Improved treatment of a brain-tumor model

Part 2: Sequential therapy with BCNU and 5-fluorouracil

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A combination chemotherapy regimen for brain tumors was developed, based on investigations of the survival of animals harboring the intracerebral 9L rat brain-tumor model and on analyses of their clonogenic tumor cells. Fischer 344 rats harboring 9L brain tumors were treated with 2-day courses of 5-fluorouracil (5-FU), in order to expose all cycling tumor cells to the drug during DNA synthesis and achieve maximum anti-tumor activity for this cell-cycle-specific anti-metabolite. Although a 74% cell kill was obtained for a total dose of 45 mg/kg or greater, animal life span was not increased over that of untreated tumor-bearing controls. However, when 5-FU (48 to 96 mg/kg total dose over 2 days) was administered after a single LD10 dose of BCNU (13.3 mg/kg), additive cell kill was suggested. In three large series, long-term animal survivors and occasional tumor cures were observed with this drug combination, a result never observed following BCNU alone. Schedule dependency was not apparent. A previously published protocol for treating recurrent malignant gliomas with sequential courses of BCNU and 5-FU was partially planned based upon these initial observations. Anti-tumor activity with the combination of drugs was superior to therapy with BCNU alone. Both animal and human studies confirm that, contrary to presently accepted oncological tenets, a chemotherapeutic agent that kills significant numbers of tumor cells but is clinically ineffective when given alone might, nevertheless, be useful in combination therapy regimens.

KEY WORDS · BCNU · 5-fluorouracil · chemotherapy · brain tumor · malignant glioma · clonogenic cell · stem cell · tumor model

PRELIMINARY studies of the relationship between tumor cell kill and animal survival in the 9L rat brain-tumor model have shown that a 90% cell kill (cytotoxic threshold) is necessary to produce an increased animal life span.19 The cytotoxic threshold is caused by delayed removal of dead cells and early proliferation of surviving clonogenic cells following treatment.7,9,19 Therefore, the 90% threshold level should represent a finite limit to any short-term treatment course, regardless of the specific therapy utilized. This suggests that, contrary to standard oncological concepts, an agent that kills less than 90% of cells and is clinically ineffective when given alone, might demonstrate anti-tumor activity when given in combination with an effective drug which had previously killed enough cells to exceed the threshold level. The pyrimidine-analog anti-metabolite 5-fluorouracil (5-FU) readily crosses the blood-brain barrier3,4 and concentrates in brain tumors,12 but is ineffective against malignant gliomas when administered alone to patients. It is an example of a cell-cycle-specific agent that kills only cells that are actively cycling and duplicating their deoxyribonucleic acid (DNA) at the time of drug administration.6 Since studies have shown that only 30% to 50% of 9L cells in a solid tumor1 and 14% to 44% of the cells in a human malignant glioma9,11 are dividing at any time, 5-FU would not be expected to achieve the 90% cell kill level.

By contrast, a cell-cycle-nonspecific agent, such as BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), can affect both cycling and noncycling cells and, therefore, has no kinetically based limit to cell kill. In previous experiments, single intraperitoneal injections of 1 × LD10 of BCNU produced a 3 to 4 log cell kill in intracerebrally implanted 9L tumors and prolonged...
animal survival.\textsuperscript{16,17,19} We have investigated our hypothesis for improving brain tumor therapy with a sequential BCNU and 5-FU treatment schedule in the 9L model system using the clonogenic cell assay and animal survival studies.

\section*{Materials and Methods}

\subsection*{Animal Model}

Our 9L brain-tumor model has been extensively described.\textsuperscript{16-19} Suspensions of 9L tumor cells were implanted with a stereotaxic technique into the left hemisphere of adult male Fischer 344 rats, each weighing 150 to 200 gm. Two weeks later, after the tumors were well established and clinical symptomatology was initially manifest, the animals were submitted to chemotherapy. Therefore, the timing of treatment for our animal model and for patients harboring malignant gliomas is comparable.

\subsection*{Drug Treatment}

\textit{5-Fluorouracil.} Maximum anti-tumor activity is achieved if 5-FU is administered while each dividing cell is in the DNA synthesis phase of its cell cycle; the duration of continuous drug exposure necessary to accomplish this maximum effect is at least as long as the entire duration of the cell cycle ($T_c$). Treatment schedules were planned to obtain a relatively continuous drug exposure of the tumor over a 2-day course because the $T_c$ of 9L tumors is approximately 20 hours.\textsuperscript{15,19} Aliquots of 5-FU* were aseptically diluted with 0.9\% NaCl just prior to intraperitoneal injections and stored at room temperature without exposure to light before each treatment. Multiple doses were given intraperitoneally by one of three essentially comparable regimens (Levin VA: personal communication, 1977): The first regimen was every 2 hours for six doses, followed by every 4 hours for nine doses; the second was every 3 hours for five doses, followed by every 12 hours for two doses; the third was every 6 hours for eight doses. For all studies, control animals received similar injections of 0.9\% NaCl alone.

\textit{BCNU.} Prior studies had determined that a single intraperitoneal BCNU dose of 13.3 mg/kg approximately constituted the LD$_{10}$ for our animal system.\textsuperscript{1} The BCNU† was administered in a 10\% ethanol solution at the LD$_{10}$ dose; control animals received injections of a 10\% ethanol solution alone.

\subsection*{Cell Survival Studies}

The complete description of the colony-forming efficiency (CFE) assay has been published.\textsuperscript{16-18} Briefly, tumor specimens are mechanically minced, then disaggregated to a single cell suspension with

\* 5-Fluorouracil obtained from Roche Laboratories, Nutley, New Jersey.

\† The BCNU was obtained from the Drug Development Branch of the National Cancer Institute.
influence the result of combination therapy with cycle phase-specific agents, z,2~ The percentage of alone is typical of the activity with a variety of cell demonstrate drug toxicity in 13 non-tumor-bearing control FU, administered 6 to 54 hours later, did not dem-

The timing of the 5-FU course did not reproducibly increase in median life span, but without long-term survivors or occasional "cures" were observed (Table 2, Figs. 1, 2, and 3). The timing of the 5-FU course did not reproducibly influence the result of combination therapy with BCNU (Table 2). The combination of BCNU and 5-

Discussion

The cytotoxicity obtained from 5-FU treatment alone is typical of the activity with a variety of cell cycle phase-specific agents. The percentage of cells killed by a 2-day course of 5-FU (74%) is slightly larger than the percentage of cells actively proliferating at the initiation of drug administration. This discrepancy is probably explained by the death of additional proliferating cells that are recruited into the cycling pool from the non-cycling pool during the 2-day course of therapy. The lack of significant increase in animal life span further supports the 90% cell kill threshold concept previously observed with BCNU treatment, and proves that survival studies may be insensitive to agents with modest, but signifi-
cant anti-tumor activity.

The addition of 5-FU to BCNU in the treatment of our animal model resulted in a suggestion of increased (additive) cell kill. The influence of a biological variability on the CFE assay requires that as many as 15 to 20 tumors must be evaluated in each of two treat-

TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Tumors Analyzed</th>
<th>Tumor Cell Kill (%)</th>
<th>Log Kill (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU alone</td>
<td>27</td>
<td>74.00</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>BCNU alone</td>
<td>4</td>
<td>99.89</td>
<td>2.99 ± 0.25</td>
</tr>
<tr>
<td>BCNU + 5-FU (6 to 54 hrs later)</td>
<td>5</td>
<td>99.97</td>
<td>3.51 ± 0.43</td>
</tr>
</tbody>
</table>

* BCNU dose: 13.3 mg/kg, once; 5-fluorouracil (5-FU) total dose for a 2-day course: 45 to 100 mg/kg (5-FU alone), or 80 mg/ kg (BCNU + 5-FU). Both drugs were given intraperitoneally. CFE = colony-forming efficiency.

TABLE 2

Survival of animals harboring 9L brain tumors treated with 5-FU and BCNU, alone and in combination*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Survival (days)</th>
<th>% ILS</th>
<th>Long-term Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none (control)</td>
<td>7</td>
<td>19</td>
<td>18-24</td>
<td>0</td>
</tr>
<tr>
<td>5-FU alone</td>
<td>17</td>
<td>20</td>
<td>14-31</td>
<td>5</td>
</tr>
<tr>
<td>BCNU alone</td>
<td>9</td>
<td>46</td>
<td>25-60</td>
<td>142</td>
</tr>
<tr>
<td>BCNU + 5-FU</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>days 3 &amp; 4</td>
<td>15</td>
<td>34</td>
<td>24-cure</td>
<td>79</td>
</tr>
<tr>
<td>days 5 &amp; 6</td>
<td>17</td>
<td>37</td>
<td>25-84</td>
<td>95</td>
</tr>
<tr>
<td>days 7 &amp; 8</td>
<td>16</td>
<td>38</td>
<td>26-cure</td>
<td>100</td>
</tr>
<tr>
<td>days 11 &amp; 12</td>
<td>18</td>
<td>44</td>
<td>28-cure</td>
<td>132</td>
</tr>
<tr>
<td>days 14 &amp; 15</td>
<td>17</td>
<td>41</td>
<td>30-75</td>
<td>116</td>
</tr>
</tbody>
</table>

Experiment 2

none (control)             | 13          | 26             | 22-39 | 0                   |
| 5-FU alone                 | 16          | 38             | 31-46 | 46                  |
| BCNU alone                 |             |                |       |                     |
| days 1 & 2                 | 18          | 37             | 30-102 | 42                 |
| days 3 & 4                 | 17          | 39             | 28-95 | 50                  |
| days 7 & 8                 | 13          | 32             | 26-40 | 23                  |
| days 11 & 12               | 17          | 33             | 28-58 | 27                  |
| days 15 & 16               | 9           | 42             | 33-59 | 62                  |

Experiment 3

none (control)             | 16          | 22             | 18-30 | 0                   |
| 5-FU alone                 | 15          | 24             | 15-30 | 9                   |
| BCNU alone                 | 16          | 34             | 23-49 | 57                  |
| BCNU + 5-FU                |             |                |       |                     |
| days 2 & 1                 | 13          | 40             | 31-104 | 82                 |
| days 1 & 2                 | 20          | 36             | 29-101 | 64                 |
| days 8 & 9                 | 17          | 36             | 30-cure | 64                 |
| days 13 & 14               | 16          | 38             | 34-67 | 73                  |

* BCNU dose: LD50 (13.3 mg/kg, intraperitoneally). 5-Fluoro-

uracil (5-FU) doses: Experiment 1 = 8-12 mg/kg every 6 hours for eight doses (total: 64-96 mg/kg); Experiment 2 = 10 mg/kg every 6 hours for eight doses (total: 80 mg/kg); Experiment 3 = 6 mg/kg every 6 hours for eight doses (total: 48 mg/kg); up to 96 mg/kg total dose was found to be nontoxic in separate experiments.

† ILS = increased animal life span, calculated as (median survival treated animals/median survival control animals - 1) × 100.

‡ Percent of animals surviving more than 60 days.

long-term survivors (Table 2). As expected, BCNU showed significant anti-tumor activity with a marked increase in median life span, but without long-term survivors or animal cures. However, when 5-FU was added to BCNU, although the median life span was not increased, long-term survivors and occasional "cures" were observed (Table 2, Figs. 1, 2, and 3). The addition of 5-FU to BCNU in the treatment of our animal model resulted in a suggestion of increased (additive) cell kill. The influence of a biological variability on the CFE assay requires that as many as 15 to 20 tumors must be evaluated in each of two treatment groups in order to document statistically signifi-
cant differences (with 95% confidence) when tumor cell kill differs by only one-half a log. We therefore concentrated our further experiments on the more clinically relevant animal survival studies. Median animal life span was not increased by the combination therapy; however, greater anti-tumor efficacy was documented by the observation of 10% to 15% long-
term survivors and occasional tumor cures. Tumor cures have never been observed following BCNU therapy alone. These results imply that agents consid-
ered ineffective when administered alone, as deter-

ined by animal survival or by a change in the size of a solid tumor, might be useful when added to treatment with an effective agent. The activity of cell cycle phase-specific agents is limited by the relatively small percentage of actively proliferating cells in solid tumors; these are among the "ineffective" agents that could be considered for combination therapy.

The inability to discover a specific 2-day course of 5-FU that was better than any other is most likely based, at least in part, on an inability to synchronize the proliferation kinetics of clonogenic cells surviving BCNU therapy in our solid brain-tumor model. Tu-

mor heterogeneity would also be expected to con-

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FIG. 1. Left: Survival curves for 116 rats harboring intracerebral 9L tumors from Experiment 1 in Table 2. The fraction of surviving animals (% surv) is plotted against days after tumor transplantation. C = controls (seven animals); 5-FU = 17 animals treated with 5-FU alone (8 to 12 mg/kg, every 6 hours for eight doses; BCNU = nine animals treated with BCNU alone (13.3 mg/kg); BCNU + 5-FU = 17 animals treated with BCNU + 5-FU combined at the noted dosages, using a representative sequential schedule. There was no increase in life span observed with 5-FU treatment alone. The BCNU therapy increased animal life span; however, long-term survivors and occasional tumor cures were seen only following combined BCNU + 5-FU treatment. Right: Survival curves for combinations of BCNU and 5-FU administered at various times, with 15 to 18 rats in each treatment group. The schedule for BCNU + 5-FU therapy did not significantly influence anti-tumor activity.

FIG. 2. Left: Survival curves for 103 rats harboring intracerebral 9L tumors from Experiment 2 in Table 2. The fraction of surviving animals (% surv) is plotted against days after tumor transplantation. C = controls (13 animals); BCNU = 16 animals treated with BCNU alone (13.3 mg/kg); BCNU + 5-FU = 18 animals treated with BCNU (13.3 mg/kg) + 5-FU (10 mg/kg every 6 hours for eight doses), using a representative sequential schedule. The group treated with BCNU alone showed increased median animal life span, but long-term survivors were observed only in the group treated with BCNU + 5-FU. Right: Survival curves for combinations of BCNU and 5-FU administered at various times, with nine to 18 rats in each treatment group. The schedule for BCNU + 5-FU therapy did not significantly influence anti-tumor activity.

Tribute to the lack of schedule dependency. As a consequence, treatment schedules that have proven successful for one or many animals should not be expected to be equally effective in all cases. Similar situations probably pertain for the treatment of human brain tumors.

Partially because of the implications of these studies, 29 patients with recurrent malignant gliomas were treated at the University of California, San Francisco, with BCNU at a single dose of 180 mg/sq m followed in 2 weeks by a 3-day continuous infusion of 5-FU (1 gm/sq m/day). The 3-day infusion was planned.
FIG. 3. Left: Survival curves for 113 rats harboring intracerebral 9L tumors from Experiment 3 in Table 2. The fraction of surviving animals (% surv) is plotted against days after tumor transplantation. C = controls (16 animals); 5-FU = 15 animals treated with 5-FU alone (6 mg/kg every 6 hours for eight doses); BCNU = 16 animals treated with BCNU alone (13.3 mg/kg); BCNU + 5-FU = 17 animals treated with BCNU + 5-FU combined at the noted dosages, using a representative sequential schedule. There was no increase in animal life span observed with 5-FU treatment alone. The BCNU increased the median animal life span; however, only the combined BCNU + 5-FU therapy was followed by long-term survival. Right: Survival curves for combinations of BCNU and 5-FU administered at various times, with 13 to 20 rats in each treatment group. The schedule for BCNU and 5-FU therapy did not significantly influence anti-tumor activity.

because the cell cycle time for human malignant gliomas has been estimated to be 45 to 75 hours. The BCNU plus 5-FU courses were repeated every 6 weeks. As previously reported, arrest of tumor progression (response or stable disease) was observed in 83% of the patients, with a median progression-free interval of 27 weeks for all patients. These results, although still unsatisfactory, were superior to therapy with BCNU alone, which showed 51% disease arrest for 14 weeks. We suggest that further advances in brain-tumor treatment will be forthcoming if the planning of therapy is rationally based upon laboratory investigations, as has been demonstrated in the present study.

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References
13. Levin VA, Crafts CD, Wilson CB, et al: BCNU (NSC-
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