benzylguanine was washed from the cells by repeated washing with serum-containing medium, regeneration of alkyltransferase activity occurred in a linear fashion with a $T_{1/2}$ of approximately 9 hr [Fig. 1B]. For this reason, we designed cytotoxicity studies which would maintain inactivation of the alkyltransferase for a prolonged period of time after removing the BCNU from the medium. This was done to prevent repair of the

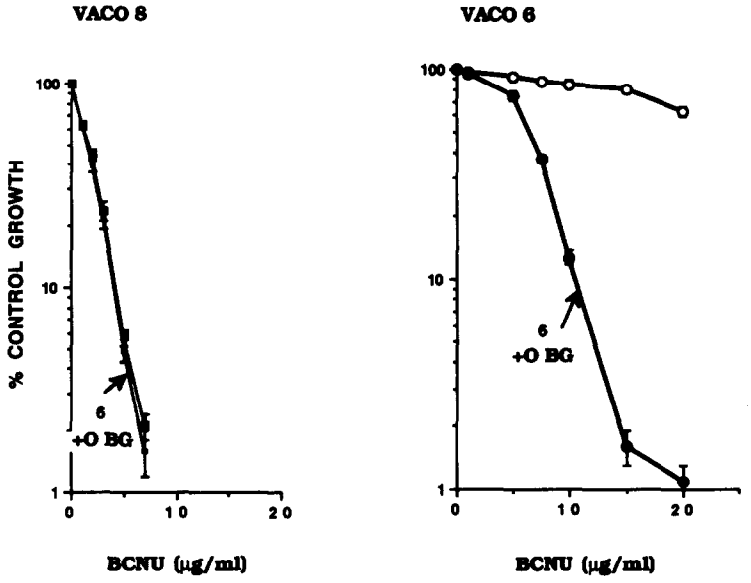


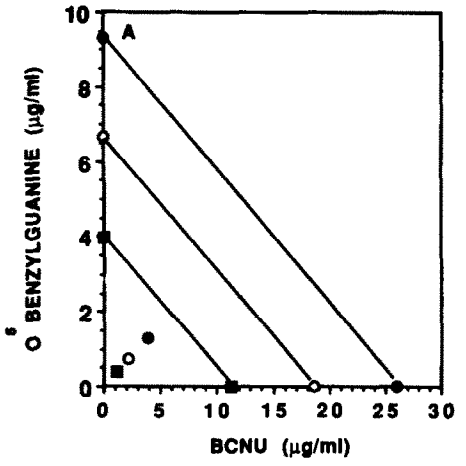
Fig. 2. Effect of O^6-benzylguanine on BCNU cytotoxicity. As described in Materials and Methods, VACO 8 or VACO 6 cells, were plated at low density in a cell regrowth assay [24, 25]. O^6-Benzylguanine 0.5 μg/mL was added to one half of the cultures followed 1 hr later by BCNU added to sets of cultures at the concentrations shown. Both drugs were removed 1 hr later, and the O^6-benzylguanine [O^6BG] was again added to the appropriate plates. Cell counts were measured at 5 days. Results indicate the means ± SD of triplicate determinations of one of three replicate experiments.

BCNU-cross-link precursor which is present for up to 8–14 hr [5]. However, because continuous exposure to 1–6 μg/mL O^6-benzylguanine for 2–5 days was cytotoxic to the VACO 6 cell line, causing between a 20 and 50% inhibition of cell growth, continuous exposure to a concentration of 0.5 μg/mL (which was nontoxic after 5 days) was used for the cytotoxicity studies.

Modulation of BCNU resistance in vitro. The effect of 0.5 μg/mL O^6-benzylguanine on cell survival after BCNU exposure was measured in the VACO 6 cell line and the VACO 8 cell line, which has undetectable alkyltransferase activity [24]. Figure 2 shows that O^6-benzylguanine markedly enhanced the cytotoxicity of BCNU in the VACO 6 cell line but not in the VACO 8 cell line. Thus, for VACO 6, the IC_{50} and IC_{90} values with and without O^6-benzylguanine were: > 20 μg/mL vs 7.6 μg/mL and >> 20 μg/mL vs 10.5 μg/mL, whereas for the VACO 8 cell line the IC_{50} and IC_{90} were 2.5 and 4 μg/mL. This indicates that complete inactivation of the alkyltransferase will modulate BCNU resistance in colon cancer cells and that the mechanism is specific because increased toxicity was not observed in the cell line which lacks alkyltransferase activity. O^6-Benzylguanine was able to reduce the resistance to BCNU in the VACO 6 cell line to within 2-fold of that of the very sensitive VACO 8 cell line. The concentration modification factor for O^6-benzylguanine combined with BCNU in the VACO 6 cell line was approximately 3- to 4-fold.

Analysis of synergy. To analyze the synergy between O^6-benzylguanine and BCNU, the two compounds were used separately or together at concentrations which represented a fixed ratio [1:3] between the two which was approximately the ratio of the IC_{50} of the two compounds alone. For these studies the IC_{50} for continuous exposure to O^6-benzylguanine was approximately 6.7 μg/mL and for exposure to BCNU was approximately 18.6 μg/mL. A separate set of experiments was performed, as outlined in Materials and Methods, and results, while consistent with, were not drawn from Fig. 2. Isobole (Fig. 3A) and combination index analyses [26] (Fig. 3B) identified the marked synergy between O^6-benzylguanine and BCNU. Isobole analysis shows that the combination of O^6-benzylguanine and BCNU produced cytotoxicity at much lower doses than expected if the drugs simply had an additive effect. In addition, the combination index was well below 1 at all concentrations of drug tested, another measure of synergy [26].

Inactivation of xenograft alkyltransferase by O^6-benzylguanine. Xenograft-bearing athymic mice were treated with O^6-benzylguanine at 30 or 60 mg/kg to develop a dosing schedule which would optimize inactivation of alkyltransferase before and after administration of BCNU. The goal was to maintain inactivation of alkyltransferase for approximately 24 hr to allow maximal BCNU-induced cross-linking to take place. In Fig. 4, the effect of a single dose of either 30 or 60 mg/kg O^6-benzylguanine was studied in the VACO 6 xenograft and in the liver of tumor bearing animals. The 60 mg/kg dose had a slightly greater effect than the 30 mg/kg dose on the duration of alkyltransferase inactivation in both xenograft and liver. The 60 mg/kg dose produced complete inactivation through



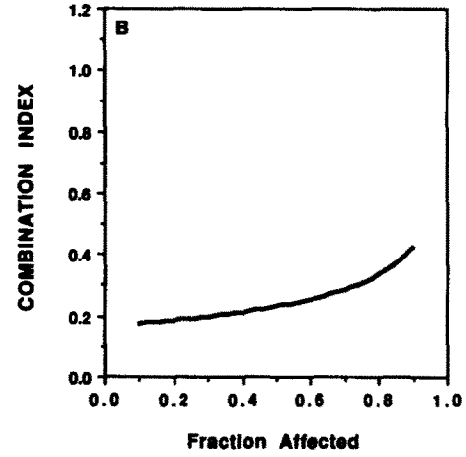
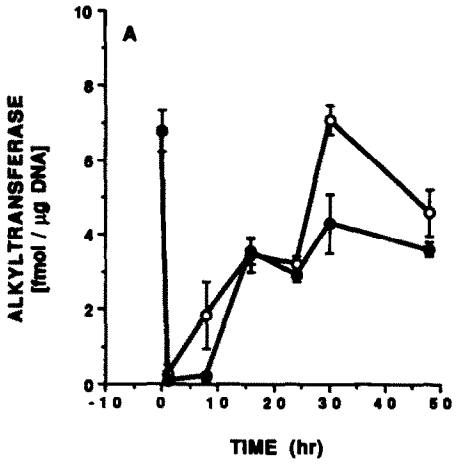


Fig. 3. Analysis of synergy between O^6-benzylguanine and BCNU in VACO 6 cells. VACO 6 cells were exposed to either O^6-benzylguanine, BCNU or the combination at a fixed ratio of 1:3 in the cell regrowth assay. A total of six different concentrations of each drug were analyzed (18 drug concentration combinations in all). Cell survival was analyzed for synergistic interactions between the two compounds and graphed as the isobole (A) and as the combination index (B). Synergy is present when the observed toxicity is greater than the line of additivity in the isobole analysis and when the observed combination index is less than 1 at all doses tested. Isoboles were calculated at the inhibitory concentration IC_{30} (■) IC_{50} (○) and IC_{70} (●) for each drug or the combination. The symbols at the lower left corner of panel A indicate the observed survival for combinations of the two drugs at the concentrations which yielded the IC_{30}, IC_{50}, and IC_{70}, respectively, and show the marked synergy between the two compounds.



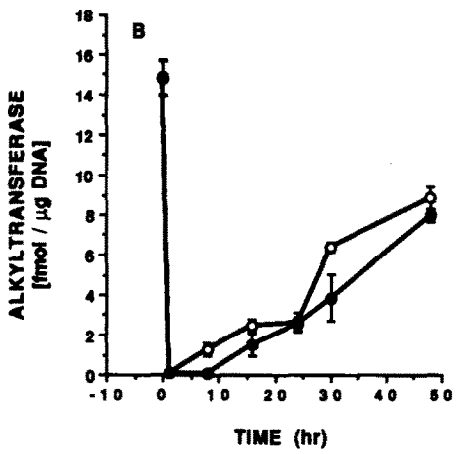


Fig. 4. Inactivation of alkyltransferase by O^6-benzylguanine in liver and the VACO 6 xenograft. VACO 6 xenograft bearing athymic mice (two xenografts per mouse) were treated with either 30 or 60 mg/kg O^6-benzylguanine at time 0 and groups of two mice were killed at each time point at each dose. Alkyltransferase activity was measured in both liver (A) and VACO 6 xenograft (B). Values indicate the means ± SEM of duplicate determinations of each of four xenografts per point. Key: (○) 30 mg/kg O^6-benzylguanine; and (●) 60 mg/kg O^6-benzylguanine.

the 8-hr time point but at 16 hr regeneration was seen in both tissues. The rate of regeneration was somewhat greater in the liver than in the xenograft, reaching 50% of control at 16 hr in the liver of mice treated at both doses of O^6-benzylguanine. In contrast, at this time point the xenograft alkyltransferase recovered to only 16% of control in mice receiving 30 mg/kg and only 10% of control in mice receiving the 60 mg/kg dose. However, the absolute levels of alkyltransferase in the tissues were similar during recovery, and the apparent selective effect on the tumor was offset by the lower basal level of activity in the liver. To prolong the inactivation of the alkyltransferase following O^6-benzylguanine exposure, which is necessary to prevent regenerating alkyltransferase from repairing the pre-cross-link lesion formed by BCNU, two doses of 60 mg/kg were administered at 0 and 8 hr. Under these conditions, xenograft and hepatic alkyltransferase was inactivated by more than 98% for 24 hr (Fig. 5), by which time BCNU-induced cross-link formation is complete [5]. This dose was chosen for

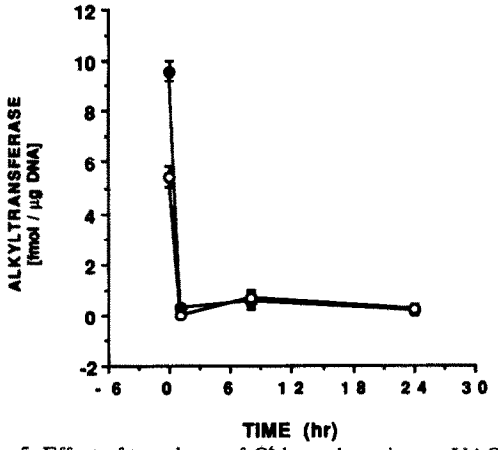


Fig. 5. Effect of two doses of O^6-benzylguanine on VACO 6 xenograft and hepatic alkyltransferase. VACO 6 xenograft bearing athymic mice were treated with 60 mg/kg O^6-benzylguanine at 0 and 8 hr and killed at the time shown. Xenografts were excised and assayed for alkyltransferase activity. Values indicate means ± SEM of duplicate determinations of four xenografts per point from one of two representative experiments. Key: (●) VACO 6 xenograft, and (○) liver.

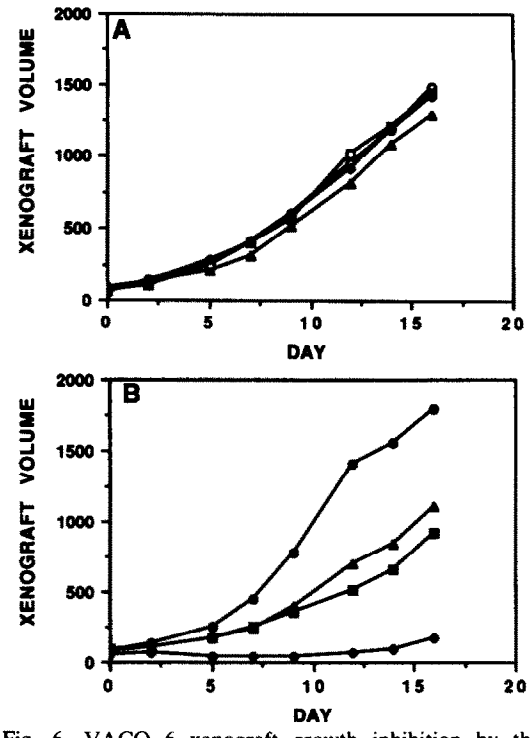


Fig. 6. VACO 6 xenograft growth inhibition by the combination of BCNU and O^6-benzylguanine in athymic mice. VACO 6 bearing athymic mice were treated as described in Materials and Methods with either vehicle, BCNU, O^6-benzylguanine or the combination. In panel A, mice received vehicle (O), or BCNU at 30 mg/m^2 (△), 50 mg/m^2 (□), or 70 mg/m^2 (◇). In panel B, mice received 60 mg/kg O^6-benzylguanine alone (●) at 0 and 8 hr, or the combination of O^6-benzylguanine and 30 mg/m^2 (▲), 50 mg/m^2 (■), 70 mg/m^2 (◆) BCNU. Points represent the means of ten xenografts. Using an exponential growth model as described in Table 1, significantly decreased growth rates were observed in xenografts of mice treated with the combination of O^6-benzylguanine and 30 mg/m^2 ($P < 0.01$), 50 mg/m^2 ($P < 0.001$), or 70 mg/m^2 ($P < 0.001$) BCNU over that of mice receiving either O^6-benzylguanine or BCNU alone.

an analysis of the efficacy of the combination *in vivo*.

Overcoming BCNU resistance in the VACO xenograft. Athymic mice bearing the VACO 6 xenograft were treated with vehicle alone, two doses of 60 mg/kg O^6-benzylguanine at 0 and 8 hr, BCNU at 30, 50, 70 or 100 mg/m^2 at 1 hr or the combination of O^6-benzylguanine and BCNU 30–70 mg/m^2, Tumor measurements were performed over a 16-day period in three separate experiments containing 5–10 mice in each group at each dose. Tumor responses are shown in Fig. 6, and the growth rate coefficient is shown in Table 1. At maximum tolerated doses of BCNU (70–100 mg/m^2), the VACO 6 xenograft remained completely unresponsive. O^6-Benzylguanine alone also had no significant effect on xenograft growth. The combination of O^6-benzylguanine and 30, 50 or 70 mg/m^2 BCNU resulted in tumor responses and significant decreases in xenograft tumor growth rates ($P < 0.01$ and $P < 0.001$, respectively) compared to O^6-benzylguanine alone. The tumor growth curves of mice treated with the combination were similar to those observed with the alkyltransferase deficient, BCNU-sensitive cell line, VACO 8 (data not shown), suggesting that BCNU resistance had been overcome. Thus, a colon cancer xenograft which is completely *resistant* to BCNU can be *sensitized* to BCNU by administration of O^6-benzylguanine.

This treatment was not without toxicity. Table 2 shows an analysis of weight loss and animal survival in each group of mice. At 70 mg/m^2 BCNU alone, there was a mean of 8.5 ± 1.7% weight loss but no deaths were seen. At 100 mg/m^2 BCNU, there was a mean of 2.9 ± 0.7% weight loss, and no deaths; and at 130 mg/m^2 BCNU all mice died. Using the combination of O^6-benzylguanine and BCNU, the maximum weight loss increased to 20.2 ± 2.0% in animals treated with 50 mg/m^2 BCNU and death was noted in 2/18 mice. More severe toxicity was noted in mice receiving O^6-benzylguanine and 70 mg/m^2 BCNU, where 11/16 mice died. Thus, as anticipated, the combination of O^6-benzylguanine and BCNU resulted in increased toxicity to the host animal. Nonetheless, tolerable doses of the combination produced significant tumor responses in a colon tumor xenograft which was otherwise completely resistant to maximum tolerated doses of BCNU.

DISCUSSION

The essence of biochemical modulation of tumor drug resistance is the ability to identify synergy *in vitro* and selectivity *in vivo*. While it has been clear that a high degree of correlation exists between alkyltransferase activity and nitrosourea resistance

Table 1. Growth inhibition of VACO 6 xenografts

Treatment*		Growth rate coefficient		
		Experiment		
O^6BG (mg/kg)	BCNU (mg/m^2)	I	II	III
0	0	0.19 ± 0.01† (5/5)‡	0.19 ± 0.01 (5/5)	
60	0	0.21 ± 0.01 (5/5)	0.22 ± 0.02 (5/5)	0.17 ± 0.01 (10/10)
60	30	0.16 ± 0.01§ (5/5)	0.15 ± 0.01§ (5/5)	
60	50	0.13 ± 0.01‖ (6/6)	0.13 ± 0.01‖ (5/5)	0.08 ± 0.01‖ (6/7)
60	70	0.06 ± 0.01‖ (4/6)		
0	30	0.19 ± 0.02 (5/5)		
0	50	0.20 ± 0.01 (5/5)		
0	70	0.18 ± 0.01 (6/6)		
0	100	0.17 ± 0.01 (10/10)		

* O^6-Benzylguanine (O^6BG) was administered at 60 mg/kg, i.p., at 0 and 8 hr; BCNU was given at 1 hr, i.p.

† Values are means ± SD.

‡ Number of survivors/number mice treated.

§,‖ Significantly different from O^6BG treatment alone: §$P < 0.01$, and ‖$P < 0.001$.

Table 2. Tolerance summary

Treatment*			
O^6BG (mg/kg)	BCNU (mg/m^2)	Number survivors/ number treated	Maximum weight loss† (% day 1 weight)
0	0	15/15	3.6 ± 0.9
0	30	10/10	4.1 ± 1.3
0	50	10/10	10 ± 2.7
0	70	20/20	8.5 ± 1.7
0	100	14/14	2.9 ± 0.7
0	130	0/4	NA
60	0	20/20	7.8 ± 1.5
60	30	10/10	7.7 ± 1.5
60	50	16/18	20.2 ± 2.0
60	70	5/16	20.7 ± 3.0
60	100	0/5	NA

* O^6-Benzylguanine (O^6BG) was administered at 60 mg/kg, i.p., at 0 and 8 hr; BCNU was given at 1 hr, i.p.

† Values are means ± SD. NA = not applicable.

in many tumor types [7, 22, 29], it has remained unclear whether biochemical modulation of alkyltransferase activity could result in synergy between the biochemical modulator and the chemotherapeutic agent, BCNU, and whether selectivity could be identified in a xenograft model with a tumor which is completely resistant to BCNU at maximum tolerated doses. Both of these issues have been addressed successfully in this study which clearly shows that O^6-benzylguanine, a potent new inhibitor of alkyltransferase activity [19, 20], potentiated the cytotoxicity of BCNU *in vitro* and promoted tumor responses in the xenograft setting.

The unique activity of the alkyltransferase suggests that it is an ideal target for biochemical modulation. Its exclusive repair of lesions at the O^6-position of guanine in double stranded DNA—which are formed by mono- and bifunctional nitrosoureas and which have been shown by a number of investigators to be a major cytotoxic lesion induced by these compounds [7, 8, 30]—allows a much better appreciation of the effects of biochemical modulation than may occur with other mechanisms of drug resistance such as those which involve such complex mechanisms as drug uptake and efflux, DNA synthesis and intermediary metabolism. Many studies have shown increased cytotoxicity following partial inactivation of the alkyltransferase by O^6-methylguanine, yet the effects have been limited by persistent activity of the alkyltransferase due to the low affinity of the alkyltransferase for O^6-methylguanine relative to that of the DNA adduct.

The recent discovery of O^6-benzylguanine as a potent inhibitor of the alkyltransferase allowed a more thorough evaluation of targeted inactivation

of the enzyme and its effects on tumor drug resistance. It is clear from our studies and those of Dolan and co-workers [20, 22] that the increased efficacy of O^6-benzylguanine is in line with its increased affinity for the alkyltransferase and its ability to inactivate the protein completely. It further suggests that the modest effects we have observed previously with O^6-methylguanine were due to persistent activity of the enzyme which was sufficient to repair persistent, potentially cytotoxic DNA adducts.

In a previous report we found a high degree of correlation between alkyltransferase activity and BCNU resistance in a series of human colon cancer cell lines [24]. Based on a linear regression analysis, we predicted that complete inactivation of the alkyltransferase should result in a decrease in the IC_{50} for the VACO 6 cell line from approximately 20 μg/mL to approximately 3–5 μg/mL. As shown in Fig. 2, the IC_{50} was reduced to approximately 7 μg/mL, suggesting that there may be other mechanisms responsible for BCNU resistance in the VACO cell line which become apparent once alkyltransferase activity is blocked. Alternatively, it suggests that there may be low level residual alkyltransferase activity and/or drug protection afforded by continued synthesis of the enzyme. In an effort to measure regeneration of the alkyltransferase, O^6-benzylguanine was washed away from the cells, and in contrast to the studies by Dolan *et al.* [19], we found that alkyltransferase in VACO 6 was regenerated with a half-life of approximately 9 hr. This difference may be due to the presence of serum in the washing medium and/or a high level of alkyltransferase mRNA and a high translation rate of alkyltransferase in VACO 6 cells. There are two implications of this observation. First, because the rate of regeneration of alkyltransferase is linear after removal of O^6-benzylguanine, it appears that the compound can be effectively removed despite its lipophilicity. Second, because alkyltransferase is constitutively synthesized in VACO 6, alkyltransferase which is newly synthesized after BCNU exposure may preferentially repair the O^6-chloroethylguanine adduct or the N^1,O^6-ethanoguanine adduct rather than be inactivated by a residual O^6-benzylguanine molecule. This may explain why the VACO 6 line remains more resistant than the VACO 8 line to BCNU, despite biochemical evidence of modulation. Based on this, we designed both *in vitro* and *in vivo* treatment schedules to maintain high concentrations of O^6-benzylguanine after BCNU exposure.

Our study provides further evidence that a tumor completely resistant to BCNU in the xenograft model can become responsive after O^6-benzylguanine treatment. While increased treatment-related toxicity was observed, including death with higher doses of the combination of BCNU and O^6-benzylguanine, the fact that significant tumor responses were seen at tolerable doses suggests that increased selectivity in tumor response has been achieved. This also suggests that other drug resistance mechanisms are not dominant in the *in vivo* setting and that *in vitro* responses are predictive of *in vivo* effects, at least for this drug resistance mechanism.

In xenograft modeling of biochemical modulation, we attempted to achieve complete inactivation of the tumor alkyltransferase for 24 hr to allow maximal levels of cross-linking to take place by BCNU-induced DNA adducts. Brent *et al.* [5] have shown previously that in cell free systems, conversion of alkyltransferase-repairable adducts to alkyltransferase-unrepairable cross-links continues to occur for a minimum of 8–14 hr after addition of the chloroethylating agent. There are no data to indicate the time course of the cross-link reaction in tissue culture cells or *in vivo* but one would anticipate a somewhat longer time course *in vivo*, perhaps due to drug sequestration and distribution. Thus, to achieve maximal effect, we chose a 24-hr depletion period. While animals tolerated 60 mg/kg O^6-benzylguanine at 0 and 8 hr, it is clear that inactivation of alkyltransferase in other organs, such as liver, was increased and prolonged as well. While we observed a small degree of tumor versus liver selectivity in alkyltransferase depletion with single doses of either 30 or 60 mg/kg O^6-benzylguanine, the two-dose schedule may have contributed to the increased toxicity observed at higher BCNU doses. Further exploitation of differences in regeneration of alkyltransferase between target tumors and normal tissues may improve the therapeutic index of this combination. In addition, studies of the effect of O^6-benzylguanine on alkyltransferase activity in other tissues which are targets for BCNU toxicity, such as lung and bone marrow, are important in efforts to optimize the use of alkyltransferase inhibitors *in vivo*.

In summary, these studies show that O^6-benzylguanine is an effective inhibitor of the alkyltransferase in human colon cancer, both *in vitro* and *in vivo*. O^6-Benzylguanine is synergistic with BCNU in inducing increased cytotoxicity and is able to induce tumor responses in a colon cancer tumor which is completely resistant to BCNU alone. Full evaluation of the selectivity of this approach and whether or not improved efficacy can be expected in clinical trials await further development of this drug and a better understanding of its pharmacokinetics, tissue distribution and tissue regeneration of alkyltransferase following its depletion.

Acknowledgements—Dr. Robert Moschel is thanked for providing generous amounts of O^6-benzylguanine, which allowed us to perform these studies. We also thank Drs. Anthony Pegg and Eileen Dolan for their very helpful discussions during the early phases of this work. This study was supported in part by Grants P01CA51183 and P30CA43703 from the National Institutes of Health and CN-34 from the American Cancer Society.

REFERENCES

1. Kohn KW, Interstrand cross-linking of DNA by 1,3-bis(2-chloroethyl)-1-nitrosourea and other 1-(2-haloethyl)-1-nitrosoureas. *Cancer Res* **37**: 1450–1454, 1977.
2. Tong WP, Kirk MC and Ludlum DB, Formation of the cross-link 1-[N^3-deoxycyctidyl], 2-[N^1-deoxyguanosinyl]-ethane in DNA treated with N,N'-bis(2-chloroethyl)-N-nitrosourea. *Cancer Res* **42**: 3102–3105, 1982.

3. Bogden JM, Eastman A and Bresnick E, A system in mouse liver for the repair of O^6-methylguanine lesions in methylated DNA. *Nucleic Acids Res* **9**: 3089–3103, 1981.
4. Pegg AE, Mammalian O^6-alkylguanine-DNA alkyltransferase: Regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* **50**: 6119–6129, 1990.
5. Brent TP, Lestrud SO, Smith DG and Remack JS, Formation of DNA interstrand cross-links by the novel chloroethylating agent 2-chloroethyl(methylsulfonyl)-methanesulfonate: Suppression by O^6-alkylguanine–DNA alkyltransferase purified from human leukemic lymphoblasts. *Cancer Res* **47**: 3384–3387, 1987.
6. Gonzaga PE and Brent TP, Affinity purification and characterization of human O^6-alkylguanine-DNA alkyltransferase complexed with BCNU-treated, synthetic oligonucleotide. *Nucleic Acids Res* **17**: 6581–6590, 1989.
7. Scudiero DA, Meyer SA, Clatterbuck BE, Mattern MR, Siolkowski CHJ and Day RS III, Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylating agents including chloroethylnitrosoureas. *Cancer Res* **44**: 2467–2474, 1984.
8. Zlotogorski C and Erickson LC, Pretreatment of human colon tumor cells with DNA methylating agents inhibits their ability to repair chloroethyl monoadducts. *Carcinogenesis* **5**: 83–87, 1984.
9. Bodell WJ, Aida T, Berger MS and Rosenblum ML, Increased repair of O^6-alkylguanine DNA adducts in glioma-derived human cells resistant to the cytotoxic and cytogenetic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea. *Carcinogenesis* **7**: 879–883, 1986.
10. Yarosh DB, Foote RS, Mitra S and Day RS III, Repair of O^6-methylguanine in DNA by demethylation is lacking in Mer$^-$ human tumor cell strains. *Carcinogenesis* **4**: 199–205, 1983.
11. Brent TP, Houghton PJ and Houghton JA, O^6-Alkylguanine-DNA alkyltransferase activity correlates with the therapeutic response of human rhabdomyosarcoma xenografts to 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea. *Proc Natl Acad Sci USA* **82**: 2985–2989, 1985.
12. Fujio C, Chang HR, Tsujimura T, Ishizaki K, Kitamura H and Ikenaga M, Hypersensitivity of human tumor xenografts lacking O^6-alkylguanine-DNA alkyltransferase to the anti-tumor agent 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea. *Carcinogenesis* **10**: 351–356, 1989.
13. Pieper RO, Futscher BW, Dong Q and Erickson LC, Effects of streptozotocin/bis-chloroethylnitrosourea combination therapy on O^6-methylguanine DNA methyltransferase activity and mRNA levels in HT-29 cells in vitro. *Cancer Res* **51**: 1581–1585, 1991.
14. Dolan ME, Morimoto K and Pegg AE, Reduction of O^6-alkylguanine-DNA alkyltransferase activity in HeLa cells treated wtih O^6-alkylguanines. *Cancer Res* **45**: 6413–6417, 1985.
15. Yarosh DB, Hurst-Calderone S, Babich MA and Day RS III, Inactivation of O^6-methylguanine-DNA methyltransferase and sensitization of human tumor cells to killing by chloroethylnitrosourea by O^6-methylguanine as a free base. *Cancer Res* **46**: 1663–1668, 1986.
16. Karran P, Possible depletion of a DNA repair enzyme in human lymphoid cells by subversive repair. *Proc Natl Acad Sci USA* **82**: 5285–5289, 1985.
17. Gerson SL and Trey JE, Modulation of nitrosourea resistance in myeloid leukemias. *Blood* **71**: 1487–1494, 1988.
18. Gerson SL, Trey JE and Miller K, Potentiation of nitrosourea cytotoxicity in human leukemia cells by chronic inactivation of O^6-alkylguanine-DNA alkyltransferase. *Cancer Res* **48**: 1521–1527, 1988.
19. Dolan ME, Moschel RC and Pegg AE, Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc Natl Acad Sci USA* **87**: 5368–5372, 1990.
20. Dolan ME, Stine L, Mitchell RB, Moschel RC and Pegg AE, Modulation of mammalian O^6-alkylguanine-DNA alkyltransferase in vivo by O^6-benzylguanine and its effect on the sensitivity of a human glioma tumor to 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea. *Cancer Commun* **2**: 371–377, 1990.
21. Mitchell RB, Moschel RC and Dolan ME, Effect of O^6-benzylguanine on the sensitivity of human tumor xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea and on DNA interstrand cross-link formation. *Cancer Research* **52**: 1171–1175, 1992.
22. Dolan ME, Pegg AE, Dumenco LL, Moschel RC and Gerson SL, Comparison of the inactivation of mammalian and bacterial O^6-alkylguanine-DNA alkyltransferases by O^6-benzylguanine and O^6-methylguanine. *Carcinogenesis* **12**: 2305–2309, 1991.
23. Phillips GL, Fay JW, Herzig GP, Herzig RH, Weiner RS, Wolff SN, Lazarus HM, Karanes C, Ross WE, Kramer B and the Southeastern Cancer Study Group, Intensive 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), NSC #4366650 and cryopreserved autologous marrow transplantation for refractory cancer: A phase I–II study. *Cancer* **52**: 1792–1802, 1983.
24. Gerson SL, Berger NA, Arce C, Petzold S and Willson JKV, Modulation of nitrosourea resistance in human colon cancer by O^6-methylguanine. *Biochem Pharmacol* **43**: 1101–1107, 1992.
25. McBain, JA, Weese JL, Meisner LF, Wolberg WH and Willson JKV, Establishment and characterization of human colorectal cancer cell lines. *Cancer Res* **44**: 5813–5821, 1984.
26. Berenbaum MC, What is synergy? *Pharmacol Rev* **41**: 93–141, 1989.
27. Dexter BU, Yamashita T, Donovan C and Gerson SL, Modulation of O^6-alkylguanine-DNA alkyltransferase in rats following intravenous administration of O^6-methylguanine. *Cancer Res* **49**: 3520–3524, 1989.
28. Gerson SL, Trey J, Miller K and Berger NA, Comparison of O^6-alkylguanine-DNA alkyltransferase activity based on cellular DNA content in human, rat and mouse tissues. *Carcinogenesis* **7**: 745–749, 1986.
29. Tsujimura T, Zhang Y-p, Fujio C, Chang H-r, Watatani M, Ishizaki K, Kitamura H and Ikenaga M, O^6-methylguanine methyltransferase activity and sensitivity of Japanese tumor cell strains to 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride. *Jpn J Cancer Res* **78**: 1207–1215, 1987.
30. Dolan M E, Pegg AE, Hora NK and Erickson LC, Effect of O^6-methylguanine on DNA interstrand cross-link formation by chloroethylnitrosoureas and 2-chloroethyl (methylsulfonyl) methanesulfonate. *Cancer Res* **48**: 3603–3606, 1988.