The effect of phenobarbital on the toxicity and tumoricidal activity of CCNU in a murine brain tumor model

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The nitrosoureas are known to be substrates for hepatic microsomal enzymes. Since phenobarbital (PB) is a potent inducer of hepatic microsomal enzymes, and since PB and other enzyme-inducing drugs are commonly used in patients with brain tumors, the authors have assessed the effect of PB pretreatment on the toxic and tumoricidal activity of CCNU in a murine model.

In C57B1/6J mice, PB pretreatment markedly reduced the lethal toxicity of high doses of CCNU. The LD₅₀ dose of CCNU was found to be 80 mg/kg given intraperitoneally. Pretreatment with PB reduced the toxic death rate of CCNU at 40, 60, 90, or 120 mg/kg to less than 10% (p < 0.01). Pretreatment with PB also reduced the tumoricidal activity of CCNU. In an intracerebral murine ependymoblastoma, intraperitoneal CCNU alone, at 30 mg/kg given on the 5th day after tumor transplantation, produced a percent increased life span (%ILS) of > 300, and 18 of 25 were long-term survivors (LTS). In contrast, after four daily doses of PB prior to the same dose of CCNU, the %ILS was reduced to 85 with no LTS among 25 mice (p < 0.01). When CCNU was given alone at 30 mg/kg intraperitoneally on Day 10, a %ILS of > 300 with 22 of 25 LTS resulted; whereas, PB pretreatment reduced the %ILS to 15, with two of 25 LTS (p < 0.01). When CCNU was administered directly into the tumors intracerebrally at 30 mg/kg on Day 10, the %ILS was > 300, with 16 of 20 LTS; whereas, PB pretreatment reduced the %ILS to 50 with five of 20 LTS (p < 0.01). Furthermore, PB pretreatment significantly reduced the ability of CCNU to retard tumor growth in a subcutaneous murine ependymoblastoma.

Thus, PB pretreatment significantly altered the activity of CCNU. Since enzyme-inducing drugs are so commonly used in patients with brain tumors, it is possible that the clinical failure of nitrosoureas in some cases may be due to an unsuspected drug antagonism.

Key Words • brain tumor chemotherapy • CCNU • phenobarbital
zyme induction on the in vivo activity of the nitrosoureas should be established.

In the present series of experiments, a murine ependymoblastoma was implanted intracerebrally or subcutaneously to assess the effect of PB pretreatment on the tumoricidal activity of CCNU. In addition, mice not bearing tumors were used to assess the effect of PB pretreatment on the toxicity of high doses of CCNU. This is the first report on the in vivo effects of PB on nitrosourea chemotherapeutic activity and toxicity.

Materials and Methods

Animals and Tumor

The experimental animals were C57B1/6J female mice weighing 16 to 18 gm.* A mouse ependymoblastoma has been maintained in our laboratory by serial subcutaneous transplantation every 2 weeks since 1963. The tumor was originally induced by the intracerebral implantation of methylcholanthrene by Zimmerman and Arnold. 32

Tumor-Cell Suspension and Tumor Implantation

For each experiment requiring tumor-bearing mice, approximately 4 gm of 14-day-old subcutaneous ependymoblastoma tumor tissue was harvested and trypsinized in phosphate-buffered saline to produce a tumor cell suspension containing 10^6 cells/μl, as described previously. 37

Penicillin (50 units/ml) and streptomycin (50 mg/ml) were added to the tumor cell suspension. A No. 30 needle attached to a micrometer syringe assembly was used to inject 6 μl of tumor cell suspension into the right cerebral hemisphere of recipient mice in a stereotaxic frame. 37 For the subcutaneous tumor implantation, 3 × 10^6 cells in 100 μl of phosphate-buffered saline were injected into the anterior abdominal wall of recipient mice.

Drugs

Phenobarbital† was purchased as sodium phenobarbital (120 mg/ml), and CCNU‡ was obtained in bulk form. The CCNU suspensions were prepared by grinding an appropriate weight of CCNU in 2 ml of 0.4% methylcellulose in a Potter-Elvehjem homogenizer for 10 minutes in an ice bath to produce a uniform suspension. Then 0.4% methylcellulose was added to the CCNU suspension to produce the required concentration. The final suspension was kept uniform by frequent mechanical mixing, and was kept in an ice bath until used. The time from drug preparation to the completion of administration did not exceed 60 minutes.

Experimental Protocol and Statistical Analysis

Toxicity Studies in Mice With no Tumor. The dose-mortality experiments were conducted on mice with no tumor. The animals were assessed daily and the death rate tabulated. The first day of drug administration was designated Day 1. Each experiment was terminated 60 days after the last death to ensure that no late mortality would be missed. After a 60-day death-free interval, the percentage death rate was calculated.

The dose-mortality relationship for groups receiving CCNU alone was compared to the groups receiving the PB-CCNU combination. The regression lines describing the best linear fit were calculated and compared (Fig. 1). When PB and CCNU were given on the same day, CCNU was always given 3 hours after the last dose of PB.

Survival Studies in Mice With Brain Tumor. In the survival studies of mice with brain tumor, all the mice in each experiment received the same tumor cell suspension. Each survival study had its own simultaneous control. Control mice received 0.4% methylcellulose in place of CCNU, and sterile water in place of PB by the appropriate route through the same gauge needle as the treatment groups. For intraperitoneal injections, the volume was 100 μl, and for intracerebral injections the volume was 6 μl administered into the right hemisphere by the micrometer syringe using the stereotaxic frame. The day of intracerebral tumor implantation was designated as Day 1. Pretreatment PB was administered in intraperitoneally in four daily doses prior to the CCNU.

The median day of death was determined graphically by plotting the percentage of cumulative deaths against survival days after intracerebral tumor implantation. Comparison was then made between the median day of death of treated and control groups, and the percentage increase in life span (%ILS) was calculated. Long-term survivors (LTS) were defined as animals living more than 100 days after intracerebral tumor implantation. The significance of survival prolongation (therapeutic result) or shortening (toxic response) was assessed from the graphs by the Kolmogorov-Smirnov test, as adapted by Tate and Clelland. 32 To test the significance of differences between proportions as in the evaluation of the number of LTS, the chi-square test with Yates' correction for continuity was used.

Volumetric Changes in Mice With Subcutaneous Tumor. All mice with subcutaneous tumor received the same tumor cell suspension, and the day of subcutaneous tumor implantation was designated as Day 1. The control procedures were identical to those used in brain-tumor-bearing mice. Thirteen days after the subcutaneous implantation of 3 × 10^6 tumor cells, the
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tumor dimensions (long and short diameters) were measured, and the tumor volumes calculated using the formula for a prolate ellipsoid as described by Simpson-Herren and Lloyd. On Day 13, the mice with subcutaneous tumor were randomized on the basis of tumor volumes into seven comparable groups. The tumor volume was calculated weekly thereafter. Phenobarbital (75 mg/kg) or sterile water was administered intraperitoneally on Days 13, 14, 15 and 16, and CCNU (10, 20, or 30 mg/kg) or 0.4% methylcellulose was administered intraperitoneally on Day 16. The mean tumor volumes on Day 36 (20 days after CCNU treatment) are tabulated in Table 6. Analysis of variance applied to the entire sample revealed highly significant (p < 0.0005) differences among the groups on Day 36. The groups that received the PB-CCNU combination were compared to the groups that received identical doses of CCNU alone by means of the Student's t-test. After 40 days, calculation of tumor volume became impractical because of central necrosis and ulceration in many of the subcutaneous tumors.

Results

Toxicity Studies in Mice Without Tumor

Figure 1 summarizes the effects of high doses of CCNU in non-tumor-bearing mice with or without PB pretreatment. The CCNU was not toxic until an intraperitoneal dose of 30 mg/kg was exceeded. At intraperitoneal doses of 40, 50, 60, 75 or 80 mg/kg, CCNU resulted in a toxic death rate of 50%, 45%, 75%, 85%, and 92%, respectively. The calculated LD₅₀ and LD₉₀ doses were 51 and 80 mg/kg, respectively. The lethal dose (LD) values are similar to those obtained by Thompson and Larson. In our series, 90% of the toxic deaths occurred within 10 days of CCNU administration.

When PB pretreatment was administered at 125 mg/kg daily for four doses prior to the CCNU, the toxic death rate was greatly reduced (p < 0.001). Even at a dose of CCNU 50% greater than the LD₉₀, the toxic death rate was only 9%. With this level of PB pretreatment, the mice slept for 3 to 6 hours after each PB injection and lost approximately 10% of their body weight during the 4 days of PB treatment.

Table 1 summarizes the effect of increasing doses of PB on the toxic death rate of the LD₉₀ dose (80 mg/kg) of CCNU. The LD₉₀ dose of CCNU in this experiment without PB pretreatment resulted in the death of all the mice. The intraperitoneal administration of PB 100 mg/kg 3 hours prior to the LD₉₀ dose of CCNU did not reduce the mortality. Two or three daily doses of the PB given before the CCNU reduced the mortality to 60% (9 of 15) and 53% (8 of 15),
Table 1: Effect of increasing doses of phenobarbital pretreatment on the survival of 15 non-tumor-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Mice</th>
<th>Day No.</th>
<th>Dose (mg/kg)</th>
<th>Total Dose (mg/kg)</th>
<th>CCNU (ip)</th>
<th>Day</th>
<th>Dose (mg/kg)</th>
<th>No. of Deaths</th>
<th>% Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td></td>
<td></td>
<td></td>
<td>1</td>
<td>80</td>
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<tr>
<td>B</td>
<td>15</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>1</td>
<td>80</td>
<td>15</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>1,2</td>
<td>100</td>
<td>200</td>
<td>2</td>
<td>80</td>
<td>9</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>1,2,3</td>
<td>100</td>
<td>300</td>
<td>3</td>
<td>80</td>
<td>8</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

*The LD99 dose of CCNU (dose-toxicity response to CCNU with phenobarbital pretreatment). ip = intraperitoneal.

respectively (p < 0.05). The regression equation describing the best linear relationship between the PB pretreatment dose and the toxic deaths caused by the LD99 dose of CCNU was Y = -0.181 + 105.40 (r = 0.924). This experiment demonstrated that two or more doses of PB, 24 hours apart, before CCNU administration significantly altered the activity of the LD99 dose of CCNU.

Survival Studies in Mice With Brain Tumor

Table 2 summarizes the results of single-dose CCNU therapy administered intraperitoneally on Day 5 after intracerebral tumor implantation. This table includes a group of mice (Group F) that received PB alone at 125 mg/kg intraperitoneally on Days 2, 3, 4, and 5. The data presented in Table 2 were compiled from 12 separate survival studies. In the control group, 97% of the mice died by the 60th day after intracerebral tumor implantation, and less than 3% died before Day 15. The %ILS after CCNU treatment at 5, 10, 20, or 30 mg/kg intraperitoneally was 12, 30, 203, and > 300, respectively. At the optimum CCNU dose (30 mg/kg intraperitoneally), 26 of 45 mice survived longer than 100 days (%LTS = 58) (p < 0.01). When PB alone, at a total dose of 500 mg/kg intraperitoneally, was administered over 4 days, there was no increase in survival.

Table 3 summarizes the effect on survival of 15 and 30 mg/kg of CCNU administered intraperitoneally on Day 5 after intracerebral tumor implantation with or without intraperitoneal PB pretreatment (75 mg/kg × 4). The survival of all the treatment groups was significantly better than the control rate. However, comparison of the groups with CCNU alone to those groups receiving an identical dose of CCNU with the addition of PB pretreatment reveals that the latter had a significantly shorter survival. For example, CCNU at 30 mg/kg intraperitoneally resulted in a %ILS of > 300 with 18 of 25 LTS. The use of PB pretreatment reduced the %ILS to 85 with none of the 25 with LTS (p < 0.01).

Table 4 summarizes the effect of CCNU at 15 and 30 mg/kg administered intraperitoneally on Day 10 after intracerebral tumor implantation with or without intraperitoneal PB pretreatment (75 mg/kg × 4). The CCNU therapy was delayed in order to allow a greater tumor burden to develop prior to therapy. When CCNU alone was given intraperitoneally at 15 and 30 mg/kg, there followed a %ILS of 30 and > 300, respectively, and the number of LTS were two of 25 and 22 of 25, respectively. The use of PB pretreatment reduced the %ILS to 33 and 38, respectively, with none of 25 and two of 25 LTS, respectively (p < 0.01). Thus, the survival rate of the

Table 2: Effect of CCNU or phenobarbital on the survival of brain-tumor-bearing mice (dose-survival response)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Mice</th>
<th>Phenobarbital (ip)</th>
<th>CCNU (ip)</th>
<th>Median Day of Death</th>
<th>%ILS</th>
<th>LTS</th>
<th>Sig.</th>
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</thead>
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<td></td>
<td></td>
<td>33.5</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>80</td>
<td></td>
<td></td>
<td>5</td>
<td>12</td>
<td>4</td>
<td>N.S.</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td></td>
<td></td>
<td>5</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>80</td>
<td></td>
<td></td>
<td>5</td>
<td>43.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>45</td>
<td></td>
<td></td>
<td>5</td>
<td>101.5</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>F</td>
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<td>2,3,4,5</td>
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<td>300</td>
<td>33</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

*ip = intraperitoneal; %ILS = percent increased life span; LTS = long-term survivors in each group; Sig. = significance of difference between treatment group and control; N.S. = not significant.
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### TABLE 3

**Effect of phenobarbital (PB) pretreatment on the survival of brain-tumor-bearing mice treated with nontoxic doses of CCNU on Day 5**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Mice</th>
<th>Phenobarbital (ip)</th>
<th>CCNU (ip)</th>
<th>Median Day of Death</th>
<th>%ILS</th>
<th>LTS</th>
<th>Sig. A</th>
<th>Sig. B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day</td>
<td>Dose (mg/kg)</td>
<td>Total (mg/kg)</td>
<td>Day</td>
<td>Dose (mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>15</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>15</td>
<td>45</td>
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<tr>
<td>C</td>
<td>25</td>
<td>2,3,4,5</td>
<td>75</td>
<td>300</td>
<td>5</td>
<td>15</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>30</td>
<td>&gt;108</td>
<td>&gt;300</td>
</tr>
<tr>
<td>E</td>
<td>25</td>
<td>2,3,4,5</td>
<td>75</td>
<td>300</td>
<td>5</td>
<td>30</td>
<td>50</td>
<td>85</td>
</tr>
</tbody>
</table>

*ip = intraperitoneal; %ILS = percent increased life span; LTS = long-term survivors in each group; Sig. A = significance of difference between treatment group and control; Sig. B = significance of difference between group receiving CCNU and group receiving PB-CCNU combination at the same CCNU dose.

PB-CCNU combination groups was no better than the control rate.

Table 5 summarizes the effect of intraperitoneal PB pretreatment (75 mg/kg × 4) on the survival of brain-tumor-bearing mice treated with the optimum dose of CCNU (30 mg/kg) administered either intraperitoneally or intracerebrally on Day 10 after intracerebral tumor implantation. In this experiment, 30 mg/kg intracranial CCNU resulted in a %ILS of >300 and 16 of 20 LTS. With PB pretreatment, the %ILS was reduced to 50 and the LTS to 5 of 20 (p < 0.01). Intraperitoneal CCNU, 30 mg/kg, resulted in a %ILS of 118 and eight of 20 LTS. With PB pretreatment, the %ILS was reduced to 17 and the LTS to two of 20 (p < 0.01). Thus, PB pretreatment greatly reduced the survival in groups receiving CCNU by either the intraperitoneal or the intracranial route of administration.

The survival studies described in Tables 3 to 5 show that the PB-CCNU combination produced a marked reduction of tumoricidal activity, as revealed by the reduction in survival of brain-tumor-bearing mice treated with the PB-CCNU combination compared to CCNU alone at identical doses.

### Volumetric Changes in Mice With Subcutaneous Tumor

Table 6 summarizes the effect of CCNU with or without PB pretreatment on mean tumor volume changes in mice with subcutaneous tumor. A significant reduction in tumor growth retardation occurred with PB pretreatment. For example, the mean tumor volume of the control group 36 days after tumor implantation was 1841 ± 349 cu mm (S.E.), which represented a 44-fold increase in tumor volume from Day 16 to Day 36 after subcutaneous tumor implantation. Intraperitoneal CCNU, 30 mg/kg, administered on Day 16 greatly retarded tumor growth. On Day 36,
TABLE 5

Effect of phenobarbital (PB) pretreatment on the survival of brain-tumor-bearing mice treated with the optimum nontoxic dose of CCNU*

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Mice</th>
<th>Phenobarbital (ip)</th>
<th>CCNU (ip or ic)</th>
<th>Median Day of Death</th>
<th>%ILS</th>
<th>LTS</th>
<th>Sig. A</th>
<th>Sig. B</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20</td>
<td>—</td>
<td>36</td>
<td>—</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>—</td>
<td>30</td>
<td>10 ic 10 ic</td>
<td>54</td>
<td>50</td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>7,8,9,10</td>
<td>54</td>
<td>10 ic 30</td>
<td>78.5</td>
<td>118</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>—</td>
<td>42</td>
<td>10 ip 30</td>
<td>42</td>
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<tr>
<td>E</td>
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<td>541</td>
<td>10 ip 30</td>
<td>42</td>
<td>17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ip = intraperitoneal; ic = intracerebral; %ILS = percent increased life span; LTS = long-term survivors in each group; Sig. A = significance of difference between treatment group and control; Sig. B = significance of difference between group receiving CCNU and group receiving PB-CCNU combination at the same dose and by the same route; N.S. = not significant.

the mean tumor volume was 170 ± 63 cu mm (S.E.), which represented only a threefold increase in tumor volume in the 20 days after CCNU treatment. With PB (75 mg/kg intraperitoneally on Days 13, 14, 15, and 16) given prior to intraperitoneal CCNU at 30 mg/kg, the mean tumor volume on Day 36 was 541 ± 91 cu mm (S.E.). This mean tumor volume is significantly greater (p < 0.005) than the mean tumor volume of the group which received 30 mg/kg of CCNU alone. The mean tumor volume of the PB-CCNU combination group had increased 18-fold in the 20 posttreatment days compared to threefold for the group with CCNU alone.

The significant reduction in tumor growth retardation that occurred when PB was administered prior to CCNU again demonstrated the detrimental effect of PB pretreatment on the efficacy of CCNU in this tumor model.

Discussion

In the present experiments, PB pretreatment resulted in a highly significant alteration in CCNU activity in vivo. For example, in the absence of PB pretreatment, the LD₉₀ and LD₉₉ doses of intraperitoneal CCNU were 51 and 80 mg/kg, respectively (Fig. 1), whereas multiple doses of PB prior to the administration of CCNU markedly reduced the lethal toxicity of the LD₉₉ dose of CCNU (Table 1). When PB pretreatment was optimum, CCNU toxicity was almost eliminated (Fig. 1), as shown by the finding that the percentage of toxic death of the 1.5 × LD₉₀ dose of CCNU (120 mg/kg intraperitoneally) was reduced to less than 10%. The finding that a single dose of PB given 3 hours prior to CCNU had no effect on the toxic death rate, while multiple doses 24 hours apart significantly reduced the toxic death rate of the LD₉₀ dose of CCNU (Table 1), suggests that activation of hepatic microsomal enzymes was responsible for the alteration in CCNU activity caused by PB pretreatment.

In brain-tumor-bearing mice, CCNU was highly effective in prolonging survival, whereas PB alone in four daily doses did not alter their survival (Table 2). Pretreatment with PB greatly reduced the tumoricidal activity of CCNU. For example, in the absence of PB pretreatment, the LD₉₀ and LD₉₉ doses of intraperitoneal CCNU were 51 and 80 mg/kg, respectively (Fig. 1), whereas multiple doses of PB prior to the administration of CCNU markedly reduced the lethal toxicity of the LD₉₉ dose of CCNU (Table 1). When PB pretreatment was optimum, CCNU toxicity was almost eliminated (Fig. 1), as shown by the finding that the percentage of toxic death of the 1.5 × LD₉₀ dose of CCNU (120 mg/kg intraperitoneally) was reduced to less than 10%. The finding that a single dose of PB given 3 hours prior to CCNU had no effect on the toxic death rate, while multiple doses 24 hours apart significantly reduced the toxic death rate of the LD₉₀ dose of CCNU (Table 1), suggests that activation of hepatic microsomal enzymes was responsible for the alteration in CCNU activity caused by PB pretreatment.

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activity of intraperitoneal CCNU (Tables 3 to 5) and intracerebral CCNU (Table 5). The alteration in tumoricidal activity of CCNU was revealed by the significantly reduced survival in the PB-CCNU groups as compared with the groups receiving CCNU alone. This PB-induced reduction in the tumoricidal activity of CCNU was also demonstrated in subcutaneous-tumor-bearing mice by the significant reduction in tumor growth retardation that occurred in the PB-CCNU combination groups compared to the groups receiving CCNU alone (Table 6).

The most probable mechanism for the PB-induced alteration in CCNU activity was hepatic microsomal enzyme induction. It has been shown by others that in vitro ring hydroxylation of CCNU was increased four to five times by PB induction of microsomal enzymes, that the hydroxylation occurred at three separate sites on the cyclohexyl moiety, that three of the six monohydroxy derivatives have little carbamoylating activity, and that carbamoylating activity correlates with nitrosourea toxicity. It has also been shown that murine microsomal enzymes act as catalysts for the denitrosation of nitrosoureas and that the denitrosation products have little antitumor activity. Since the probable mechanism of this drug interaction (PB-CCNU) is hepatic microsomal enzyme induction, and since there are many compounds that act as enzyme inducers, it is quite possible that drug interactions of considerable clinical significance are taking place in patients with malignant brain tumors who are undergoing nitrosourea chemotherapy.

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