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Correlations between Experimental Chemotherapy in the Murine Glioma and Effectiveness of Clinical Therapy Regimens*

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Summary. *Intracerebral murine glioma 26 was used as a model system for evaluating two-drug combinations of antitumor agents. BCNU was combined with either procarbazine, dianhydrogalactitol, or ellipticine. CCNU was combined with procarbazine. All combinations were more active than the individual drugs alone. The most potent combinations achieved 85–100% tumor "cure" at 120 days, with combined toxicity indices of 0.25 (CCNU-procarbazine) to 1.30 (BCNU-dianhydrogalactitol). The experimental data were compared to clinical studies with CCNU, procarbazine, and vincristine, and BCNU-procarbazine.*

Introduction

Although animal tumor model systems have been used for the experimental evaluation of potential anticancer agents for many years, seldom have they been used routinely for preclinical testing of drug combination regimens. At present, this approach has few advocates among clinicians, and it is uncommon for clinical studies of poly-drug therapies to be initiated on the basis of results obtained from animal experimentation.

The major purpose of this study is to demonstrate the utility of testing several two-drug combinations on the intracerebral glioma 26 mouse tumor model as a prelude to some types of clinical trials. As an initial effort, we evaluated: (1) 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) combined with either procarbazine (PCB), dianhydrogalactitol (DAG), or ellipticine (ELP); and (2) 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) combined with PCB.

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Methods and Results

Glioma 26 Tumor Model

Male C57 BL/6 mice weighing 18–22 g were purchased from Simmonson, Inc. (Gilroy, California). Glioma 26 tumors, originally obtained from Microbiologic Associates (Bethesda, Maryland) through a contract with the National Cancer Institute, were carried in the flank of C57 BL/6 mice.

For intracerebral (i.c.) inoculation, one or more relatively firm tumors of approximately 1–1.5 cm in diameter were excised under semisterile conditions. The tumors were minced with scissors in a sterile Petri dish. The minced tumor was suspended in sterile Hank's balanced salt solution and filtered through a fine stainless steel mesh into a sterile test tube. The cells were counted and the density of cells was adjusted to 4×10^4 cells/0.01 ml by the addition of sterile Hank's solution.

Prior to tumor inoculation, the animals were lightly anesthetized with open-drop ether. The tumor cells (4×10^4 in 0.01 ml) were inoculated into the centrum ovale of the right parietal lobe using a 25-gauge needle connected to a 0.5-ml Hamilton syringe and repeating dispenser. The needle had a brass stop to allow penetration to a depth of no more than 3.0 mm. The acute mortality from this procedure, as measured from the time of implantation to 5 days later, was less than 5%.

The average median day of death for untreated animals was 27.6 ± 1.4 (SE) days. The percentage of animals dying of an i.c. tumor was 98–100% in all control groups.

Survival vs Day of Treatment

Beginning on day 1 after tumor implantation, and on successive days thereafter until day 20, eleven groups of

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10 animals each were treated daily with a single intraperitoneal LD_{10} dose of BCNU (34 mg/kg). Figure 1 graphically demonstrates the relationship of the survival among groups, as measured by the ratio of median treated survival time/median control survival time (T/C), as a function of time of BCNU treatment. The highest T/C occurred between days 12–14; the relationship of T/C to day of treatment is modal. Because of these results, day 12 was picked as the "optimum" day for BCNU and CCNU treatment in all further studies.

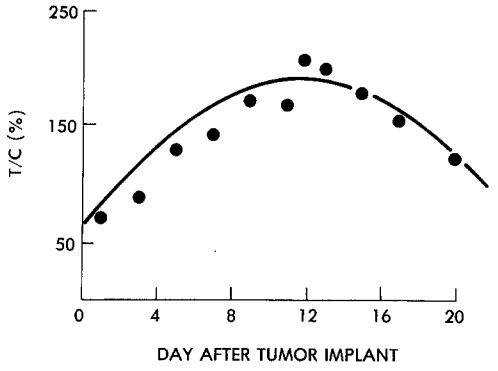

Fig. 1. Plot of ratio of median day of death for BCNU-treated group/control group (T/C) versus day of treatment with LD_{10} dose of BCNU (34 mg/kg). Each point represents T/C for 10 treated and 20 control animals. The line is a parabola fitted by the method of least squares

LD_{10} Studies

The LD_{10} values for BCNU and CCNU were obtained from the literature (Shapiro et al., 1970; Shapiro, 1971). The LD_{10} values for the other drugs used were determined in our laboratory using 5 animals/dose and a range of 4 doses. Animals were observed for 42 days. The LD_{10} values were calculated by Dr. Lloyd Harris, Southern Research Institute (Birmingham, Alabama), using the method of Litchfield and Wilcoxon (1949). Table 1 lists the LD_{10} doses for the drugs used in these studies.

For each two-drug combination a "combined toxicity index" (CTI) was computed from the LD_{10} values obtained for each individual drug. Table 2 summarizes

Table 1. LD_{10} values for C57 BL/6 mice

Drug	NSC-No.	Vehicle	Dose schedule (mg/kg)
BCNU (Shapiro et al., 1970)	409962	ETOH, Water	34.0 i.p., q.d. × 1
CCNU (Shapiro, 1971)	79037	Emulfor[a], Water	55.0 i.p., q.d. × 1
Dianhydrogalactitol (DAG)	132313	Saline	5.5 i.p., q.d. × 1
Ellipticine (ELP)	71795	Klucel[b]	140.0 i.p., q.d. × 1
Procarbazine (PCB)	77213	Saline	152.0 i.p., q.d. × 5

[a] Polyethoxylated vegetable oil and ethanol
[b] Hydroxypropyl cellulose (Glogan and Co., Inc., Melrose Park, Illinois)

Table 2. Combined toxicity index (CTI)[a, b]

Nitrosourea mg/kg	Procarbazine (PCB)		Dianhydrogalactitol (DAG)		Ellipticine (ELP)	
	10 mg/kg (0.07)	20 mg/kg (0.13)	2 mg/kg (0.36)	4 mg/kg (0.71)	50 mg/kg (0.36)	100 mg/kg (0.71)
BCNU:						
20 (0.59)	0.66	0.72	0.95	1.30	0.95	1.30
30 (0.88)	0.95	1.01	1.24	1.59	1.24	1.59
CCNU:						
10 (0.18)	0.25	0.31				
20 (0.36)	0.43	0.49				
30 (0.54)	0.61	0.67				
40 (0.73)	0.80	0.86				
50 (0.91)	0.98	1.04				

[a] Computed theoretical CTI based on single agent LD_{10} values
[b] Parentheses indicate fraction of LD_{10} value for single agents

the CTI values computed for each drug and combination. This CTI differs from that used by Skipper (1974), who determined the values experimentally.

Design of Two-Drug Studies

Figure 2 shows the general design of a two-drug study. To restrict variables to a workable number, only three controlled variables were considered: the dose of drug A (e.g., a nitrosourea); the dose of drug B (procarbazine, dianhydrogalactitol, ellipticine); and t, the time drug B is given. In all cases, drug A was given on day 12. Except for the CCNU-PCB combination, only two dose levels were used for each drug. Drugs A and B given singly form a part of the design that is not shown in Figure 2.

There were 20 animals in each control group, 13–15 animals in each two-drug treatment group, and more than 60 animals in each group that received single therapy doses of BCNU and CCNU.

Since effective long-term regimens were being sought, the probability of survival to 120 days was chosen as the major endpoint for our studies. Autopsies were performed at 120 days on animals chosen randomly from the various groups to evaluate the presence or absence of tumor. It was uncommon to find tumor at autopsy, although needle track scars were evident.

Statistical Methods

The efficacy of each two-drug combination was compared to that of each drug when it was used singly at the appropriate dose level; all two-drug combinations were compared to each other. For this purpose, the nonparametric survival analysis of Gehan (1965) was used to compare individual pairs of experimental populations. This analysis, which adjusts for termination of the experiment before all animals have died, also accounts for tied observations.

The BCNU and CCNU studies were also analyzed to obtain a *P*-value according to the null hypothesis that the probability of 120-day survival for any combination of drug A with drug B will be the same as that for drug A alone. The *P*-value was defined to be the lowest significance level supported by applying the Bonferroni adjustment to the six one-sided Fisher exact tests (Miller, 1966) for each BCNU group and the two lowest dose combinations for CCNU.

BCNU-Procarbazine (PCB)

Table 3 summarizes the results with this combination. The results of this study indicated that: 1. except for a slight increase in survival with higher BCNU doses, there was no relationship of survival to PCB dose or

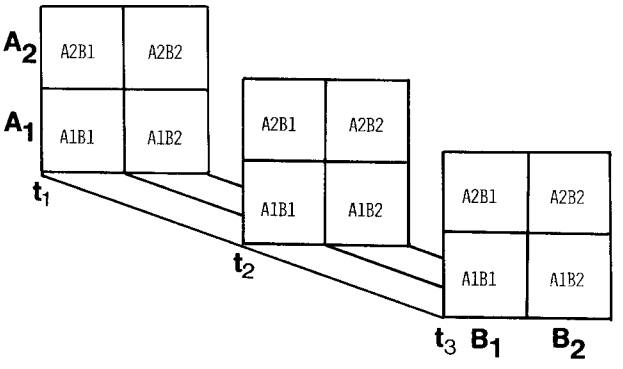

Fig. 2. Design model for the two drug combinations. A_1 and A_2 are two dose levels of BCNU (five for CCNU); B_1 and B_2 are two dose levels for the second drug (procarbazine, ellipticine, or dianhydrogalactitol); t_1, t_2, and t_3 are the three times drug B was administered. Drug A was always given on day 12 after tumor implantation

Table 3. BCNU-procarbazine (PCB): percentage of animals surviving more than 120 days

BCNU[a, c]	PCB[b]					
	Day 12–16		Day 15–19		Day 18–22	
	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg
20 mg/kg	62	62	46[d]	62	62	69
30 mg/kg	46[d]	85	92	85	69	62

[a] Treatment i.p. on day 12
[b] Treatment i.p. on day designated
[c] No PCB alone survivors at 120 days and only 3–16% BCNU survivors on day 120; *P*-values for null hypothesis at 20 mg/kg = 0.016 and at 30 mg/kg < 0.0001
[d] All combinations but these two were statistically superior to BCNU alone by the Gehan modification of the Wilcoxon rank sum analysis ($P < 0.01$)

Table 4. CCNU-procarbazine (PCB): percentage of animals surviving more than 120 days

CCNU[a]	PCB[b]						
	Day 12–16		Day 15–19		Day 18–22		No PCB[e]
	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg	
10 mg/kg	92[c]	92[c]	92[c]	100[c]	92[c]	85[c]	8 (50)
20 mg/kg	75	92	92	100	92	100	38 (34)
30 mg/kg	91	25[d]	76	76	100	84	40 (30)
40 mg/kg	29	7[d]	29	0[d]	7[d]	43	52 (23)
50 mg/kg	0	0[d]	50	7[d]	43	14[d]	39 (18)

[a] Treatment i.p. on day 12
[b] Treatments i.p. on days designated
[c] Best at day 120 by ANOVA and Gehan modification of the Wilcoxon rank sum analysis at $P < 0.01$ for table of CCNU doses of 10 mg/kg alone and with PCB
[d] Inferior to CCNU alone with $P < 0.01$ by Wilcoxon rank sum analysis
[e] Parentheses indicate number animals

Table 5. BCNU-ellipticine (ELP): percentage of animals surviving more than 120 days

BCNU[a, c]	ELP[b]					
	Day 11.92		Day 12.04		Day 13	
	50 mg/kg	100 mg/kg	50 mg/kg	100 mg/kg	50 mg/kg	100 mg/kg
20 mg/kg	85	85	92	71	92	78
30 mg/kg	92	71	92	0[d]	92	50[d]

[a] Treatment i.p. on day 12
[b] Treatment i.p. on day designated
[c] No ELP alone survivors at 120 days and only 3–16% BCNU survivors on day 120; P-value for null hypothesis at 20 mg/kg < 0.005 and at 30 mg/kg < 0.0001
[d] Only two values significantly different from the rest of the table by ANOVA at days 45, 75, and 120 with $P = 0.01$. All remaining values statistically better than BCNU alone at $P < 0.005$

timing; and 2. of the 12 combinations, 5 had 69–92% survival, as compared to BCNU alone, which achieved 3–16% survival at day 120; and 3. the combinations were equally effective at CTI of 0.66–1.01, using the CTI computed on the basis of the individual drug LD_{10} values (Table 2).

CCNU-Procarbazine (PCB)

Table 4 summarizes a study using five doses of CCNU in combination with two doses of PCB. From the studies performed with CCNU alone, it is apparent that glioma 26 is very sensitive to CCNU. For this reason, the efficacy of CCNU-PCB combinations could not be evaluated adequately until the dose of CCNU was reduced to 10 mg/kg, or 18% of the LD_{10}. From our results, we concluded that: 1. at any given CTI less than 1.0, CCNU doses of 40 and 50 mg/kg were more toxic in combination with PCB than were BCNU doses in combination with PCB, especially when PCB was given on days 12–17 (45-day survivors = 120-day survivors) and 2. the best results were obtained when initiation of PCB therapy was delayed until day 15; and 3. a 120-day survival of 100% was achieved at CTI values of 0.25–0.49.

BCNU-Ellipticine (ELP)

Table 5 shows the results of this matrix study. Since ELP is a lipophilic compound that can intercalate between DNA bases, and has the prerequisites for adequate brain capillary transport (Kohn et al., 1975; Levin

Table 6. BCNU-dianhydrogalactitol (DAG): percentage of animals surviving more than 120 days

BCNU[a, c]	DAG[b]					
	Day 11		Day 12.2		Day 13	
	2 mg/kg	4 mg/kg	2 mg/kg	4 mg/kg	2 mg/kg	4 mg/kg
20 mg/kg	100[d]	100[d]	100[d]	92	85	100[d]
30 mg/kg	92	85	50	92	92	100[d]

[a] Treatment i.p. on day 12
[b] Treatment i.p. on days designated
[c] No DAG alone survivors at 120 days and only 3–16% BCNU survivors on day 120; P-value for null hypothesis at both doses of BCNU was < 0.0005
[d] Best at days 45, 75, 120 by ANOVA at $P = 0.01$. All combinations better than BCNU alone at $P < 0.001$

et al., 1976c; Liss and Kensler, 1976), we evaluated this drug 2 h before, 1 h after, and 24 h after BCNU treatment. The timing of drug administration was designed to evaluate the possible toxic effects of the two drugs, as well as the potentially additive activity of the two drugs in inhibiting DNA repair. The conclusions drawn were: 1. high doses of BCNU and ELP are toxic at a CTI of 1.59, particularly when ELP treatment follows within 24 h of BCNU administration; and 2. there was no statistical difference in 120-day survivors for the remaining dose regimens (71–92% of animals survived 120 days). The 92% 120-day survivors occurred at CTIs of 0.95–1.24, and only with low dose ELP (50 mg/kg).

BCNU-Dianhydrogalactitol (DAG)

Table 6 summarizes the results of this combination, which is of particular interest because glioma 26 is quite resistant to the antitumor effects of DAG (Levin et al., 1976b). The study indicated that: 1. the best results were obtained when either dose of DAG was combined with either dose of BCNU, and the results were independent of timing relative to BCNU; and 2. the optimal CTI was 1.26 (range 0.95–1.59).

Discussion

Survival vs Day of BCNU Treatment

The results reported here are similar to those obtained for BCNU treatment of the 9L i.c. gliosarcoma in the rat (Tel et al., 1974). In the earlier studies, treatment at day 16 was found to be superior to treatment at days 10, 14, or 20 after tumor implantation. Results of our previous work on many anatomical, physiological, and cell kinetic features of the 9L tumor model are used in the following attempt to explain the results of both studies. At day 6, the 9L tumor showed no vasculature, as determined by anatomical studies after the injection of India ink into the left heart. Some evidence for early vessel formation was apparent by day 9, but vascular development was not maximal until days 14–16.[1] Initially (day 6), the tumor cells were compact and formed a tumor that was nearly spherical in appearance. Later (days 16–18), the tumors began to outgrow their blood supply; they were distinctly necrotic by day 20.

Since BCNU is highly lipophilic and of small molecular size, it has "infinite" brain-capillary permeability, and its delivery to the normal brain, brain adjacent to tumor, and tumor are limited only by blood flow (Levin et al., 1975). In addition, because of its lipophilicity and susceptibility to rapid chemical breakdown, it is likely that the concentration of BCNU becomes significantly lower as the drug diffuses further from the capillary (Levin et al., 1976d). These facts, coupled with the steepness of the dose-response curve for BCNU-induced cell kill of 9L cells, suggests that the ineffectiveness of BCNU therapy before adequate vascularization of the tumor occurs is a result of inadequate drug delivery to a sufficient number of tumor cells. Late therapy failure after day 16 may be, to an exent, the result of limited drug perfusion and sequestered cell populations. However, the size of the tumor at that time may make therapeutic intervention impossible for other reasons. In all tumors, but particularly in older and larger ones, the nitrosoureas kill primarily by cross-linking DNA (Kohn, 1977) and causing sterility in daughter cells; tumor growth may, however, continue for 2–3 generations, and may increase the bulk of the i.c. tumor to a critical mass that causes dislocation of vital brain structures (e.g., herniation) and death to the rats.

[1] Levin, V. A.: Unpublished observations, 1975

Table 7. Procarbazine, CCNU, and vincristine (PCV) regimens

	PCV No. 1	PCV No. 2	PCV No. 3
CCNU	75 mg/m² day 1	75 mg/m² day 1	110 mg/m² day 1
Procarbazine	100 mg/m² days 1–14	100 mg/m² days 8–21	60 mg/m² days 8–21
Vincristine	1.4 mg/m² days 1, 8	1.4 mg/m² days 8, 29	1.4 mg/m² days 8, 29
Repeat cycle	q. 4 weeks	q. 6–8 weeks	q. 6 weeks

Table 8. BCNU-procarbazine combination

BCNU	100 mg/m²	day 1
Procarbazine	100 mg/m²	days 1–14
Repeat cycle	4 weeks then	q. 6 weeks

BCNU-PCB and CCNU-PCB Combinations

The original stimulus for these studies was a clinical trial of a PCB, CCNU, and vincristine (PCV No. 1) combination that was therapeutically active, but highly toxic (Gutin et al., 1975). BCNU-PCB was therapeutically less active, but more toxic, than expected. In view of the demonstrated activity of BCNU, CCNU, and PCB against recurrent gliomas of the brain (Levin and Wilson, 1975), it was important that we try to maximize potential antitumor activity and minimize toxicity.

Glioma 26 was chosen as a model system because it was not highly sensitive to either PCB or BCNU, although its sensitivity to CCNU made the studies with this drug more difficult to conduct.

The PCV No. 1 dose regimen used in patients is shown in Table 7. CCNU was given at 0.58 of the MTD, and PCB at approximately 0.33 of the MTD (computed on the basis of an MTD of 150 mg/m² for 28 days, extrapolated to a 14-day schedule in PCV No. 1 of 100 mg/m² for 14 days). Responding malignant glioma patients, most of whom were adults, usually had evidence of tumor regrowth at dose reductions of 25–50% of the starting dose. The children with recurrent medulloblastoma exhibited an even greater initial toxicity to PCV No. 1, since many had previous neuraxis radiation. As a result, few tolerated beginning therapy at the full dose. Without exception, however, all medulloblastoma patients who responded to PCV No. 1 had tumor regrowth only during dose reduction, and not while they were receiving either the full or the highest dose tolerated during the first several courses.[2]

The CCNU-PCB glioma-26 studies (Table 4) demonstrated that reduced toxicity could be achieved by initiating PCB treatment several days after the first CCNU dose was administered. As a result, we modified PCV No. 1 therapy to that shown in Table 7, with PCB beginning on day 8 following CCNU. PCV No. 2 has been in use nearly two years and, although it is too early to make a complete report of our findings, preliminary results are encouraging. To date, seven patients with recurrent medulloblastomas and seven with nonrecurrent tumors treated two months following radiation therapy have been given PCV No. 2. Some of the recurrent patients had been on PCV No. 1 and showed evidence of tumor regrowth, as determined by their worsening radionuclide scans, increasing glucocorticoid requirements, and clinical neurological deterioration following dose reduction due to myelotoxicity. After the institution of PCV No. 2 therapy in these patients, tumor progression was halted and dose escalation became possible.

Because of the slightly better response of malignant-glioma patients to CCNU as compared to PCB (Levin and Wilson, 1975) and the observation, in these studies, that a low dose of PCB was sufficient to potentiate CCNU activity against the i.c. glioma 26 tumor, we instituted PCV No. 3 therapy for patients with recurrent malignant gliomas. The results with the first 22 patients who have received this therapy also indicates reduced myelotoxicity and good antitumor activity compared to PCV No. 1. In fact, a number of patients who failed to respond to BCNU did respond to this therapy. Another year of evaluation will be necessary to determine whether PCV No. 2 and PCV No. 3 will be significantly better than PCV No. 1; at present, the toxicity data and response patterns are encouraging.

BCNU-PCB

Before these studies were completed, we had undertaken a clinical trial of BCNU-PCB (Levin et al., 1976a). The rationale for the study was that BCNU was a more potent anticancer agent than CCNU for malignant gliomas (Levin and Wilson, 1975). We fully expected that BCNU-PCB would be superior to PCV No. 1; however, the duration of response for BCNU-PCB was shorter and the number of responders was smaller than we had anticipated. In addition, this combination was strikingly myelotoxic. Table 8 summarizes the doses and times of treatment. From the results of our glioma-26 studies (Table 3) it was clear that delaying PCB therapy would reduce toxicity as the PCV No. 2 protocol demonstrated. We have not had sufficient time to test this hy-

[2] Crafts, D. C., Wilson, C. B., Levin, V. A., Boldrey, E. B., Enot, K. J.: Chemotherapy of recurrent medulloblastoma with combined procarbazine, CCNU and vincristine. Submitted to J. Neurosurg.

pothesis in a clinical trial, but the reduced toxicity observed with PCV No. 2 in these studies provides supporting evidence for clinical use.

BCNU-DAG

Uncontrolled pilot studies in our laboratory have shown that DAG, a hexitol epoxide that crosses the blood–brain barrier (Levin et al., 1976b), has only limited antitumor activity against primary malignant brain tumors, but Geran et al. (1974) have shown that DAG was extremely active against the i.c. murine ependymoblastoma. Our findings confirm their results, but indicate that DAG is not effective against two other i.c. tumors, rat 9L and murine glioma 26 (Levin et al., 1976b).

The basis for this drug resistance in one of two closely related murine tumors, which are histologically similar and are grown in the same animal species, is unresolved. Theoretically, two mechanisms of resistance are possible: defective intracellular transport or differential mechanisms for tumor-DNA repair. We anticipated that combining DAG with another alkylating agent, BCNU, might lessen repair capabilities in the glioma 26 tumor cell and result in synergistic antitumor activity.

The results of our study (Table 6) clearly demonstrate that BCNU-DAG combinations have a synergistic action, regardless of whether DAG is given before or shortly after BCNU. DAG administered 24 h before BCNU has a highly synergistic effect, implying that cellular transport mechanisms do not account for the differential antitumor responses observed in the murine ependymoblastoma and glioma 26 tumor lines, since very little DAG is present in plasma 24 h after injection (Kimura et al., 1977; Levin et al., 1976b). These results do allow for the possibility that a different mechanism for DNA repair is the explanation for our observations, although this hypothesis remains to be proved.

No clinical trials of BCNU-DAG have been instituted as far as we know, but the data suggest that such a trial might be worthwhile, in spite of the fact that DAG used alone has not proven to be effective against malignant glioma tumors in humans.[3]

BCNU-ELP

There were two specific reasons for carrying out this study. First, the ellipticine family comprises the only lipophilic DNA intercalators known to us that both cross the blood–brain barrier (Liss and Kensler, 1976) and also show antitumor activity against i.c. glioma 26.[4] Second, because a major toxic side effect of these drugs is red-cell hemolysis, there has been little enthusiasm for ellipticine in the United States, although the methoxy analog has been used in Europe (Mathé et al., 1970). We hypothesized that if the drug could be combined with a potent alkylating agent such as BCNU, it might improve the BCNU response at a lower dose than that which causes clinically significant hemolysis.

The times used to evaluate ELP therapy were chosen so that intercalation would either proceed, be simultaneous with, or follow BCNU therapy. Intuitively, these three time periods seemed to be the logical choices in the attempt to maximize DNA damage and minimize DNA repair. The results (Table 5) show that the combination is clearly more active than either drug alone, and may be synergistic at the doses used. When ELP followed BCNU by 24 h, it was more toxic, particularly at the high dose of each. All other times and doses achieved reasonably similar results (71–92% 120-day survivors).

Although these data do not provide a basis for clinical trials at this time, we hope that other investigators will be stimulated to validate and extend our observations in other animal models. The potential antitumor activity of a lipophilic DNA intercalator that can cross the blood–brain barrier certainly warrants further investigation.

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