Improved treatment of a brain-tumor model

Part 1: Advantages of single- over multiple-dose BCNU schedules

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Clonogenic cell and animal survival studies were used to determine the most effective BCNU therapy schedule in the 9L rat brain-tumor model. Survival of tumor cells following a single LD10 dose of BCNU (13.3 mg/kg intraperitoneally) was compared to cell survival after one to four daily 0.5 × LD10 doses. The posttreatment kinetics of surviving clonogenic cells were investigated at various times after BCNU was given in single doses of 0.25 to 1 × LD10 and in two daily doses of 0.5 × LD10. The cell kill was greater, time to reinitiation of cell growth was later, posttreatment rate of clonogenic cell proliferation was slower, and the interval to total repopulation of the clonogenic cell pool was longer with a single LD10 dose as compared to the multiple-dose schedules. Animal survival studies confirmed that a single LD10 dose of BCNU was at least as effective as a cumulative level of up to 1.69 times that amount when treatment was administered in smaller doses, regardless of the fractionation schedule.

Clinical experience with patients harboring malignant brain tumors has shown that a single BCNU dose of 185 to 200 mg/sq m is tolerated well. Results of these animal experiments suggest that this therapy should have anti-tumor activity at least equivalent to the more commonly employed schedule of 80 mg/sq m/day given for 3 days. Although direct comparison of treatment efficacy using the two schedules is not possible, no adverse clinical effects have been observed with the recently adopted single-dose schedule. Furthermore, the duration of patient hospitalization for chemotherapy has decreased.

Key Words · BCNU · chemotherapy · brain tumor · malignant glioma · clonogenic cell · stem cell · tumor model

Even though clinical results are still unsatisfactory, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) is considered to be an effective agent for chemotherapy of malignant brain tumor. The drug crosses the blood-brain barrier readily, has a powerful alkylating and carbamoylating action, and does not have a significant cell-cycle stage dependency. Phase III trials have shown that a combined treatment with BCNU and irradiation is more effective in glioma patients than chemotherapy or radiotherapy alone. In addition, small fractionated doses of BCNU have been reported to show additivity or synergism with radiation treatments in vitro.

The present studies expand upon previous preliminary investigations on BCNU dose schedules in the treatment of brain tumors in vivo. We have used studies of animal and clonogenic cell survival to determine the most effective schedule of BCNU chemotherapy in the in vivo 9L-tumor model. A direct relationship between the assayed tumor-cell kill and the animals' life span following various doses of BCNU has validated the assay as a sensitive, reliable measure of in vivo therapeutic efficacy.

Materials and Methods

Complete descriptions of the animal brain-tumor model and of the general procedure for the clonogenic cell assay have been published previously. Briefly, 9L gliosarcoma cells are implanted intracerebrally with a stereotaxic technique in adult male Fischer 344 rats, each weighing 150 to 200 gm. An easily removable solid tumor is first evident about 7 days postimplantation.
to 8 days later. The median life expectancy of untreated animals is approximately 20 to 22 days after cell transplantation. Animals are treated intraperitoneally with BCNU when their tumors are well established, and when they display minimal neurological deficits (after approximately 2 weeks). This sequence is roughly comparable to that used in the treatment of human brain tumors. Each experiment also includes untreated tumor-bearing control animals. The LD<sub>10</sub> dose is 13.3 mg/kg.

After a specified posttreatment interval, animals are sacrificed and the tumors surgically removed, minced, and enzymatically disaggregated to a single-cell suspension. Various cell dilutions are plated in 60-mm Petri dishes and processed for colony formation; they are incubated for 2 weeks at 37°C in a mixture of 5% CO<sub>2</sub> and 95% air, with an enriched medium found to be optimal for the in vitro growth of this tumor-cell line. The colony-forming efficiency is determined as the ratio between the number of colonies formed and the number of cells plated, and the surviving fraction of clonogenic brain-tumor cells is calculated as the ratio between the colony-forming efficiency of treated and untreated tumor cells. The surviving fractions are multiplied by the relative total number of tumor cells at each posttreatment interval, to eliminate the influence of dead-cell removal on the analysis of the clonogenic cell population.

**TABLE 1**

<table>
<thead>
<tr>
<th>Experiment &amp; BCNU Dose</th>
<th>No. of Daily Doses</th>
<th>No. of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 x LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1, 2, 3, or 4</td>
<td>35</td>
</tr>
<tr>
<td>LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td>cell kinetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 to 1 x LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1</td>
<td>167</td>
</tr>
<tr>
<td>0.5 x LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>2</td>
<td>77</td>
</tr>
<tr>
<td>animal survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 to 1 x LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1</td>
<td>36</td>
</tr>
<tr>
<td>0.5 x LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1, 2, or 4</td>
<td>62</td>
</tr>
<tr>
<td>no BCNU (control)</td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>
Single- versus multiple-dose BCNU treatment schedule

The experimental groups are summarized in Table 1. Split-dose experiments were performed to compare the cell kill achievable with single large and multiple small doses of BCNU. Tumors from 35 animals were analyzed 6 to 24 hours after one, two, three, or four daily 0.5 × LD_{10} doses of BCNU. Cell survival was determined by colony-forming efficiency and was compared to cell survival evaluated in 38 animals for 4 days following a single LD_{10} dose of BCNU.

The posttreatment kinetics of surviving clonogenic cells were investigated in 167 animals at various times after single BCNU doses of 0.25 to 1 × LD_{10}. Similar experiments were performed on 77 animals that received two daily 0.5 × LD_{10} doses of the drug. The cell kill, time to reinitiation of cell growth, posttreatment rate of clonogenic cell proliferation, and interval to total repopulation of the clonogenic cell pool were compared for the single- and multiple-dose schedules.

Animal survival studies were performed on 98 animals with single (0.5 to 1 × LD_{10}) and multiple (0.5 × LD_{10}) doses of BCNU administered at intervals of 1, 2, and 4 days. Thirty-two untreated animals served as controls.

Results of the split-dose experiments are shown in Figs. 1 and 2. The first dose resulted in approximately a 2-log cell kill, but the second gave only a 1-log kill, and a third and fourth showed no further activity. The plateau observed was at the same level as for a single BCNU dose of 0.75 × LD_{10} (as reported in a previous study), with a surviving fraction consistently higher than after a single LD_{10} dose (Fig. 2).

Tumor-cell growth after treatment was dose-dependent (Table 2). After a lag period of approximately 1 to 4 days, the surviving clonogenic cells proliferate, with a doubling time of 15, 21, and 38 hours for single 0.25, 0.5, and 1 × LD_{10} doses, respectively; the repopulation interval ranged between 4 days (for 0.25 × LD_{10} doses) and 23 days (for LD_{10} doses). Investigations on the posttreatment kinetics of clonogenic cells surviving two daily 0.5 × LD_{10} doses of BCNU (Figs. 3 and 4, and Table 2) showed that repopulation begins on Day 2, with a cell-doubling time of 26 hours and a repopulation interval of approximately 10 days. A comparison of these results for two doses with those obtained with a single LD_{10} dose confirms a marked therapeutic advantage for the latter, despite the administration of the same total amount of the drug (Fig. 4).

**FIG. 3.** Posttreatment kinetics of surviving clonogenic cells following two daily 0.5 × LD_{10} doses of BCNU. Each point represents a single tumor; different symbols indicate results of different experiments. The curve is drawn by best eye fit.

Evaluation of the changes in tumor size following animal treatment with one LD_{10} and two 0.5 × LD_{10} doses demonstrates a difference in therapeutic response (Fig. 5). Tumor weights appeared to increase slightly for the first 3 to 4 days after an LD_{10} dose of BCNU, followed by a decrease to pretreatment levels by the end of the 1st week and a further decrease during the 2nd week; a nadir of approximately 40% of pretreatment size was reached on Day 14. By contrast, the tumor weight never decreased after treatment with two 0.5 × LD_{10} doses, although the rate of tumor growth was less than for untreated animals.

Animal survival studies showed an increased life span (ILS) of 90% after a single LD_{10} dose of BCNU, as compared to 48% and 86% following two and three

**TABLE 2**

<table>
<thead>
<tr>
<th>BCNU Dose (fraction of LD_{10})</th>
<th>Tumor Cell Kill (%)</th>
<th>Log Cell Kill</th>
<th>Proliferation Lag (days)</th>
<th>Clonogenic Cell-Doubling Time (hrs)</th>
<th>Repopulation Interval (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>96.37</td>
<td>1.44</td>
<td>1</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>0.5</td>
<td>97.82</td>
<td>1.66</td>
<td>1-2</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>0.50 × 2</td>
<td>99.83</td>
<td>2.77</td>
<td>1</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>99.96</td>
<td>3.40</td>
<td>2-4</td>
<td>38</td>
<td>23</td>
</tr>
</tbody>
</table>

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Fig. 4. Comparison of the posttreatment kinetics of surviving clonogenic cells following two daily 0.5 \times \text{LD}_{10} doses of BCNU or a single \text{LD}_{10} dose. Each point is derived from the measured surviving fraction of 10 to 12 tumors and their relative total number of cells; error bars represent standard errors. Despite the administration of the same total amount of BCNU, the bottom curve (one \text{LD}_{10} dose) shows a slower posttreatment repopulation.

daily doses of 0.5 \times \text{LD}_{10}, respectively (Table 3, Group A). A separate experiment determined that a single \text{LD}_{10} dose yielded an ILS of 105%, which was markedly superior to multiple (two to four) doses of 0.5 \times \text{LD}_{10} given at intervals of 2 and 4 days (maximum ILS of 38%) (Table 3, Group B). Toxicity was observed for four daily 0.5 \times \text{LD}_{10} doses, in the form of delayed hepatic failure and consequent death of the animal.

Discussion

Animal survival studies using the 9L brain-tumor model have shown that single \text{LD}_{10} doses of BCNU are at least as effective as up to 1 1/2 times that amount given as split treatments, regardless of the schedule.

**TABLE 3**

Animal survival studies comparing single- and multiple-dose BCNU therapy of a rat brain tumor (9L)

<table>
<thead>
<tr>
<th>Treatment Schedule</th>
<th>Total BCNU Dose (fraction of \text{LD}_{10})</th>
<th>No. of Rats</th>
<th>Median Life Span (days)</th>
<th>ILS* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU Dose (fraction of \text{LD}_{10})</td>
<td>Retreatment Interval</td>
<td>No. of Treatments</td>
<td>(fraction of \text{LD}_{10})</td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>1.0</td>
<td>untreated</td>
<td>1</td>
<td>1.0</td>
<td>18</td>
</tr>
<tr>
<td>0.5</td>
<td>daily</td>
<td>2</td>
<td>1.0</td>
<td>15</td>
</tr>
<tr>
<td>0.5</td>
<td>daily</td>
<td>3</td>
<td>1.5</td>
<td>15</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>1.0</td>
<td>untreated</td>
<td>1</td>
<td>1.0</td>
<td>9</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>0.5</td>
<td>2 days</td>
<td>2</td>
<td>1.0</td>
<td>8</td>
</tr>
<tr>
<td>0.5</td>
<td>2 days</td>
<td>3</td>
<td>1.5</td>
<td>8</td>
</tr>
<tr>
<td>0.5</td>
<td>4 days</td>
<td>2</td>
<td>1.0</td>
<td>8</td>
</tr>
<tr>
<td>0.5</td>
<td>4 days</td>
<td>3</td>
<td>1.5</td>
<td>8</td>
</tr>
</tbody>
</table>

* ILS = increased life span, calculated by [(median survival of treated animals/median survival of control animals) - 1] \times 100.
Single- versus multiple-dose BCNU treatment schedule

for the fractionated-dose chemotherapy. The reason for this observation is explained by our analyses of clonogenic tumor cells. The time to total repopulation of the clonogenic cell pool, which is equivalent to the period of prolongation of the animal's life span, depends upon three variables: the extent of the cell kill, the proliferation lag, and the repopulation rate. The administration of single large doses of BCNU results in a larger tumor-cell kill, longer proliferation lag, and slower repopulation rate than with smaller split doses.

Several possible factors might explain the differences observed in posttreatment clonogenic tumor-cell kinetics. First, since the transport of BCNU into tumors is based on concentration-dependent diffusion,\(^5,17,18,34,25\) it is possible that the larger LD\(_{10}\) dose kills cells at a greater distance from capillaries than the smaller 0.5 × LD\(_{10}\) dose. A decreased environmental concentration of oxygen and glucose at the greater distances from functioning capillaries might result in the longer lag and slower proliferation of clonogenic cells after the LD\(_{10}\) dose.\(^5,29\) Second, the cells surviving a large BCNU dose might be genetically or phenotypically different from the cells surviving smaller doses. Recent investigations have shown that the production of BCNU-resistant cells is dose-dependent, and these cells have been observed occasionally following an LD\(_{10}\) dose (unpublished data). In fact, BCNU-resistant cells have frequently shown a slower rate of growth than their BCNU-sensitive parent cell line (unpublished data). Finally, extensive destruction of the tumor mass as a result of single large doses of the agent might facilitate the recognition of, and tumor infiltration by, the host's immunocompetent cells. Such host factors might influence the proliferation and the repopulation rate of the surviving clonogenic cells.\(^3\)

If we assume that similar situations pertain for human brain tumors, our results with this experimental brain-tumor model suggest that the administration of a single BCNU dose of 160 mg/sq m should be at least as effective as the usual treatment course of 80 mg/sq m given daily for 3 days.\(^4,15,30,31\) In fact, aside from an increased frequency of emesis, clinical trials have shown that single BCNU doses up to 250 mg/sq m are tolerable,\(^7\) although single doses of 185 to 200 mg/sq m result in toxicity that is more equivalent to three daily doses of 80 mg/sq m.\(^36\) Indeed, even larger doses (over 600 mg/sq m) may be administered relatively safely when autologous bone marrow rescue is performed.\(^30,37\) Over the past several years, we at the University of California San Francisco have treated more than 150 patients with single large doses of BCNU (185 to 200 mg sq m) for their malignant brain tumors; patient toxicity levels have been acceptable, as noted in several past reports.\(^13,14,16\)

Although, to our knowledge, no studies have directly compared anti-tumor activity using different dose schedules, results of multimodality treatment protocols used at our institution showed no deleterious effects at the time we changed our BCNU administration protocol from 80 mg/sq m/day administered for 3 days to the single larger dose. Our clinical observations and the results of the present investigation in the 9L brain-tumor model might suggest that human treatment protocols could show greatest efficacy from BCNU when single large doses are given. At least, the length and expense of hospitalization would be shortened by conversion to a single-dose protocol.

References

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