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Preclinical Antitumor Activity of Temozolomide in Mice: Efficacy against Human Brain Tumor Xenografts and Synergism with 1,3-Bis(2-chloroethyl)-1-nitrosourea[1]

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ABSTRACT

Temozolomide, a methylating agent with clinical activity against brain tumors, demonstrated excellent antitumor activity following p.o. administration to athymic mice bearing human brain tumor xenografts. In the early stage s.c. implanted SNB-75 astrocytoma model, a 400-mg/kg dose administered on Day 5 produced 10 of 10 Day 54 tumor-free mice. In later staged s.c. U251 and SF-295 glioblastoma models, a single 600-mg/kg dose produced 9 of 10 Day 86 and 2 of 10 Day 40 tumor-free mice, respectively. In the latter group, a tumor growth delay of >315% was attained. Similar levels of activity were attained with equal total doses on schedules of daily for 5 doses and every fourth day for 3 doses. A single 40-mg/kg i.v. dose of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) also demonstrated excellent activity, producing 9 of 10 tumor-free mice in the SNB-75 model and growth delays of 283 and 301% in the U251 and SF-295 models, respectively. Temozolomide was also highly effective against intracerebral implants of the U251 and SF-295 glioblastomas. Administration of either 600 mg/kg on Day 1 or 200 mg/kg on Days 1, 5, and 9 produced 7 of 9 Day 90 tumor-free mice in the U251 model. In the SF-295 model, a single 400-mg/kg dose or three 200-mg/kg doses produced 3 and 4 of 10 Day 90 tumor-free mice, respectively, and prolonged survival by 127%. A single 40-mg/kg i.v. dose of BCNU was more effective than temozolomide in the intracerebral SF-295 model, and less effective in the intracerebral U251 model. The synergistic potential of temozolomide and BCNU in combination was evaluated in an advanced stage s.c. implanted SF-295 model. When temozolomide was administered 2 h after BCNU on a single treatment day, a dramatic synergistic therapeutic effect was observed in two experiments. For example, single agent doses of temozolomide (600 mg/kg) and BCNU (60 mg/kg) and a combination (400 mg/kg + 27 mg/kg) demonstrating equivalent toxicity produced growth delays of 190, 258, and >492% (includes 5 of 10 Day 51 tumor-free mice), respectively. Analysis of the data by a quadratic dose response model indicated synergism with significance at $P = 0.0001$ in both experiments. Synergism also was demonstrated by the isobole method. The reverse sequence was more toxic, but at lower combination doses a synergistic effect was still observed ($P = 0.0001$). Using the quadratic model, no confirmed modulation of the antitumor activity of temozolomide was demonstrated by i.p. administration of either 10 or 30 mg/kg O^6-benzylguanine 1 h before or 1 h after temozolomide. These data provide a rationale for the clinical evaluation of temozolomide and BCNU combinations in patients with brain tumors.

INTRODUCTION

Temozolomide[3] is an imidazotetrazinone derivative that has demonstrated preclinical antitumor activity against a broad spectrum of murine tumors *in vivo*, including leukemias, lymphomas, and solid tumors (1). Activity was observed following both i.p. and p.o. treatments, and pharmacokinetic data indicated good bioavailability and rapid absorption following p.o. administration to mice (1). Temozolomide is believed to exert its antitumor effects through formation of MTIC, the putative active metabolite of the structurally related clinical dimethyltriazene, DTIC (1, 2). However, unlike DTIC, which is transformed to MTIC by oxidative *N*-demethylation, an enzymatic process subject to species differences, temozolomide forms MTIC through chemical decomposition under mild alkaline conditions (1, 2). Although the mechanism of action has not been elucidated fully, it is assumed that DNA methylation is involved and that alkylation of specific base positions is more critical for cytotoxicity than the overall level of reaction with DNA (3). Comparative studies conducted with sublines of L1210 leukemia cells sensitive and resistant to BCNU and treated with temozolomide indicated that DNA alkali-labile sites, possibly caused by removal of 7-methylguanine by 7-methylguanine-DNA glycosylase, were not crucial to the cytotoxic effects of temozolomide (4). However, differences in AGT activity between the two sublines suggested that methylation of the O^6 position of guanine was the important determinant for cytotoxicity (4). The importance of O^6-methylation also was indicated in other studies in which sensitivity of several murine and human tumor cell lines (5, 6) and blasts from acute myelogenous leukemia patients (7) correlated with low levels of AGT. Measurements of 3-methyladenine-DNA glycosylase in tumor cell lines suggested that methylation of adenine was not an important cytotoxic lesion (8).

Clinical evaluation of temozolomide is in progress. In the United Kingdom, activity was detected in a Phase I trial involving p.o. administration daily for 5 doses (9). Included among the responders were two patients with recurrent high-grade gliomas. In a subsequent Phase II trial involving patients with primary brain tumors, major improvements in computer tomography scans were noted in 5 of 10 patients with recurrent astrocytomas and in 4 of 7 patients with newly diagnosed high-grade astrocytomas (10). Irrespective of the mechanism by which temozolomide exerts its antitumor effects, this promising activity observed in patients with brain tumors will make temozolomide a candidate for combination chemotherapy, especially with chloroethylating nitrosoureas. The nitrosoureas are the most effective drugs currently used to treat brain tumor patients but, although their effect is palliation of the disease by shrinking of tumors, the responses are of relatively short duration and current combination chemotherapy does not produce convincing evidence of improved activity (11). The combination of chloroethylnitrosoureas with a methylating agent has precedence. The streptozotocin/BCNU combination is being evaluated in patients because of

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[3] The abbreviations used are: temozolomide, 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazine-4(3*H*)-one, also known as NSC 362856, CCRG 81045, and SCH 52365; MTIC, 5-(3-methyl)-1-triazen-1-yl)imidazole-4-carboxamide; DTIC (dacarbazine), 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide; AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; BCNU (carmustine), 1,3-bis(2-chloroethyl)-1-nitrosourea; DTP, Developmental Therapeutics Program; NCI, National Cancer Institute; i.c., intracerebral.

the ability of streptozocin to methylate the O^6 position of guanine (12). Rapid repair of the streptozocin-formed O^6-methylguanine by AGT depletes AGT levels allowing BCNU-induced DNA cross-links to form, thereby sensitizing cells to BCNU. The ability of temozolomide to sensitize tumor cells to diethyl-1,3-(2-chloroethyl)-3-nitrosoureidoethyl phosphonate and to BCNU, presumably through the same mechanism, has been demonstrated *in vitro* (13, 14). AGT levels also affect temozolomide-induced cytotoxicity (4–6). Thus, AGT-depleting agents might augment the antitumor effects of temozolomide. Sensitization of tumor cells to temozolomide has been demonstrated by O^6-methylguanine and BG *in vitro* (5, 6).

The studies presented in this paper were conducted to explore the potential antitumor efficacies of combinations involving temozolomide. Initially, the response of human brain tumor xenografts to temozolomide was determined in athymic nude mice. Upon demonstration of antitumor efficacy in these models, the synergistic potential of temozolomide in combination with either BCNU or BG was evaluated.

MATERIALS AND METHODS

Temozolomide was supplied by the Pharmaceutical Resources Branch, DTP, Division of Cancer Treatment, NCI following synthesis by Aerojet Strategic Propulsion, Sacramento, CA, under a contract to DTP. BCNU was supplied by the Drug Synthesis and Chemistry Branch, DTP, and BG was kindly supplied by Dr. R. C. Moschel, NCI-Frederick Cancer Research and Development Center, Frederick, MD. For administration to mice, temozolomide was prepared fresh daily as a suspension in aqueous hydroxypropyl cellulose (Klucel) and used within 15 min. Klucel was prepared free of silicon dioxide and was kindly donated to DTP by Hercules Powder Company, Inc., Wilmington, DE. BCNU was prepared fresh daily in a 0.9% NaCl solution containing 2% ethanol. Solutions were kept on ice and used within 45 min. BG was solubilized in aqueous 40% PEG 400 (Sigma).

Mice and Tumors. Athymic NCr-*nu/nu* mice were obtained from the NCI animal program, Frederick, MD, and housed in sterile, polycarbonate, filter-capped microisolator cages (Lab Products, Inc.). They were maintained in a barrier facility on 12-h light/dark cycles, and they were provided with sterilized food and water *ad libitum*.

Human tumor lines were obtained from the NCI Tumor Repository, Frederick, MD. Frozen cells were thawed, cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone), and harvested for i.c. or s.c. implantation into athymic mice. The resulting solid tumors from s.c. implants were maintained by serial *in vivo* passage of tumor fragments.

***In Vivo* Models.** Antitumor evaluations were conducted through the DTP *in vivo* screening program. For s.c. models, tumor fragments (about 30 mg each) were implanted into the axillary region of athymic mice on Day 0. Treatment was initiated either when tumors reached specified weight ranges indicated in the tables (advanced stage model) or when tumors were just palpable (early stage model). Tumor weights were calculated from *in situ* caliper measurements of tumor length and width in mm using the formula for a prolate ellipsoid (15). Tumor size and body weights were recorded approximately twice a week. Generally, tumor size was monitored until an upper weight limit approaching 5000 mg was attained. For i.c. models, a 25-gauge needle was used to inject either 10^6 U251 or 10^5 SF-295 human glioblastoma cells into the right cerebral hemisphere. Treatment was initiated on Day 1. Body weights were recorded approximately twice weekly until 4 days after treatment had ended. Mortality was monitored daily, and mice were sacrificed if moribund. Titration groups were included to establish a tumor-doubling time for use in $\log_{10}$ cell kill calculations (16).

Temozolomide and BCNU were administered by gavage and by i.v. injection, respectively, at multiple dose levels according to schedules and time intervals indicated in the tables. BG was administered i.p. at 30 and 10 mg/kg. The former BG treatment has been shown to augment the activity of BCNU against s.c. implanted human glioblastomas (17). In the combination studies, mice receiving a single agent were also given the vehicle of the second agent such that all mice received identical handling. All treatments were administered on the basis of exact body weight (0.1 ml/10 g) to groups of 5 or 10 mice; 20 vehicle-treated tumored control mice were included in each experiment.

Evaluation of Anticancer Activity. For s.c. models, antitumor activity was assessed on the basis of tumor growth delay, tumor-free survivors, and for the advanced stage model only, tumor regressions. Growth delay was expressed as a percentage by which the treated group was delayed in attaining a specified number of doublings compared to controls using the formula

$$100 \times \frac{(T - C)}{C}$$

where T and C are the median times in days for treated and control tumors, respectively, to attain the specified number of doublings. Both complete regressions and tumor-free survivors were defined by instances in which tumor weight decreased to below measurable limits (<63 mg) during the experimental period. The two parameters differed by observation of either tumor regrowth (complete regression) or no regrowth (tumor-free) prior to the final observation day. For i.c. models, antitumor activity was assessed from the percentage of increase in life span (based on median survival times of dying mice) and long-term survivors. For both models, an estimate of the number of net $\log_{10}$ units of cells killed at the end of treatment were calculated as

$$\frac{[(T - C) - \text{duration of treatment}] \times 0.301}{\text{Median doubling time}}$$

where the doubling time either is calculated from the titration curve and T and C are median days of death for the treated and control mice, respectively (i.c. models) or is the time for tumors to increase in size from 200 to 400 mg and T and C are the median times for tumors to reach a specified number of doublings (s.c. models). If the duration of treatment is 0, then it can be seen from the formulae for net log cell kill and percentage of growth delay that log cell kill is proportional to percentage of growth delay.

Statistical Analysis. A quadratic regression analysis was performed to examine the changes in the percentage of growth delay with respect to the dose levels of the two drugs including their interaction (18). The model is

$$y = a_1d_1 + a_2d_2 + a_3d_1^2 + a_4d_2^2 + a_5d_1d_2 + e$$

where y is the percentage of growth delay, d_1 and d_2 are, respectively, the dose levels for BCNU and temozolomide, and e is the random error term. The model is based on the hypothesis that the tumor size is reduced to a particular fraction of its original size during treatment, that after treatment the tumor regrows at the same rate as the control tumors, and that it takes a particular time (measured by $T - C$) for the tumor size of the treated mice to "recover" to the original size of the controls (after which it takes an additional time C to reach the specified number of doublings). Using an end point of time to 3 doublings, a drug that results in a 100% growth delay, according to this model, must be reducing tumor size by a factor of 8. If a drug results in a 100% growth delay and a second drug, which acts independently of the first one, also produces a 100% growth delay, then both drugs acting together would result in a reduction in the tumor size by a factor of 64 (and would result in a 200% growth delay), where the tumor size reductions combine multiplicatively and the percentage of growth delays combine additively. This model is known as the "survivor fraction multiplication" model (19). The rationale for including the quadratic terms in the model is that increasing the dosage of the individual drugs beyond a certain level may be associated with a less than additive treatment effect. Inclusion of the interaction term in the model allows one to accommodate or test for potential antagonism or synergy between the two drugs.

The model-independent isobole method (19) was also used to evaluate the synergistic effects of the drug combinations. Further explanation regarding this method is provided with the presentation of results from the BCNU/temozolomide combination sequence.

RESULTS

Single Agent Antitumor Activity. The antitumor effects of temozolomide administered by gavage and i.v.-administered BCNU against 3 human brain tumors implanted s.c. in athymic mice are documented in Tables 1 and 2. Both agents produced 60–100% tumor-free mice in the SNB-75 astrocytoma xenograft model follow-

Table 1 *Activity of p.o. temozolomide and i.v. BCNU against early stage s.c. implanted SNB-75 astrocytoma xenografts*

Approximately 30-mg tumor fragments were implanted s.c. into the axillary region of athymic NCr-*nu/nu* mice on Day 0. Treatment was initiated on Day 5 when median tumor weights per group ranged from 63 to 75 mg, and 78% of the individual tumors ranged from 63 to 88 mg while the remainder were <63 mg. All 20 vehicle-treated control tumors grew well with a median doubling time of 2.65 days and a median time of 14.8 days to reach 500 mg.

	Treatment Day 5		Treatment Days 5, 9, 13	
Compound	Dose (mg/kg/day)	Tumor-free on Day 54	Dose (mg/kg/day)	Tumor-free on Day 54
p.o. temozolomide	600 (LD_{30})[c]	7/10	200[a]	10/10
	400	10/10	133	8/10
	270	9/10	90	7/10
	180	6/10	60	6/10
i.v. BCNU	40 (LD_{10})	9/10	27[b]	10/10
	27 (LD_{10})	8/10	18	10/10
	18	10/10	12	10/10

[a] Maximum mean net body weight loss of 11% measured on Day 12.
[b] Maximum mean net body weight loss of 23% measured on Day 19.
[c] LD_{30}, LD_{10}, dose lethal to 30 and 10% of animals, respectively.

ing single-bolus administration at several dose levels either on Day 5 only or on Days 5, 9, and 13 (Table 1). The U251 glioblastoma xenografts (Table 2) were slightly less responsive to treatment. All but 3 of 60 small tumors regressed completely following treatment with the two highest dosage levels of temozolomide administered on three schedules, but tumor regrowth was observed in the majority of the mice receiving the lower of the two dosages. Lower dosages were less effective with, at maximum, 2 of 10 tumor-free mice per group (data not shown). A good percentage of tumor growth delays were attained following BCNU treatment, but no mice attained tumor-free status. Results from a second s.c. U251 study indicated that larger U251 glioblastomas were still highly responsive to a single treatment of temozolomide. When treatment was delayed until tumors had grown to be about 300 mg, 600-, 400-, and 270-mg/kg doses produced 10, 9, and 8 of 10 Day 71 tumor-free mice, respectively (data not shown). The SF-295 glioblastomas were the least sensitive of the three brain tumors evaluated (Table 2). Although good tumor growth delays were attained, few mice remained tumor-free at the end of the experiment.

The effects of temozolomide and BCNU against i.c. implants of the SF-295 and U251 glioblastomas are summarized in Table 3. Both agents were highly effective, producing a number of long-term (90-day) survivors. As was the case with the s.c. models, the U251 tumor xenografts appeared to be more sensitive than the SF-295 tumors.

In the preceding experiments, temozolomide demonstrated no significant schedule dependence following p.o. administration to tumorous mice. Single bolus treatments on a single day, every fourth day for 3 doses, and daily for 5 dose schedules demonstrated similar levels of activity, and maximally tolerated total doses were equivalent. The maximally tolerated dose for single p.o. administration was approximately 600 mg/kg. In 13 experiments (not all shown), this dosage level caused 15 of 105 (14%) drug-related deaths in tumorous athymic mice. On the every fourth day for 3 dose schedule, the maximally tolerated dose was 200 mg/kg/day, causing 4 of 75 (5%) drug-related deaths in 9 experiments.

Combinations of BCNU Followed by Temozolomide. The s.c. implanted SF-295 glioblastoma xenograft model was selected for combination studies because it appeared to be the least sensitive of the 3 s.c. implanted brain tumor models. Mice received single doses of either p.o. temozolomide alone, or i.v. BCNU alone, or combinations of the two with BCNU administered either 2 h before or 2 h after temozolomide. In the first trial involving 5 mice/group and BCNU preceding temozolomide, enhancement of antitumor activity was observed in groups of mice treated with several combination dosage levels. While neither single agent produced any partial or complete regressions, regressions were attained in several combination treated groups. Effects of optimal single agent and combination treatments on tumor growth are illustrated in Fig. 1. The 600-mg/kg dose of temozolomide alone caused 1 of 5 drug-related deaths on Day 15 and

Table 2 *Activity of p.o. temozolomide and i.v. BCNU against staged s.c. implanted SF-295 and U251 glioblastoma xenografts*

Approximately 30-mg tumor fragments were implanted s.c. into the axillary region of groups of 10 (20 controls) athymic NCr-*nu/nu* mice on Day 0. Treatment was initiated when tumor sizes ranged from 63 to 294 mg (SF-295 model) or from 63 to 108 mg (U251 model). All vehicle-treated control tumors grew well with median doubling times of 1.4 and 2.5 days for the SF-295 and U251 experiments, respectively. Median time to 4 doublings was 7.8 days for SF-295 control tumors. Median time to 2 doublings was 8.3 days for U251 control tumors.

Compound	Route and treatment schedule	Dose (mg/kg/day)	No. of drug deaths	No. of complete regressions	No. tumor free[a]	Growth delay[b] (%)	Net log_{10} cell kill[b]
SF-295 glioblastoma experiment							
Temozolomide	p.o. Day 6	600	2/10	3/10	2/10	>315	>5.3
		400	0/10	0/10	1/10	237	4.0
	p.o. Days 6, 10, 14	200	1/10	0/10	2/10	295	3.2
		133	0/10	1/10	1/10	232	2.2
	p.o. Days 6–10	120	0/10	1/10	0/10	>336	>4.8
		80	1/10	0/10	0/10	182	2.2
BCNU	i.v. Day 6	40	0/10	3/10	1/10	301	5.1
		27	0/10	0/10	1/10	121	2.0
	i.v. Days 6, 10, 14	27	2/10	0/10	7/10	>336	>3.9
		18	0/10	1/10	0/10	276	2.9
U251 glioblastoma experiment							
Temozolomide	p.o. Day 7	600	0/10	1/10	9/10	675	6.7
		400	0/10	4/10	6/10	478	4.8
	p.o. Days 7, 11, 15	200	2/10	3/10	5/10	640	5.4
		133	0/10	8/10	2/10	548	4.5
	p.o. Days 7–11	120	0/10	3/10	7/10	694	6.5
		80	1/10	6/10	3/10	624	5.8
BCNU	i.v. Day 7	40	0/10	10/10	0/10	283	2.8
		27	0/10	9/10	0/10	196	2.0
	i.v. Days 7, 11, 15	27	1/10	9/10	0/10	460	3.6
		18	0/10	8/10	0/10	281	1.8

[a] Day 40 for SF-295, Day 86 for U251.
[b] Excludes tumor-free mice.

Table 3 *Therapeutic response of i.c. implanted SF-295 and U251 glioblastoma xenografts to p.o. temozolomide or i.v. BCNU*

Athymic NCr-*nu/nu* mice were inoculated i.c. with either 10^5 SF-295 or 10^6 U251 tumor cells on Day 0. Treatment with p.o. temozolomide or i.v. BCNU was started on Day 1 according to the schedules and dosages shown. The median days of death of vehicle-treated control mice were Day 22 for the SF-295 experiment and Day 17 for the U251 experiment. There were no 90-day survivors among the 20 SF-295 control mice and 2 among the 19 U251 controls.

	Treatment Day 1				Treatment Days 1, 5, 9			
Compound	Dose (mg/kg/day)	ILS[a] (%)	Net $\log_{10}$ cell kill	Day 90 survivors/total	Dose (mg/kg/day)	ILS (%)	Net $\log_{10}$ cell kill	Day 90 survivors/total
SF-295 glioblastoma experiment								
p.o. temozolomide	600 (LD_{40})	−36		3/10	200 (LD_{10})	127	2.4	4/10
	400	127	3.3	3/10	133	93	1.5	2/10
	270	68	1.8	2/9	90	61	0.6	1/9
i.v. BCNU	40	168	4.4	9/10	27	159	3.2	6/8
	27	232	5.1	8/10	18	175	3.6	8/10
	18	64	1.6	4/10	12	91	1.4	4/10
U251 glioblastoma experiment								
p.o. temozolomide	600	−6	−0.1	7/9	200	338	3.9	7/9
	400	324	4.3	5/9	133	268	3.0	3/9
	270	359	4.8	4/9	90	279	3.1	6/10
i.v. BCNU	40	182	2.5	3/10	27	100	0.7	8/10
	27	241	3.2	1/8	18	324	3.7	5/10
	18	150	2.0	2/10	12	182	1.8	1/10

[a] ILS, increase in life span, based on median day of death of dying mice only (survivors excluded); LD_{40}, LD_{10}, dose lethal to 40 and 10% of animals, respectively.

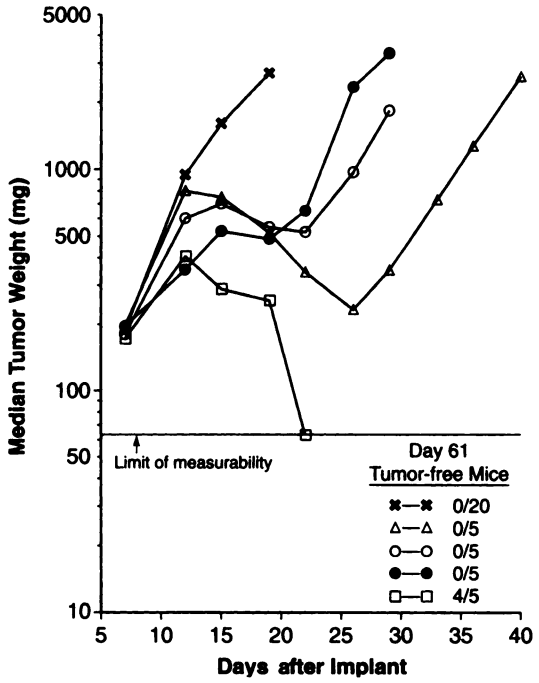

Fig. 1. Response of advanced stage s.c. implanted human SF-295 glioblastoma xenografts following treatment with p.o. temozolomide and/or i.v. BCNU on Day 7. ×, treated control; △, 600 mg/kg temozolomide; ○, 400 mg/kg temozolomide; ●, 40 mg/kg BCNU; □, 27 mg/kg BCNU followed 2 h later by 400 mg/kg temozolomide. Median tumor weights per group are plotted over time. Individual weights on Day 7 ranged from 108 to 256 mg. Median doubling time and time to 3 doublings for control tumors were 2.15 and 7.2 days, respectively.

produced a 329% growth delay in the remaining 4 mice. The 400-mg/kg dose was well tolerated and produced a 164% growth delay. BCNU, at doses of 40 and 27 mg/kg, produced growth delays of 140 and 11%, respectively. In the mice receiving 400-mg/kg temozolomide plus 27 mg/kg BCNU, all 5 tumors regressed completely, and 4 of 5 mice were still tumor free when the experiment was terminated on Day 61 (>650% growth delay). To verify these results, a second experiment was conducted using 10 mice/treatment group. The percentages of tumor growth delays are documented in Table 4. Various dosage levels of the combination produced a percentage of growth delays which exceeded the percentage of growth delay (including their sum) for corresponding dosage levels of the individual drugs. Only one partial tumor regression was attained in 80 mice receiving single agent therapy (at 40 mg/kg BCNU). By comparison, 9 partial regressions, 5 complete regressions, and 19 tumor-free mice were observed in 8 of 9 combination groups, including 5 of 10 tumor-free mice on Day 51 in groups receiving either 400 mg/kg temozolomide plus 27 mg/kg BCNU or 180 mg/kg temozolomide and 40 mg/kg BCNU.

Results of the statistical analysis of the second experiment (based on the individual animal tumor growth) are presented in Table 5. A

Table 4 *Median percentage of tumor growth delay attained following treatment of advanced stage s.c. human SF-295 glioblastoma xenografts with p.o. temozolomide and/or i.v. BCNU: BCNU/temozolomide sequence*

Treatment was initiated on Day 9 when individual tumor weights ranged from 100 to 343 mg. Temozolomide was administered 2 h after BCNU. Median doubling time and median time to 3 doublings for control tumors were 1.7 and 7.1 days, respectively. Growth delays include tumor-free survivors on Day 51 but exclude drug-related deaths. Except where noted, all treatments were equal to, or below, maximally tolerated doses.

BCNU (mg/kg)	Temozolomide (mg/kg)				
	0	180	270	400	600
0		1	39	28	190
18	17	244	228	380	NT[a]
27	59	401	444	>492	NT
40	242	>492	443[b]	>492[c]	NT
60	258	NT	NT	NT	NT

[a] NT, not tested.
[b] Toxic treatment, 4 of 10 apparent drug-related deaths.
[c] Toxic treatment, 3 of 10 apparent drug-related deaths.

Table 5 *Regression analysis of data from the BCNU/temozolomide combination sequence*

Individual percentage of tumor growth delays from the experiment summarized in Table 4 were analyzed by the quadratic dose-response model.

Variable	Coefficient estimate	Change in % of growth delay[a]	*P*
d_1	$a_1 = 0.46$	+46	0.005
d_2	$a_2 = 7.54$		0.0001
d_1^2	$a_3 = -0.00025$	−23	0.44
d_2^2	$a_4 = -0.069$		0.03
$d_1 d_2$	$a_5 = 0.013$	+52	0.0001

[a] Change in percentage of growth delay for an additional 100 mg/kg of temozolomide at 40 mg/kg of BCNU and 400 mg/kg of temozolomide.

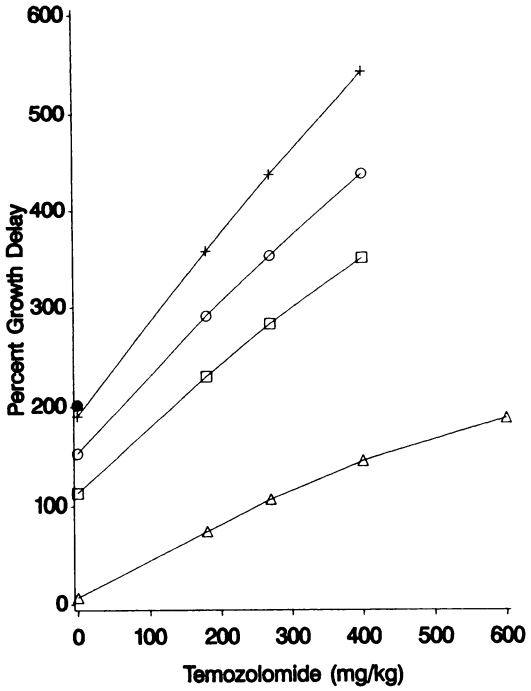

Fig. 2. Predicted percentage of growth delays for temozolomide based on analysis of the BCNU/temozolomide combination sequence using the quadratic dose-response model. *Lines*, growth delays predicted by the model for various doses of temozolomide combined with BCNU at either: △, 0 mg/kg; □, 18 mg/kg; ○, 27 mg/kg; +, 40 mg/kg; or ●, 60 mg/kg. See Tables 4 and 5.

similar analysis using log cell kill instead of percentage of growth delay would have produced the same results since log cell kill is proportional to percentage of growth delay. The parameter estimate for the interaction term of the two drugs is positive (d_1d_2 = 0.013) with P = 0.0001. This indicates that there is a synergistic effect which is statistically significant. To determine the magnitude of this effect, the change in percentage of growth delay predicted by the model for an additional 100 mg/kg of temozolomide (at 40 and 400 mg/kg of BCNU and temozolomide, respectively) is included in Table 4. The change in percentage of growth delay attributable to the effect of the additional temozolomide alone is 23 [46 + (−23), the linear plus the quadratic effects], compared to a 75 (23 + 52) change in percentage of growth delay if the synergistic effect of the BCNU is included. Thus, there is an increase in effect by a factor of at least 3. In this analysis, 83% of the total variation in the data is explained by the model.

The predicted values for the percentage of growth delay calculated using the model above are plotted with respect to the dosages in Fig. 2. The synergistic effect of the interaction also is demonstrated in Fig. 2. An increasing effect, ranging up to approximately 170% growth delay, is observed when temozolomide is given alone at increasing doses up to 600 mg/kg. The slopes of the temozolomide dose-response curves are increased substantially when BCNU is added. The effect of temozolomide when the BCNU dose is 40 mg/kg ranges from about 180% growth delay (for the BCNU alone) to 580% growth delay (for the BCNU plus the temozolomide at 400 mg/kg), giving a span of 400% growth delay. Hence, there is an increase in span by a factor of at least 2, from the first curve where no BCNU is given, to the last curve where the BCNU dose is 40 mg/kg, as the dose levels of the temozolomide are increased up to 400 mg/kg. In the absence of any interaction effect, these dose-response curves would have been parallel and the spans would have been equal.

The same analysis was performed on the first experiment (data not shown). The synergistic effect of the combination was very dramatic. There was an increase in the effect of the temozolomide by a factor of at least 3 when the BCNU dose levels were increased.

To provide an additional demonstration of synergism, in a model-independent fashion, the isobole method (19) was used: (*a*) the separate dose survival curves for temozolomide and BCNU were determined, by fitting separate quadratic models to the growth delays observed for the individual animals; (*b*) a graph was drawn with the dose range for the two drugs appearing on the two axes and with diagonal isoboles plotted to connect points on the axes which result in identical cell survivals; (*c*) finally, the tested drug combinations were plotted on the graph, and the observed growth delays which they gave were compared to the growth delays associated with the isoboles upon which they lay. The assumption is that if the drugs act independently, then the observed growth delays will be approximately equal to the isobole growth delays; likewise, drug combination growth delays which are consistently higher than the growth delays associated with the isoboles upon which they lie indicate synergy. The justification for the isobole method is that it is the unique method which will always yield independent action if we imagine that the two drugs in the combination are, in fact, the same. Fig. 3 shows the dramatic synergistic effect of the combination of the two drugs using the isobole method.

Combinations of Temozolomide Followed by BCNU. In an initial trial using the s.c. SF-295 model and 5 mice/group, at least additive toxicity was observed when BCNU was administered i.v. 2 h after temozolomide was administered p.o. on Day 8 post-tumor implant (data not shown). While the 400-mg/kg dose of temozolomide alone caused 1 of 5 apparent drug-related deaths, the same dose in combination with BCNU doses of 27 to 8 mg/kg caused 4 or 5 of 5 drug-related deaths. Similarly, while a 27-mg/kg dose of BCNU alone caused a maximum net body weight loss of 14.8% but no deaths, the same dose in combination with temozolomide doses of 400 to 120 mg/kg caused 4 or 5 of 5 drug-related deaths. Also, lethality was observed following 270 and 180 mg/kg temozolomide with 18 mg/kg BCNU. However, at lower nontoxic combination dosage levels, enhancement of antitumor effects were observed. Similar results were attained when the study was repeated using 10 mice/group (Table 6).

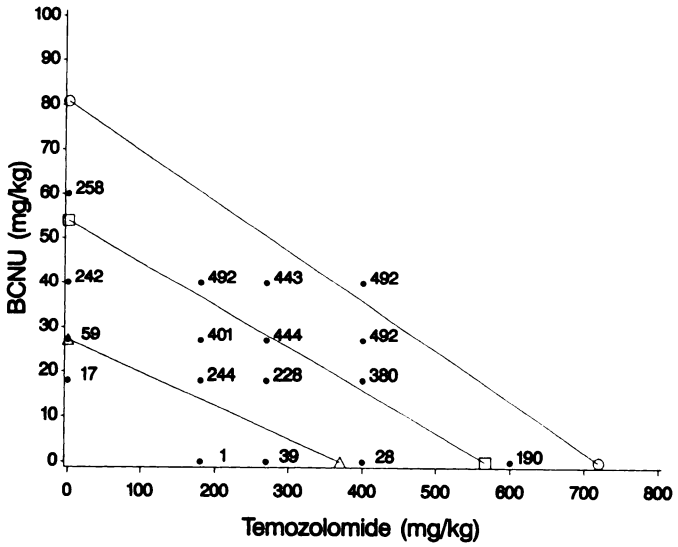

Fig. 3. Isoboles for the BCNU/temozolomide combination sequence. *Lines*, isoboles (isoeffect) for predicted percentage of growth delays of 100% (△), 200% (□), and 300% (○) for the drug combinations which lie on them, assuming no synergy or antagonism between BCNU and temozolomide. The points (●) represent the median percentage of growth delays observed for the various tested combinations of the two drugs (see Table 4).

Table 6 *Median percentage of tumor growth delay attained following treatment of advanced stage s.c. human SF-295 glioblastoma xenografts with p.o. temozolomide and /or i.v. BCNU: temozolomide/BCNU sequence*

Treatment was initiated on Day 6 when individual tumor weights ranged from 162 to 245 mg. Temozolomide was administered 2 h before BCNU. Median doubling time and median time to 3 doublings for the control tumors were 1.5 and 5.3 days, respectively. Growth delays include tumor-free mice on Day 41.

BCNU (mg/kg)	Temozolomide (mg/kg)				
	0	180	270	400	600
0		49	125	270	274
8	−2	183	345	377	NT[a]
12	−2	294	370	Toxic[b]	NT
18	9	374	Toxic[b]	Toxic	NT
27	8	Toxic	Toxic	Toxic	NT
40	185	NT	NT	NT	NT
60	49	NT	NT	NT	NT

[a] NT, not tested.
[b] Five of 10 drug-related deaths; 9 or 10 of 10 deaths in the remaining toxic treatment groups.

Table 7 *Regression analysis of data from the temozolomide/BCNU combination sequence*

Individual percentage of tumor growth delays from the experiment summarized in Table 6 were analyzed by the quadratic dose response model.

Variable	Coefficient estimate	Change in % of growth delay[a]	*P*
d_1	0.71	+71	0.0001
d_2	3.13		0.0114
d_1^2	−0.00038	−24	0.1325
d_2^2	−0.02		0.4630
d_1d_2	0.044	+79	0.0001

[a] Change in percentage of growth delay for an additional 100 mg/kg of temozolomide at 18 mg/kg of BCNU and 270 mg/kg of temozolomide.

Using the quadratic dose response model (based on the individual animal tumor growth), both sets of results demonstrate a synergistic effect for the lower combination dosage levels. Results for the second experiment are presented in Table 7. The parameter estimate for the interaction term of the two drugs is positive (0.044) with $P = 0.0001$, a statistically significant synergistic effect. To determine the magnitude of this effect, the change in percentage of growth delay predicted by the model for an additional 100 mg/kg of temozolomide (at 18 and 270 mg/kg of BCNU and of temozolomide, respectively) is included in Table 7. The change in percentage of growth delay attributable to the effect of the additional temozolomide alone is 47, compared to a 126 change in percentage of growth delay if the synergistic effect of the BCNU is included. Thus, there is an increase in effect by a factor of about 2.7. In this analysis, 85% of the total variation in the data is explained by the model. The synergistic effect was confirmed by the isobole method of analysis (data not shown).

Combinations of Temozolomide and BG. The advanced stage s.c. implanted SF-295 glioblastoma xenograft model also was used to evaluate the modulating effects of BG on the antitumor efficacy of temozolomide. BG, administered i.p. at dosage levels of 10 and 30 mg/kg 1 h before p.o. temozolomide, demonstrated no modulating activity (data not shown). The quadratic dose response model indicated a statistically nonsignificant negative interaction ($d_1d_2 = -0.00043$, $P = 0.9455$). The reverse sequence, with BG administered 1 h after temozolomide, demonstrated no confirmatory synergistic interaction (data not shown). In the initial study involving 5 mice/treatment group, there appeared to be a positive interaction that was barely significant ($d_1d_2 = 0.012$, $P = 0.0479$) but, in the followup study involving 10 mice/treatment group, the data indicated a nonsignificant negative interaction ($d_1d_2 = -0.0027$, $P = 0.2698$). In both experiments involving the temozolomide/BG sequence, the toxicity of temozolomide appeared to be augmented by the addition of BG. The highest dose of temozolomide evaluated, 525 mg/kg, when administered alone, caused 2 of 15 apparent drug-related deaths. The same dose in combination with 30 mg/kg of BG caused 5 of 15 deaths.

DISCUSSION

Although chemotherapy is able to have a palliative effect on brain tumor patients by shrinking tumors and prolonging survival time in some instances, there is really no satisfactory chemotherapy currently available for recurrent primary brain tumors (11). Thus, reports that p.o. administered temozolomide demonstrated activity against recurrent high-grade gliomas and newly diagnosed astrocytomas in its early clinical evaluations (9, 10) have potential importance for the treatment of patients with brain tumors. The observation of clinical responses also raises questions concerning potential augmentation of activity through use of combination chemotherapy involving other agents with demonstrated activity against brain tumors, *e.g.*, BCNU. The data presented in this report provide support for evaluating the combination of temozolomide and BCNU clinically. The data show that temozolomide is highly effective in the treatment of experimental models involving human brain tumors implanted in athymic mice. Following administration by gavage, temozolomide produced either long-term, tumor-free survivors or substantial growth delays in three s.c. implanted and two i.c. implanted brain tumor models (Tables 1–3). Based on this agreement with clinical results, one of these models, the advanced stage s.c. implanted SF-295 glioblastoma model, was used to evaluate the efficacy of temozolomide in combination with BCNU. Strong evidence of synergy was found (Figs. 1–3; Tables 4–7). The sequence of administration of the two cytotoxic agents may be important. While synergism was attained with temozolomide administered both 2 h before and 2 h after BCNU, the former combination sequence produced fewer tumor-free mice and demonstrated greater toxicity to the host. In other studies, augmentation of toxicity also was observed when other methylating agents, O^6-methylguanine (13) and DTIC (14), were administered to mice before BCNU treatment.

It is believed that temozolomide exerts its antitumor effects through spontaneous decomposition to the active metabolite MTIC (1, 2) and subsequent alkylation damage to DNA through methylation of O^6-guanine residues in DNA (20). In the latter study, MTIC produced differential cytotoxicity between O^6-alkylguanine repair-proficient (Mer^+) and deficient (Mer^-) cells, with increased cytotoxicity attributed to reduced repair associated with low levels of AGT (Mer^- cells). The cytotoxic effects of temozolomide in murine and human tumor cell lines and blasts from acute myelogenous leukemia patients also correlated with low levels of AGT (4–7). Low levels of AGT may be the underlying reason for the activity demonstrated by temozolomide against the brain tumor xenografts reported here. AGT activity was low in fragments of tumors serially passaged in mice; 3.7 ± 1.7, 3.0 ± 2.8, and 7.4 ± 3.7 fmol/mg protein for the SNB-75, U251, and SF-295 tumors, respectively.[4]

The antitumor activity of BCNU and other chloroethylating agents is limited by AGT repair of monoadducts at the O^6-position of guanine residues in one DNA strand prior to formation of the potentially lethal interstrand cross-links (21). Depletion of AGT activity in tumor cells by exposure of the cells to substrates for the protein, such as BG (22), enhances the cytotoxicity of BCNU in tissue culture (23) and increases the antitumor activity of BCNU in mice (24). Temozolomide also depletes AGT activity in tumor cells (5, 6, 14) and increases the sensitivity of human HT-29 colon cancer cells to BCNU

[4] M. Eileen Dolan, personal communication.

in vitro (14). Thus, the observed synergistic activity of the BCNU/temozolomide combinations against the SF-295 glioblastomas probably is related to the depletion of AGT in the tumors by temozolomide, although the mechanism of action is likely to involve saturation of the AGT repair system through formation of O^6-methylguanine-DNA adducts rather than a direct interaction with the protein. The same mechanism for augmenting the cytotoxicity of chloroethylating agents has been proposed for the methylating agents streptozotocin (25) and O^6-methylguanine (13).

The most marked synergy was observed when temozolomide was administered 2 h after BCNU. Following p.o. administration to mice, temozolomide levels in plasma peaked within 0.5 h (1), corresponding to 2.5 h after BCNU treatment in the current studies. A 25-mg/kg dose produced peak levels of about 101 μM and levels of approximately 21 μM at 3 h (1). If the pharmacokinetics for p.o. administered temozolomide is linear with dose, the 400-mg/kg-dosage level used in the combination studies should produce plasma levels of approximately 330 μM at 3 h. In cell culture assays, complete depletion of AGT activity was attained following a 3-h exposure of Raji cells to 300 μM temozolomide (5) or of MCF-7, LOVO, and MAWI cells to 200–300 μM drug (6). Four h following i.p. administration of a related triazene, DTIC, to nude mice bearing s.c. implants of HT-29 colon tumors, AGT levels in the tumors were significantly depleted (14). These data suggest AGT depletion in the s.c. SF-295 glioblastoma implants in mice at approximately 5.5–6.5 h after BCNU administration for those experiments in which temozolomide was given 2 h post-BCNU. This time would coincide with that for increased BCNU cross-linking (26). However, it is possible that the synergism observed with the BCNU/temozolomide combination may be independent of AGT depletion. AGT activity is low in the SF-295 glioblastoma, and a number of *in vitro* studies demonstrated that the cytotoxic effects of alkylating agents on cells with low AGT activity (<10 fmol/mg protein) were not enhanced by BG (6, 23), O^6-methylguanine (13), or temozolomide (13).

The low level of AGT activity in SF-295 cells may be the reason why no enhancement of temozolomide antitumor activity was observed in the combination studies involving BG. While BG potentiated the cytotoxicity of temozolomide to human tumor cell lines *in vitro,* the degree of enhancement was dependent on the level of AGT activity (6), and BG did not sensitize ZR-75-1 cells expressing low levels of AGT (6).

Based on the observed synergistic activity of temozolomide and BCNU in the experimental brain tumor model and the activity of each single agent in patients with brain tumors, the combination of temozolomide and BCNU should be explored in clinical trials. However, the highly interesting preclinical results presented here should be tempered by the knowledge that methylation of O^6-guanine residues in DNA by methylating agents, such as temozolomide, is associated with mutagenicity as well as cytotoxicity (27).

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REFERENCES

1. Stevens, M. F. G., Hickman, J. A., Langdon, S. P., Chubb, D., Vickers, L., Stone, R., Baig, G., Goddard, C., Gibson, N. W., Slack, J. A., Newton, C., Lunt, E., Fizames, C., and Lavelle, F. Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res., *47:* 5846–5852, 1987.
2. Tsang, L. L. H., Quarterman, C. P., Gescher, A., and Slack, J. A. Comparison of the cytotoxicity *in vitro* of temozolomide and dacarbazine, prodrugs of 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. Cancer Chemother. Pharmacol., *27:* 342–346, 1991.
3. Bull, V. L., and Tisdale, M. J. Antitumour imidazotetrazines—XVI. Macromolecular alkylation by 3-substituted imidazotetrazinones. Biochem. Pharmacol., *36:* 3215–3220, 1987.
4. Catapano, C. V., Broggini, M., Erba, E., Ponti, M., Mariani, L., Citti, L., and D'Incalci, M. *In vitro* and *in vivo* methazolastone-induced DNA damage and repair in L-1210 leukemia sensitive and resistant to chloroethylnitrosoureas. Cancer Res., *47:* 4884–4889, 1987.
5. Tisdale, M. J. Antitumour imidazotetrazines—XV. Role of guanine O^6 alkylation in the mechanism of cytotoxicity of imidazotetrazinones. Biochem. Pharmacol., *36:* 457–462, 1987.
6. Baer, J. C., Freeman, A. A., Newlands, E. S., Watson, A. J., Rafferty, J. A., and Margison, G. P. Depletion of O^6-alkylguanine-DNA alkyltransferase correlates with potentiation of temozolomide and CCNU toxicity in human tumour cells. Br. J. Cancer, *67:* 1299–1302, 1993.
7. D'Atri, S., Bonmasser, E., Franchi, A., Piccioni, D., Tuorto, V., Del Poeta, G., and Papa, G. Repair of DNA-methyl adducts and sensitivity to temozolomide of acute myelogenous leukemia (AML) cells. Exper. Hematol. (NY), *19:* 530, 1991.
8. Deans, B., and Tisdale, M. J. Antitumour imidazotetrazines XXVIII. 3-Methyladenine DNA glycosylase activity in cell lines sensitive and resistant to temozolomide. Cancer Lett., *63:* 151–157, 1992.
9. Newlands, E. S., Blackledge, G. R. P., Slack, J. A., Rustin, G. J. S., Smith, D. B., Stuart, N. S. A., Quarterman, C. P., Hoffman, R., Stevens, M. F. G., Brampton, M. H., and Gibson, A. C. Phase I trial of temozolomide (CCRG 81045: M & B 39831: NSC 362856). Br. J. Cancer, *65:* 287–291, 1992.
10. O'Reilly, S. M., Newlands, E. S., Glaser, M. G., Brampton, M., Rice-Edwards, J. M., Illingworth, R. D., Richards, P. G., Kennard, C., Colquhoun, I. R., Lewis, P., and Stevens, M. F. G. Temozolomide: a new oral cytotoxic chemotherapeutic agent with promising activity against primary brain tumours. Eur. J. Cancer, *29A:* 940–942, 1993.
11. Steward, D. J. The role of chemotherapy in the treatment of gliomas in adults. Cancer Treat. Rev., *16:* 129–160, 1989.
12. Panella, T. J., Smith, D. C., Schold, S. C., Rogers, M. P., Winer, E. P., Fine, R. L., Crawford, J., Herndon, J. E., II, and Trump, D. L. Modulation of O^6-alkylguanine-DNA alkyltransferase-mediated carmustine resistance using streptozotocin: a Phase I trial. Cancer Res., *52:* 2456–2459, 1992.
13. D'Incalci, M., Taverna, P., Erba, E., Filippeschi, S., Potenza, D., Mariani, L., Citti, L., and Catapano, C. V. O^6-Methylguanine and temozolomide can reverse the resistance to chloroethylnitrosoureas of a mouse L1210 leukemia. Anticancer Res., *11:* 115–122, 1991.
14. Mitchell, R. B., and Dolan, M. E. Effect of temozolomide and dacarbazine on O^6-alkylguanine-DNA alkyltransferase activity and sensitivity of human tumor cells and xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Chemother. Pharmacol., *32:* 59–63, 1993.
15. Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. M., and Abbott, B. J. Protocols for screening chemical agents and natural products against animal tumors and other biological systems, Ed. 3. Cancer Chemother. Rep., Part 3, *3:* 51, 1972.
16. Schabel, F. M. Jr., Griswold, D. P. Jr., Laster, W. R., Jr., Corbett, T. H., and Lloyd, H. H. Quantitative evaluation of anticancer agent activity in experimental animals. Pharmacol. Ther., *1:* 411–435, 1977.
17. Dolan, M. E., Pegg, A. E., Moschel, R. C., Vishnuvajjala, B. R., Flora, K. P., Grever, M. R., and Friedman, H. S. Biodistribution and effectiveness of O^6-benzylguanine administered in PEG 400 or cremophor vehicles in human brain tumor xenografts. Proc. Am. Assoc. Cancer Res., *35:* 319, 1994.
18. Draper, N., and Smith, H. *In:* Applied Regression Analysis, Ed. 2. New York: Wiley-Liss, Inc., p. 219, 1981.
19. Berenbaum, M. C. What is synergy? Pharmacol. Rev., *41:* 93–139, 1989.
20. Gibson, N. W., Hartley, J., La France, R. J., and Vaughan, K. Differential cytotoxicity and DNA-damaging effects produced in human cells of the Mer^+ and Mer^- phenotypes by a series of alkyltriazenylimidazoles. Carcinogenesis (Lond.), *7:* 259–265, 1986.
21. Erickson, L. C., Laurent, G., Sharkey, N. A., and Kohn, K. W. DNA cross-linking and monoadduct repair in nitrosourea-treated human tumour cells. Nature (Lond.), *288:* 727–729, 1980.
22. Dolan, M. E., Moschel, R. C., and Pegg, A. E. 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.
23. Dolan, M. E., Mitchell, R. B., Mummert, C., Moschel, R. C., and Pegg, A. E. Effect of O^6-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. Cancer Res., *51:* 3367–3372, 1991.
24. Mitchell, R. B., Moschel, R. C., and Dolan, M. E. 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 Res., *52:* 1171–1175, 1992.
25. Futscher, B. W., Micetich, K. C., Barnes, D. M., Fisher, R. L., and Erickson, L. C. Inhibition of a specific DNA repair system and nitrosourea cytotoxicity in resistant human cancer cells. Cancer Commun., *1:* 65–73, 1989.
26. Erickson, L. C., Bradley, M. O., Ducore, J. M., Ewig, R. A. G., and Kohn, K. W. DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. Proc. Natl. Acad. Sci. USA, *77:* 467–471, 1980.
27. Hall, J., and Montesano, R. DNA alkylation damage: consequences and relevance to tumour production. Mutat. Res., *233:* 247–252, 1990.

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