The Uptake, Distribution, and Antitumor Activity of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea in the Murine Glioma

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SUMMARY

The antitumor activity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) against the murine ependymoblastoma, glioma 26, and glioma 261 was compared with the uptake and distribution of the 14C-labeled drug in the murine ependymoblastoma. With a modified Wilcoxon rank-sum test, it was found that CCNU increased life-span in tumor animals from 2- to 4-fold, depending on the dose used and the particular tumor type studied. Uptake and distribution studies of CCNU-14C in intracerebral and subcutaneous tumors, brain adjacent to tumor, and distant (normal) brain indicated that the parent drug had relatively constant tissue/plasma ratio levels. Of the ether-soluble fractions, CCNU had a tissue/plasma ratio of greater than 4 and accounted for 15% of the ether-soluble radioactivity. From the data, it was concluded that the antitumor activity of CCNU is probably independent of preferential uptake or distribution between normal and tumor tissue. Although few conclusions concerning the mode of action of CCNU on gliomatous tumors can be made, nevertheless further consideration of its use in the treatment of human gliomas seems warranted.

INTRODUCTION

Previous studies have demonstrated that brain tumors are more permeable to water-soluble substances than is normal brain (1, 7). Water-soluble substances enter tumors and distribute in a space which is at least 30% larger than the extracellular space of normal brain (4, 8). The purpose of this study was to determine the uptake and distribution of a lipid-soluble drug into a murine glioma and normal mouse brain. In previous studies, BCNU1 (NSC 409962) has demonstrated chemotherapeutic effectiveness against both i.c. L1210 leukemia in mice (6, 12) and i.c. murine gliomas (2, 13). BCNU is lipid soluble, but its molecular symmetry makes it difficult to study its metabolic fate. A closely related compound, CCNU, (NSC 79037), is also lipid soluble and its asymmetric configuration is more conducive to labeled metabolic studies. The tumor and brain distribution of CCNU and its chemotherapeutic efficacy against murine brain tumors were studied. It was hoped that a preferential pattern of localization might be found to help elucidate the mode of action of this drug. It is thought that BCNU and probably CCNU work as alkylating agents through an intermediary diazohydroxide compound (10).

Because of the importance of the present work to the chemotherapy of brain tumors, the following study is being presented prior to the publication of a larger work which describes, in detail, the development of the murine ependymoblastoma chemotherapy screen (J. I. Ausman, W. R. Shapiro, D. P. Rall, in preparation). The tumor uptake and distribution studies are extensions of techniques developed by Ausman and Levin (1), which have been presented in preliminary form recently.

MATERIALS AND METHODS

Tumors. The technique for the rapid i.c. implantation of carcinogen-induced murine gliomas has been described previously (2, 13). Fragments of ependymoblastoma, glioma 261, and glioma 26 (carried by s.c. transplant for years) were inoculated into the brain of C57BL/6 male mice. Although differing somewhat in their biological behavior, all 3 tumors are histologically indistinguishable from an ependymoblastoma.

Nine ependymoblastoma-bearing mice were used for the uptake studies, and 10 normal mice were used for the plasma clearance determinations.

All 3 tumors were utilized in the chemotherapy studies. In each experiment, 25 tumor-bearing animals were not treated and served as controls. Fifty tumor-bearing animals were divided into 5 groups of 10 animals per group; each group

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1The abbreviations used are: BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; i.c., intracerebral; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; LD50, 10% lethal dose; ILS, increased life-span.
received a different dose of drug. Fifty nontumor-bearing mice were similarly divided into 5 groups and treated with the same dose of drug; these served as drug controls. The 5 groups received, respectively, 20, 30, 40, 50, and 70 mg/kg of CCNU i.p. on Day 2 after tumor inoculation. The LD₀₁₀ of CCNU was approximately 60 mg/kg. The drug was diluted with steroid-suspending vehicle² to yield dose concentrations which could be injected at the constant volume of 0.01 ml/g of body weight.

Chemotherapy results were analyzed by 3 methods. (a) Treated animals at each dose level were compared with the untreated control group by a modification of the Wilcoxon rank-sum test (5, 16) to determine whether significant ILS had been afforded by the drug. (b) The median survival time of the treated groups was compared with that of the untreated controls to establish the degree of ILS. (c) Treated animals surviving more than 60 days after tumor implantation were tabulated as long-term survivors. The chance of a tumor no-take by 60 days has previously been established as less than 1 in 100 (13).

Five chemotherapy experiments involving 75 mice in each experiment were done. One utilized ependymoblastoma, 2 used glioma 261, and 2 used glioma 26.

Isotope. The CCNU, obtained from the Cancer Chemotherapy National Service Center, was uniformly labeled with ¹⁴C on the cyclohexyl ring. The compound was prepared by reduction of ¹⁴C-aminobenzyl analog to cyclohexylamine. Reaction of the latter with 2-chloroethyl isocyanate followed by nitrosation yielded CCNU with a specific activity of 1.13 mCi/mmol.³ The radiopurity of the compound was estimated to be in excess of 95% as determined by thin-layer chromatography in a chloroform: 95% ethanol (98:2, v/v) system. Before being used, it was stored as a powder at the temperature of liquid nitrogen. For animal use, the radioactively labeled CCNU was suspended in a mixture of propylene glycol and 95% ethanol (4:2, v/v) and administered i.p. as 0.08 mM CCNU in 0.05 ml volume.

Plasma Clearance of CCNU-¹⁴C. A Hamilton syringe (PB 600) was used to administer a constant amount of drug to each animal. At timed intervals of 3, 6, 9, 12, and 30 min, animals were anesthetized, and blood was drawn from the axillary vessels. The plasma was rapidly separated from the erythrocytes by centrifugation and placed into tared counting bottles. After reweighing, the samples were solubilized with 0.3 ml NCS (Nuclear-Chicago Solubilizer, Nuclear-Chicago Corp., Des Plaines, Ill.) and counted with Liquifluor (New England Nuclear Corp., Boston, Mass.) in toluene in a Packard Tri-Carb scintillation spectrometer. The counting efficiency for ¹⁴C in these samples was 75%, and the radioactive counts were well above background. The cpm/mg of plasma, corrected for body weight, were plotted against time on semilogarithmic paper. The time required for the radioactivity, representing intact CCNU, to drop 50% from its peak plasma level was designated as the t₁/₂. With this value, an injection schedule was determined to maintain the plasma level of CCNU-¹⁴C relatively constant for 25 min. This required an initial injection and a second injection, which was 25% of the first, given 12 min later.

Technique of Tumor and Brain Sampling. This technique has been described previously (1, 7). Twenty-four hr prior to study, all tumor-bearing animals received 0.2 ml of a 2% solution of trypan blue i.p. to demarcate the tumor from nontumor brain. At intervals of 7, 14, and 24 min after the initial injection, animals were anesthetized with ether, and axillary blood samples were taken. Following this the animals were removed into liquid nitrogen for 20 sec. The animals were removed, and the skull was rapidly sawed in the coronal plane with a Stryker saw. The i.c. tumor, scalp tumor, brain adjacent to tumor, and normal brain were removed under direct vision and placed in tared counting vials, reweighed, and placed on Dry Ice. The entire procedure, from time of plasma sampling to the placing of the last sample on Dry Ice, took 5 min. Samples were stored at −20° until chromatographed, as described below.

CCNU Extraction and Chromatography. Tissues were finely minced with scissors prior to ether extraction. Tissues and plasma were extracted 3 times with 1 to 1.5 ml of ether to remove CCNU quantitatively. Each time, the samples were mixed 30 sec on a Vortex mixer. All ether aspirates were saved and pooled for future chromatography. The pooled ether extracts were evaporated under a stream of nitrogen, and the ether-soluble residue was dissolved in 0.13 ml of 95% ethanol by agitation on a Vortex mixer for 30 sec. Duplicate 50-μl samples of the ether-soluble fraction were spotted on silica gel strips at the origin and at a point well above the migrating solvent front. A sample of pure reference CCNU was spotted for comparison next to the ether fraction. The precoated 5- x 20-cm plastic sheets were commercially prepared MS-Polygram Sil S-HR from the Machery-Nagel Company, Düran, Germany. The solvent system was chloroform:95% ethanol, (98:2, v/v). The silica gel strips were scanned for radioactivity in a Baird-Atomic Deluxe Radiochromatograph Scanner to locate the sites of peak activity. At intervals of peak activity, the plastic strips were cut and placed in Liquifluor and toluene for scintillation counting (see "Materials and Methods"). The most migration radioactive peak (Rf, 0.9) coincided with the known CCNU sample. The other 2 regions, which accounted for almost all of the remaining radioactivity, were located at, or extremely near, the origin and were not considered for the purposes of this study.

Radioactivity in the CCNU fraction of plasma, i.e., tumor, s.c. tumor, brain adjacent to tumor, and distant brain was corrected for dilution and normalized to cpm/mg, wet weight. The ratio of activity of each tissue CCNU fraction to plasma for each animal was then derived.

²Composition of steroid-suspending vehicle: benzyl alcohol, 9 mg; Tween 80, 4 mg; sodium carboxymethyl cellulose, 5 mg; sodium chloride, 9 mg; and sterile water sufficient to make 1 ml.
³Details of the radioactive synthesis may be obtained from Dr. Robert R. Engle, Drug Development Branch, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. 20014.
RESULTS

Chemotherapy Studies. Table 1 shows the results of administering CCNU on the survival time of mice bearing i.c. gliomas. Significant increased survival occurred at 4 doses at or below the LD₁₀, with ILS values ranging from as low as 27% for glioma 26 to greater than 426% for ependymoblastoma. The ILS values were cut off at 100 days in those groups in which less than one-half the animals had died. This occurred for all 4 doses in the case of glioma 261, and a dose-response relationship was not seen. For both ependymoblastoma and glioma 26, increased dosages generally resulted in increased ILS values. However, glioma 261 appeared to be the most sensitive and glioma 26 the least sensitive to the drug. With optimum doses of CCNU for each tumor, approximately one-half the animals survived longer than 60 days.

CCNU Tumor Levels in Vivo. The CCNU₁⁴C fraction in each tissue is summarized in Table 2. The values for each tissue represent means of animals sacrificed at 7, 14, and 24 min. They were so presented because of the lack of any clear-cut linear or logarithmic relationship. This suggests that “steady-state” levels of drug were achieved in the tissue studied by 7 min.

DISCUSSION

The pharmacological distribution and mode of action of BCNU has been studied (3, 9, 10, 14, 15), although it has

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**Table 1**

Effect of CCNU, i.p. Day 2 only, against 3 i.c. murine gliomas

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Tumor</th>
<th>Experiment No.</th>
<th>Median day of death (treated/controls)</th>
<th>Significance of Wilcoxon test</th>
<th>ILS (%)¹</th>
<th>Survivors &gt;60 days</th>
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<tr>
<td>20 Ependymoblastoma</td>
<td>1 28/19 p&lt;0.0001 47 0/10</td>
<td></td>
<td></td>
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<td>Glia 261</td>
<td>1 &gt;100/23 p&lt;0.0001 &gt;338 6/10</td>
<td>2 &gt;100/26 p&lt;0.00011 &gt;285 6/10</td>
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<td></td>
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<tr>
<td>Glioma 26</td>
<td>1 27/30 N.S.¹</td>
<td>2 32/25 p&lt;0.0001 28 1/10</td>
<td></td>
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<tr>
<td>30 Ependymoblastoma</td>
<td>1 41.5/19 p&lt;0.0001 118 1/10</td>
<td></td>
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<tr>
<td>Glia 261</td>
<td>1 &gt;100/23 p&lt;0.00011 &gt;338 7/10</td>
<td>2 &gt;100/26 p&lt;0.00011 &gt;285 10/10</td>
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<tr>
<td>Glioma 26</td>
<td>1 38/30 p&lt;0.001 27 2/10</td>
<td>2 33/25 p&lt;0.0001 32 1/10</td>
<td></td>
<td></td>
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<tr>
<td>40 Ependymoblastoma</td>
<td>1 61/19 p&lt;0.00001 221 4/10</td>
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<td>Glia 261</td>
<td>1 &gt;100/23 p&lt;0.00001 &gt;338 10/10</td>
<td>2 &gt;100/26 p&lt;0.001 &gt;285 7/10</td>
<td></td>
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<tr>
<td>Glioma 26</td>
<td>1 57.5/30 p&lt;0.00001 92 3/10</td>
<td>2 35/25 p&lt;0.001 40 2/10</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>50 Ependymoblastoma</td>
<td>1 &gt;100/19 p&lt;0.001 &gt;426 8/10</td>
<td></td>
<td></td>
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<tr>
<td>Glia 261</td>
<td>1 &gt;100/23 p&lt;0.01 &gt;338 6/10</td>
<td>2 &gt;100/26 p&lt;0.001 &gt;285 8/10</td>
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<tr>
<td>Glioma 26</td>
<td>1 70.5/30 p&lt;0.00001 135 4/10</td>
<td>2 57/25 p&lt;0.00001 128 5/10</td>
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<tr>
<td>70 Ependymoblastoma</td>
<td>1 &gt;100/19 p&lt;0.001 &gt;426 8/10</td>
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<tr>
<td>Glia 261</td>
<td>1 40.5/23 p&lt;0.05 76 2/10</td>
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<td>2 7/25 N.S.¹</td>
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</table>

¹ILS values were cut off at 100 days in those groups where less than one-half the animals had died.

²N.S., not significant by Wilcoxon test.
not been clearly elucidated. Metabolism studies (11) of the analog, CCNU, have indicated that little difference exists between the physiological disposition of this agent and BCNU in experimental animals. What is known of the effects of BCNU on nucleic acid synthesis suggests that it may be an alkylating agent (14) exerting its action via a catabolic-Intermediate, diazohydroxide compound (10). It has been shown to (a) inhibit formate incorporation into purines, (b) inhibit ribonucleotide conversion to DNA and, to a lesser extent, RNA, and (c) inhibit DNA synthesis (14). It has also been shown to prolong the S phase in L1210 leukemia cells (17).

BCNU has been found effective in the treatment of i.c. L1210 leukemia in mice (6, 12) and i.c. murine glioma (13), while CCNU, in the present study, was shown to be at least as effective as BCNU against the same i.c. gliomas. Because of the importance of the nitrosoureas in the treatment of i.c. tumors, we believed that it was necessary to study their distribution and uptake characteristics in the murine ependymoblastoma. Since it was technically easier to study CCNU-14C than BCNU, we chose the former compound.

The efficacy of CCNU in prolonging the survival of mice bearing i.c.-implanted gliomas has been demonstrated by the results shown in Table 1. This model has been used extensively in testing other cancer chemotherapeutic agents, such as BCNU, cyclophosphamide, mithramycin, and methotrexate (13). Of the compounds tested, only BCNU and to a lesser extent cyclophosphamide have demonstrated therapeutic efficacy. Although all of the drugs have not been used in the same experiment, CCNU and BCNU have been most effective; of these, CCNU has thus far been the most efficacious. Since many water-soluble compounds enter brain tumors more readily than normal brain (1, 7), it is not yet clear why the lipid-soluble drugs have thus far proved more effective. Lipid-soluble compounds enter and distribute in both brain tumor and normal brain to about the same extent. In some manner, this may permit the tumor cells to have a longer exposure to the drugs than would be the case with water-soluble agents which might be expected to diffuse rapidly away from the tumor and/or return to the plasma.

The data summarized in Table 2 suggest that CCNU concentrates in the tumor to the same degree that it does in normal brain and brain adjacent to tumor. The tissue/plasma ratios indicate a greater than 4-fold level of CCNU in tumor and brain compared to plasma. On the basis of absolute radioactive counts, the amount of CCNU entering the tumor is only 15% of the radioactivity in the ether-soluble fraction. Further studies are required to delineate the chemical nature of the radioactivity remaining in the ether-soluble fraction. These studies indicate that the antitumor activity of CCNU, and probably other nitrosourea compounds, does not particularly relate to differences in uptake or distribution between normal and tumor tissue. CCNU distributes similarly in i.c. and s.c. tumor, brain adjacent to tumor, and distant brain. The intrinsic carcinostatic activity of the drug, while still obscure, may be dependent on the rate of nucleic acid metabolism in tumor cells (14, 17). If this is true, then CCNU would be expected to be effective against disseminated glial tumors as well as tumors of low permeability such as low-grade astrocytomas. Further clinical studies seem indicated to explore the possible uses of this family of drugs in the treatment of gliomatous tumors.

ACKNOWLEDGMENTS

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