Use of Phenobarbital and High Doses of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea in the Treatment of Brain Tumor-bearing Mice

Paul J. Muller, Charles H. Tator, and Martin L. Bloom

Division of Neurosurgery, University of Toronto, and Sunnybrook Medical Centre, Toronto, Ontario, M4N 3M5, Canada

ABSTRACT

It has been shown that the nitrosoureas are substrates for hepatic microsomal enzymes in vitro and that phenobarbital (PB) administered in multiple doses prior to nitrosourea administration significantly reduces the activity of the nitrosoureas in murine brain tumor models.

In the present study, the effect of PB on 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) was assessed by determining the CCNU dose which would result in the long-term survival of 50% of the treated mice, and the CCNU dose which would result in the toxic death of 50% of the treated mice, with or without PB pretreatment in C56BL/6J mice. The therapeutic index, the CCNU dose which would result in the long-term survival of 50% of the treated mice, per the CCNU dose which would result in the toxic death of 50% of the treated mice, without PB pretreatment was 2.1; the therapeutic index of CCNU after PB pretreatment was 1.7. There is no significant difference between the therapeutic indices. Thus, the reduction in the tumoricidal activity of CCNU after PB pretreatment was restored by increasing the dose of CCNU without a significant change in its lethal toxicity.

INTRODUCTION

The nitrosoureas are the most effective agents for the chemotherapy treatment of malignant brain tumors because of their lipid solubility and ability to cross the blood-brain barrier. However, their lipid solubility and ability to cross the blood-brain barrier significantly reduce the in vivo tumoricidal activity of BCNU and administered in multiple doses prior to nitrosourea administration significantly reduces the activity of the nitrosoureas in murine brain tumor models.

In the present study, the effect of PB on 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) was assessed by determining the CCNU dose which would result in the long-term survival of 50% of the treated mice, and the CCNU dose which would result in the toxic death of 50% of the treated mice, with or without PB pretreatment in C56BL/6J mice. The therapeutic index, the CCNU dose which would result in the long-term survival of 50% of the treated mice, per the CCNU dose which would result in the toxic death of 50% of the treated mice, without PB pretreatment was 2.1; the therapeutic index of CCNU after PB pretreatment was 1.7. There is no significant difference between the therapeutic indices. Thus, the reduction in the tumoricidal activity of CCNU after PB pretreatment was restored by increasing the dose of CCNU without a significant change in its lethal toxicity.

In the present series of experiments, we have assessed the effect of CCNU on brain tumor-bearing and normal mice with or without PB pretreatment in order to ascertain whether the reduction in tumoricidal activity of CCNU by PB pretreatment can be restored by increasing the dose of CCNU.

MATERIALS AND METHODS

Animals and Tumor. Female C56BL/6J mice weighing 16 to 18 g were obtained from The Jackson Laboratory, Bar Harbor, Maine. A mouse ependymoblastoma has been maintained in our laboratory by serial s.c. transplantation since 1963. The tumor was originally induced by the i.c. implantation of methylcholanthrene by Zimmerman and Arnold (39).

Tumor Cell Suspension and Tumor Implantation. Approximately 4 g of 14-day-old s.c. ependymoblastoma tumor tissue were harvested and trypsinized in phosphate-buffered saline to produce a tumor cell suspension containing 10⁶ cells/ml as described previously (35). Penicillin (50 units/ml) and streptomycin (50 mg/ml) were added to the tumor cell suspension. A 30-gauge 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 by means of a stereotactic frame (35).

Drugs. PB was purchased from Sterilab, Downsview, Ontario, Canada, as sodium pentobarbital (120 mg/ml). CCNU was generously provided in bulk form by the Drug Development Branch, National Cancer Institute, Bethesda, Md. The CCNU suspensions were prepared by grinding an appropriate weight of CCNU in 2 ml of 0.4% methylcellulose in an ice bath using a Potter-Elvehjem homogenizer for 10 min to produce a uniform suspension. 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 use. The time from drug preparation to the completion of administration did not exceed 60 min.

Experimental Protocol. All mice in each survival study received the same tumor cell suspension, and each survival study had its own simultaneous control. Control mice received 0.4% methylcellulose in place of CCNU and received sterile water in place of PB by the i.p. route through the same gauge needle as that used for the treatment groups. The i.p. injection volume was 100 μl. The day of i.c. tumor implantation was designated as Day 1. PB was administered i.p. in daily doses prior to the CCNU. The last dose of PB was given 3 hr before CCNU.

The median day of death was determined graphically by plotting the percentage of cumulative deaths against survival days after i.c. tumor implantation. Comparison was then made between the median day of death of control and treatment groups, and the percentage of increased life span was calculated. LTS were defined as animals surviving >100 days after i.c. tumor implantation. The percentage of LTS is an index of treatment efficacy, and the relationship between CCNU dose and LTS was used to determine the ED₅₀ of CCNU given on Day 5, 10, or 15 without PB pretreatment and Day 10 with PB pretreatment. In no survival study was the control percentage of LTS >15%. In those groups in which the control tumor-bearing mice had a LTS percentage of 5 to 15%, this LTS percentage of the control was subtracted from the LTS percentage of the treatment groups to give the percentage of LTS corrected. The toxic death rate in brain tumor-bearing mice was defined as the number of mice dying in the
interval between CCNU administration in the drug-treated group and the first deaths in the tumor control group not receiving CCNU. The toxic death rate is given as the number of deaths over the number of mice in the group. The relationship between CCNU dose and percentage of toxic deaths was used to determine the LD$_{50}$ with or without PB pretreatment.

**Statistical Assessments.** The significance of survival prolongation (therapeutic) or shortening (toxic) as measured by the percentage of increased life span was assessed from the graphs by the Kolmogorov-Smirnov test as adapted by Tate and Clelland (29). To test the significance of differences between proportions as in LTS or toxic deaths, the $\chi^2$ test with Yates correction for continuity was used. In order to define the ED$_{50}$ and LD$_{50}$ values, standard regression analysis was used.

**RESULTS**

Table 1 summarizes the relationship between high doses of CCNU and toxic death rate in C57BL/6J mice. The best linear relationship was

$$y = 1.6195x - 32.5900 \quad (r = 0.934; p < 0.005)$$

The LD$_{50}$ and LD$_{90}$ were 50.9 and 81.3 mg/kg, respectively. These results are similar to those described by others (33).

Table 2 summarizes the effect of CCNU administered i.p. on Day 5, 10, or 15 after i.c. tumor implantation. In 250 control mice, the percentage of LTS was 3%. The best linear relationships in the groups receiving CCNU on Day 5, 10, or 15 were

$$y = 2.7150x - 10.8689 \quad (r = 0.858; p < 0.005)$$

and

$$y = 3.1774x - 28.7143 \quad (r = 0.827; p < 0.005)$$

respectively. The values of the ED$_{50}$ for CCNU given on Day 5, 10, or 15 are 22.4, 24.8, and 24.7, respectively. The time of CCNU administration in the first 15 days after i.c. tumor implantation does not appear to influence the ED$_{50}$.

Table 3 summarizes the relationship between high doses of CCNU administered on Day 10 and the toxic death rate in brain tumor-bearing mice after PB pretreatment (75 mg/kg i.p. daily for 4 days). The best linear relationship between CCNU dose and percentage of death rate was

$$y = 0.5896x - 18.4767 \quad (r = 0.968; p < 0.02)$$

The LD$_{50}$ and LD$_{90}$ were 116.1 and 199.3 mg/kg, respectively.

Table 4 summarizes the relationship between high doses of CCNU and the percentage of LTS corrected in brain tumor-bearing mice. All groups received CCNU on Day 10 except Groups 1 and 3 which received CCNU on day 5. The best linear relationship was

$$y = 1.1125x - 24.7064 \quad (r = 0.924; p < 0.02).$$

The ED$_{50}$ was 67.2 mg/kg. The therapeutic index, LD$_{50}$/ED$_{50}$, for CCNU administered i.p.
on Day 10 without PB pretreatment was 2.1; and, the therapeutic index for CCNU administered on Day 10 with PB pretreatment was 1.7. This difference is not significant.

**DISCUSSION**

The nitrosoureas are substrates for hepatic microsomal enzymes in vitro (10, 14, 15). It has been shown that in vitro ring hydroxylation of CCNU was increased 4- to 5-fold by PB induction of microsomal enzymes in vitro (15), that hydroxylation occurred at 3 separate sites on the cyclohexyl moiety (20), and that 3 of the 5 monohydroxy derivatives have little carbamoylating activity (16). It has been deduced also that carbamoylating activity correlates with toxicity (38). Thus, one might predict that PB induction of hepatic enzymes would reduce CCNU toxicity. Although the nitrosoureas, including CCNU, have a very short chemical half-life (7, 20), the rate of hepatic enzyme degradation is fast enough to metabolize large portions of the administered dose before chemical decomposition occurs (10). The microsomal hydroxylation of CCNU results in products which retain their tumoricidal activity (11) unlike the hepatic denitrosation of BCNU which results in products of negligible tumoricidal activity (19). A reduction in the *in vivo* tumoricidal activity by PB pretreatment could thus be predicted for BCNU, but not for CCNU. Levin et al. (14) demonstrated in the 9L rat glioma that chronic p.o. PB pretreatment eliminated the tumoricidal effect of BCNU and significantly reduced the tumoricidal effect of PCNU. Muller et al. (18) have shown, in C56BL/6J mice bearing i.e. epedynoblastoma, that PB pretreatment significantly reduced survival when the CCNU was administered i.p. on Day 5 or 10 at doses less than the LD<sub>50</sub>.

In the present series of experiments, the ED<sub>50</sub> for CCNU administered on Day 10 was 24.8 mg/kg. With PB pretreatment, the ED<sub>50</sub> changed 2.7-fold to 67.2 mg/kg. This represents a highly significant loss of tumoricidal activity which might not be expected if the hepatic enzymatic degradation products were highly carcinogenic.

The 2.3-fold increase in the LD<sub>50</sub>, from 50.9 mg/kg without PB pretreatment to 116.1 mg/kg with PB pretreatment, closely mirrors the change in the ED<sub>50</sub> resulting in a similar therapeutic index with or without PB treatment. It can thus be concluded that the tumoricidal activity of CCNU can be restored by increasing the CCNU dose. Levin et al. (14) found that a 25% increase over the optimal dose of a BCNU in their model did not restore tumoricidal activity after PB pretreatment. In our experiments, it was necessary to double the dose of CCNU in order to fully restore the tumoricidal effect (Table 4).

Alberts and von Daalen Wetters (1), using a mouse leukemia model, showed that PB pretreatment reduced the tumoricidal activity of CPM without decreasing the toxicity of CPM on normal marrow stem cells. They concluded that increasing the dose of CPM would not overcome the reduction in therapeutic index. In our present series of experiments with CCNU, the therapeutic index was restored after PB pretreatment by increasing the dose of CCNU.

In the present experiments, PB has been shown to reduce the tumoricidal activity and lethal toxicity of CCNU; similar effects have been described with BCNU and PCNU (14). Enzymatic induction by PB is the mechanism by which this alteration of nitrosourea activity occurs (14, 15). There are many drugs known to induce hepatic enzymes including the barbiturates and other hypnotics, analgesics, tranquilizers, antihistamines, p.o. hypoglycemic agents, and anticoagulants (5, 6). It would seem appropriate that oncologists using the nitrosoureas for the treatment of malignant brain tumors be aware of the possible interaction between nitrosoureas and hepatic enzyme-inducing drugs. If this type of drug interaction is identified in patients, it may be possible to overcome it by increasing the dose of the nitrosourea, since the decline in tumoricidal activity is closely matched by a decline in toxicity.

**REFERENCES**


Use of Phenobarbital and High Doses of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea in the Treatment of Brain Tumor-bearing Mice

Paul J. Muller, Charles H. Tator and Martin L. Bloom


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/5/2068

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.