Post-Treatment Kinetics of BCNU in a 9L Rat Brain Tumor Model*

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Summary

After *in vivo* treatment with BCNU at 13.3 mg/kg, the dose lethal to 10% (LD₁₀), rats implanted with 9L brain tumors exhibited an increased life span (ILS) of 56%. The growth fraction (GF) of treated tumors, as measured by fractionated doses of ³H-thymidine administered over 40 h, decreased for the first 2 to 10 days post-treatment, then increased and overshot the baseline (though by no more than 10%) by Day 21. A second treatment with a LD₁₀ of BCNU either 5, 10, or 14 days after the initial treatment increased the ILS to 126–128% and resulted in long-term survival (ILS > 300%) in 20% to 30% of animals, but did not produce a remarkable change in GF beyond that resulting from the initial treatment. Histological examinations revealed increased numbers of giant cells 2 to 6 days after the initial treatment; however, mitosis decreased immediately following treatment and remained low for the next 8 days. These results imply that (in this animal model, at least) a cell cycle non-specific (CCNS) drug is more effective as a second treatment after initial treatment with a CCNS drug than is a cell cycle specific one.

Key words: Brain tumor chemotherapy, BCNU, animal model, perturbation by BCNU, post-treatment tumor kinetics

Introduction

BCNU, one of the nitrosourea derivatives, is a cell cycle non-specific (CCNS) drug useful in treating brain tumors that have a low growth fraction (GF)⁸⁾ as well as limited drug transportation.⁹⁾ Rosenblum has shown that, using a 9L rat brain tumor model, the BCNU dose lethal to 10% of animals (LD₁₀) produces an increased life span (ILS) of 50–100% and a 3-log cell kill as measured by the colony-forming efficiency (CFE) assay.^{13,14)} Despite its marked effect on experimental brain tumors, however, BCNU by itself offers little hope for tumor cure because of such common problems of malignant tumors as the deleterious effects it has on the immune system of the host, the heterogeneity of the tumor cell population it

would need to eradicate, and the potential development of drug resistance by the tumor. The proposed solutions to this dilemma are combination chemotherapy or multimodality therapy, spaced for maximum therapeutic effect. Treatment with one anticancer agent will, of course, create a perturbed tumor cell population that represents an entirely uncharacterized milieu for a chemotherapeutic agent used subsequently. Thus, for all drugs used in combination chemotherapy protocols, it is necessary to determine the extent of the resultant apparent synchronization and recruitment of nonproliferating (G₀) cells into the proliferating pool as a result of such perturbations as cell death or accumulation of cells in particular phases of the cycle.

We used autoradiographic procedures as well as histological examinations to study the post-treatment kinetics of BCNU and, in particular, the effect of a second BCNU treatment administered after various intervals following the initial treatment.

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Materials and Methods

I. Cell line and animal model

A rat brain tumor induced by N-methylnitrosourea was transplanted into the brains of 200-gm adult male Fischer rats, using a technique reported previously.¹⁾ The original tumor was described as a malignant astrocytoma;^{4,16)} but as a result of characteristics acquired during animal- and cell-culture passage, the 9L tumor is now classified as a gliosarcoma.¹⁾ About 1 week after transplantation, a readily extirpable tumor appears. The parameters of its subsequent growth include a tumor weight doubling time of 40 h, a cell cycle time of 19 h, a GF of 0.47, and a cell loss factor of 0.12.

II. Effect of BCNU on rat survival

BCNU was obtained in vials each containing 100 mg of drug, which was reconstituted by adding 3 ml of absolute ethanol and then 27 ml of sterile water to each vial. The drug was administered immediately upon reconstitution. To determine the effectiveness of BCNU in prolonging survival, 68 tumor-bearing rats were randomly divided into five groups of 12 to 14 animals each. Group 1, the control group, was not treated; Groups 2, 3, 4, and 5 were treated by i.p. administration of a LD₁₀ (13.3 mg/kg) of BCNU 14 days after tumor implantation. The same BCNU treatment was administered a second time to Groups 3, 4, and 5 on post-treatment Day 5, 10, and 14, respectively. Animals in all groups were observed daily and weighed every 4 or 5 days. The day of animal death was recorded and used to calculate the median survival time (MST) and ILS for each group. Lungs, kidneys, and testes of rats that survived more than 80 days were examined microscopically.

III. Autoradiographic analysis

In a second experiment, rats were implanted with 9L brain tumor and were then either used as controls or treated on Day 14 after implantation with an i.p. LD₁₀ of BCNU; then on Day 2, 3, 7, 14, 17, and 21 after treatment, three tumor-bearing rats each time were given ³H-thymidine (³H-TdR), 1 µg/gm of body

weight injected i.p. 5 times at intervals of 8 h. Another group of rats were treated with a second LD₁₀ of BCNU 10 days after the first treatment; and on Day 4, 7, and 17 after the second treatment, groups of rats were given the same fractionated injections of ³H-TdR. These fractionated injections spanned approximately two cell cycles during which normally dividing cells could be labeled with ³H-TdR. Thirty min after the last injection, the animals were sacrificed; the tumors were removed, and half of each was fixed in 10% formalin for pathological examination. The other half was disaggregated for 10 min at 37°C with 0.25% trypsin into a single-cell suspension, from which the dead cells were removed by the Isopaque Ficoll method described below, and the remaining viable cells were treated for 5 min with a hypotonic solution, fixed, and spread on a slide. From these slides, autoradiographs were obtained by a method detailed in a previous report.¹¹⁾ The percentage of cells labeled with ³H-TdR (the labeled population) for each tumor was determined using between 1,000 and 3,000 cells from each slide. Cells were counted as labeled if they contained more than four grains per nucleus.

IV. Removal of dead cells

The procedure used to separate white cells from red cells and dead cells was detailed in a previous report.⁵⁾ In brief, it consisted of adding 20 µl of 25% (v/v) sodium azide to 5.0 ml of tissue culture medium (final solution: 0.1%) containing from 10⁷ to 5 × 10⁸ tumor cells, then incubating the mixture for 3 min in a water bath 37°C to bring it to 20°C. This mixture (Isopaque Ficoll), which had a density of 1.09, was stored at 4°C and protected from light until used. After the tumor cell suspension was layered on the Isopaque Ficoll, the tubes were spun for 15 min at 2,000 g in a centrifuge prewarmed to 20°C. The supernatant above the Isopaque Ficoll interface was discarded, and the live cell layer at the interface was collected, as was all of the separating medium above the dead-cell pellet. Viability of the cells was checked by the trypan blue exclusion test.¹⁰⁾ The tumor cell preparation was then diluted with 10 ml of cold medium and mixed, and the live cells were pelleted by centrifugation at 300 g for 10 min at 4°C. The pelleted cells

Table 1 Results of multiple treatments with BCNU (LD_{10} dose) for rat brain tumor

Effect	No. of rats	Median survival	Increased life span	Long term survivors	
				60 days (%)	80 days (%)
Treatment					
Control	12	23 days	—	0	0
Once	12	36 days	56.5%	0	0
Twice (5 day interval)	14	52.5 days	128.3%	6(43)	3(21)
Twice (10 day interval)	14	52.5 days	128.3%	4(23)	3(21)
Twice (14 day interval)	12	52 days	126.1%	5(42)	4(33)

were washed once more with the medium before they were used for the autoradiographic study.

Results

I. Effect of BCNU on rat survival

Survival of the various treatment groups is summarized in Table 1. All untreated controls died during the fourth week, resulting in an MST of 23 days for Group 1. For animals in Group, 2 which received a single LD_{10} of

BCNU, the MST was 36 days, which constituted an ILS of 56%. Animals receiving a second LD_{10} of BCNU had the same MST regardless of the treatment schedule, with the range among Groups 3, 4, and 5 between 52.0 and 52.5 days (ILS=126–128%). This amounted to only a modest increase in life span (calculated from the mean life span) beyond that achieved by a single LD_{10} , but the retreated groups included a considerable portion (ranging between 20% and 30%) of long-term survivors (animals surviving more than 80 days: ILS

Fig. 1 Relative weights to pretreatment weights of each treated group are plotted. Black arrows show the day when LD_{10} BCNU was administered to each group of rats indicated by symbols. ★: Group 2, △: Group 3, ■: Group 4 and □: Group 5. (See text for detail).

Fig. 2 Bizarre shaped giant cell indicated by arrow. H. and E. $\times 132$

300%). Weight changes in each group are shown in Fig. 1, which shows that weight loss was significantly faster for Groups 2 (single LD₁₀) and 3 (second LD₁₀ 5 days after the first) than for Groups 4 and 5 (second LD₁₀ after 10 and 14 days, respectively). Histological examination of long-term survivors revealed some congestive and atelectatic changes in lung tissue, with edema, as well as emphysematous portions; interstitial fibrosis was present but minimal. However, morphologically bizarre giant cells (Fig. 2) were occasionally observed in the lungs. Kidneys and testes appeared normal.

II. Autoradiographic analysis

After treatment with one LD₁₀ of BCNU: The labeled cells separated by the Isopaque Ficoll method from tumors receiving ³H-TdR on Day 0, 2, 14, 17, or 21 after the single LD₁₀ were counted. The percentage of labeled cells in tumors at the various intervals is shown in Fig. 3. The dye exclusion test showed that, for all groups, between 90% and 95% of the cells in interphase were alive, and 15% to 20% of the cells in the pellet were viable. The labeled population (LP) for untreated control tumors was 38%. In the treated tumors, the LP decreased almost to 20% three days after treatment, remained at that level until Day 7,

Fig. 3 Change of labeled population (LP) of the tumor following administration of a single dose of LD₁₀ BCNU. An open circle represents an average LP of the individual tumor studied.

Fig. 4 Change of labeled population (LP) of the tumor following two LD₁₀ doses of BCNU spaced at 10 days. Closed circles represent an average LP of the individual tumor studied.

and then gradually increased until it reached 45% on Day 21.

After treatment with two LD₁₀ of BCNU, 10 days apart: The LP in tumors receiving ³H-TdR on the respective Days 0, 3, 7, 14, 21, or 27 after the first treatment (the second treatment having been given on Day 10) is plotted in Fig. 4. The LP of untreated tumors was 38%. On Day 3, the LP decreased to 20% and remained there until Day 14, thereafter increasing to 45%.

III. Pathological findings

Figure 5A shows the morphology of an untreated tumor: the cells were usually bipolar, with well stained cytoplasm. In contrast, tumor cells treated with BCNU (Fig. 5B) lost cytoplasmic detail and contained swollen cells and single or multinucleated giant cells. Figure 6 shows the morphological changes by Day 40 after the initial LD₁₀ of BCNU in a tumor that was treated a second time on Day 10: viable cells were found only around the tumor vessels, and there were large necrotic areas, which were never observed in the untreated tumors at that size. The detailed histology of BCNU-treated brain tumor is summarized in Fig. 7. By Day 2 after the initial treatment, the number of abnormal giant cells had increased from 1.0% (on Day 0, the baseline) to about 5%; the percent of mitoses had decreased from the baseline of 1.2% to about 0.5% by Day 2, and thereafter it gradually increased to 1.5% by Day 14, at which level it remained.

A

B

Fig. 5 Morphological appearance of untreated (A) and BCNU-treated (B) glioma. $\times 400$

Fig. 6 Morphological appearance by Day 40 after first treatment with two LD_{10} of BCNU, 10 days apart; viable cells only around the tumor vessels and large necrotic areas were recognized. $\times 100$

Fig. 7 Mitotic index (X) and percent of abnormally giant cells (*) are plotted following LD_{10} dose of BCNU.

Discussion

Information concerning cell population kinetics should provide a more rational basis of the design of drug schedules in tumor chemother-

apy, and animal models have been valuable in testing a number of novel strategies on experimental tumors. The response of the 9L rat brain tumor model has been extensively investigated and a good deal is known about its kinetics. A single LD_{10} of BCNU adminis-

tered to this tumor produced an ILS of 50% to 100% and resulted in a 3-log cell kill as determined by an *in vitro* CFE assay.^{13,14)} Post-treatment kinetics revealed that the maximum cell kill from an i.p. LD₁₀ of BCNU occurred 30 min after administration, and there was no apparent change in the mean measured fraction of surviving cells over the next 4 days; then over the following 10 days it gradually increased to around 0.1, where it plateaued.¹⁴⁾ On the basis of this information, a combination of BCNU with a 2-day course of 5-FU—while the repopulation of clonogenic cells was active—seemed to be a likely way to increase the cell kill.¹⁵⁾

When the combination protocol with 5-FU was tried in animal studies, only a modest additional cell kill was obtained by courses administered as much as 5 days following treatment with BCNU; there were, however, a few long-term survivors, which were never found after treatment with BCNU alone.¹⁵⁾ These results imply that the GF does not increase during tumor repopulation and/or that not all of the clonogenic cells were cycling during this period. Autoradiographic studies revealed that the GF of cycling cells with a cell cycle time of less than 40 h (which is double that for untreated 9L cells) was low (~20%) for the first 10 to 15 days after treatment with BCNU and increased only to 45% on Day 21. This would imply that between 20% and 45% of additional tumor cells might be killed by adjunctive cell cycle specific (CCS) drugs during this period. Although some of the labeled cells would die in any case and thus lower the GF,⁶⁾ the therapeutic effects of CCS drugs would nevertheless be tremendous if the GF really includes all of the clonogenic cells.

Another important finding of this study is that the CFE started to recover on Day 4 after BCNU treatment,¹⁴⁾ while the GF remained low (~20%) until Day 10. Between Days 2 and 4, there were remarkable histological changes, such as giant cell formation, abnormal mitosis, and focal necrosis. It was during this period that actual cell death took place: The increase in clonogenic cells when there was no increase in the proliferating fraction suggests that some cells recovered from potentially lethal damage (PLD), even though recovery of BCNU treated 9L cells

from PLD has not been observed *in vitro* (personal communication from K. T. Wheeler). Also, PLD usually occurs within a day or two after treatment rather than 4 to 25 days after treatment when the apparent recovery was observed in this experiment.³⁾

When a second treatment with BCNU was administered at an interval of from 5 to 24 days after the initial treatment, the ILS was more than doubled in all groups, and there were some long-term survivors. Moreover, these actually started to regain weight 50 days after tumor implantation and did not show any drastic weight loss until their death. Among the animals receiving a second treatment, animals in Group 3, which received the second dose after an interval of 5 days, showed more severe weight loss than those in Groups 5 and 6, receiving the second dose after intervals of 10 and 14 days respectively. This may indicate that the toxic effect of two LD₁₀'s is more pronounced when the second dose closely follows the first one.

Though autopsy material from long-term survivors revealed some pathological changes in the lung, these animals did not show the marked interstitial fibrosis of lung that has been observed in both rats and human patients after chronic treatment with BCNU.^{2,7)} Since it was from the effects of the tumor that the animals died, it would appear that they were able to tolerate two LD₁₀'s of BCNU.

This study demonstrates (1) that recovery of CFE is not only due to proliferation of clonogenic cells that escaped the effect of the drug, (2) that the 3-log cell kill induced by a LD₁₀ of BCNU is not enough to cause any significant portion of the remaining cells to move into the cycling pool, so a second treatment using another CCNS agent with different toxic effects than BCNU might be more effective than treatment with a CCS drug following treatment with BCNU, and (3) that cells surviving or repopulating after an initial treatment with BCNU are, therefore, not resistant to BCNU.

These results favor the use of a repeated course of BCNU at LD₁₀ on this model, though some treatment protective against the toxic effects of repeated courses of BCNU should be sought.

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References

- 1) Barker, M., Hoshino, T., Gurcay, O., et al.: Development of an animal brain tumor model and its response to therapy with 1, 3-bis (2-chloroethyl)-1-nitrosourea. *Cancer Res* 33: 976-986, 1973.
- 2) Barker, M., Deen, D. F. and Baker, D. G.: BCNU and x-ray therapy of intracerebral 9L rat tumors. *Int J Radiat Oncol Biol Phys* (in press).
- 3) Barranco, S. C.: In vitro responses of mammalian cells to drug-induced potentially lethal and sublethal damage. *Cancer Treat Rep* 60: 1799-1816, 1976.
- 4) Benda, P., Someda, K., Messer, J., et al.: Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. *J Neurosurg* 34: 310-323, 1971.
- 5) Davidson, W. F. and Parish, C. R.: A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J Immunolog Method* 7: 291-300, 1975.
- 6) Ehman, U. K. and Wheeler, K. T.: Cinemicrographic determination of cell progression and division abnormalities after treatment with 1, 3-bis (2-chloroethyl)-1-nitrosourea. *Eur J Cancer* 15: 461-473, 1979.
- 7) Holoye, P. Y., Jenkins, D. E. and Greenberg, S. D.: Pulmonary toxicity in long-term administration of BCNU. *Cancer Treat Rep* 60: 1691-1694, 1976.
- 8) Hoshino, T., Wilson, C. B., Rosenblum, M. L., et al.: Chemotherapeutic implications of growth fraction and cell cycle time of glioblastoma. *J Neurosurg* 43: 127-135, 1975.
- 9) Levin, V. A.: Pharmacological principles of brain tumor chemotherapy. pp 315-325, In Thompson, R. A. and Green, J. R. (eds): *Advances in Neurology*. Vol. 15. Raven Press, New York, 1976.
- 10) McLimans, W. F., Davis, E.V., Glover, F. L., et al.: The submerged culture of mammalian cells: The spinner culture. *J Immunol* 79: 428-433, 1957.
- 11) Nomura, K., Hoshino, T., Knebel, K., et al.: BCNU-induced perturbations in the cell cycle of 9L rat brain tumor cells. *Cancer Treat Rep* 62: 747-754, 1978.
- 12) Rall, P. and Homan, E. R.: Possible approaches to selective toxicity: New concepts. *Cancer Chemother Rep* 51: 247-251, 1967.
- 13) Rosenblum, M. L., Wheeler, K. T., Wilson, C. B., et al.: In vitro evaluation of in vivo brain tumor chemotherapy with 1, 3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Res* 35: 1387-1391, 1975.
- 14) Rosenblum, M. L., Knebel, K. D., Vasquez, D. A., et al.: In vitro clonogenic tumor cell kinetics following 1, 3-bis(2-chloroethyl)-1-nitrosourea brain tumor therapy. *Cancer Res* 36: 3718-3725, 1976.
- 15) Rosenblum, M. L., Wheeler, K. T., Barker, M., et al.: In vivo clonogenic tumor cell kinetics following BCNU brain tumor therapy. *Proc Am Assoc Cancer Res* 17: 219, 1976.
- 16) Schmidek, H. H., Nielson, S. L., Schillel, A. L., et al.: Morphological studies of rat brain tumors induced by N-nitrosomethylurea. *J Neurosurg* 34: 335-340, 1971.