Factors Influencing the Survival of Rat Brain Tumor Cells after *in Vitro* Treatment with 1,3-Bis(2-chloroethyl)-1-nitrosourea

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**SUMMARY**

The shape of dose-response curves obtained for asynchronous, exponentially growing 9L rat brain tumor cells treated *in vitro* with 1,3-bis(2-chloroethyl)-1-nitrosourea changed as a function of the drug exposure time. For short treatment times (<1 hr), the dose-response curves had shoulders, indicating that the cells may accumulate sublethal damage; however, after longer treatments (>1 hr), little if any shoulder was apparent. The slope of the exponential portion of the dose-response curve increased progressively with treatment periods from 15 min to 2 hr. Longer exposure times (up to 24 hr) produced no further changes in the cell-kill kinetics. Cell survival was directly related to the BCNU exposure dose \(\int_0^t C_d(t)dt\) and to the amount of bound BCNU per cell. Extrapolation of the curves for these two variables indicated that some BCNU damage accumulates before death occurs. The amount of serum and cell products available in the medium to bind BCNU affected the level of survival; however, there was no evidence that extracellular spontaneous breakdown products or chemical transformation products were involved in the cell-killing mechanism.

**INTRODUCTION**

Since BCNU is one of the most effective single chemotherapeutic agents used against human brain tumors (17), investigations of the mechanisms by which this drug produces its lethal effects on cells are important for the design of brain tumor therapy schedules. Because few solid tumor systems have been adapted for *in vivo* to *in vitro* investigations that appear in the literature.

**MATERIALS AND METHODS**

9L cells from an N-methylnitrosourea-induced rat brain tumor (tumor line originally supplied by W. H. Sweet, P. T. Kornblith, J. R. Messer, and B. O. Whitman, Massachusetts General Hospital, Boston, Mass.) (11) were grown in monolayer cultures in complete medium composed of BME supplemented with 10% FCS, 1% L-glutamine, 1% nonessential amino acids, 1% vitamins, and antibiotics (penicillin, 80.5 units/ml; streptomycin, 80.5 µg/ml) (Microbiological Associates, Albany, Calif.). All cultures were maintained at 37° in a humidified 5% CO₂ atmosphere. Approximately 24 hr before treatment with BCNU, stock cultures were trypsinized [0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.) + 20 mg/100 ml EDTA (University of California Pharmacy)] and 2 to 3 x 10⁶ cells were seeded in 75-sq cm Falcon tissue culture flasks, each containing 13.5 ml of medium. Therefore, all cultures were asynchronous exponentially growing cultures with a doubling time of 18 to 20 hr (1) when treated with BCNU.
BCNU (supplied by Dr. Robert Engle, Drug Research and Development Branch, National Cancer Institute, Bethesda, Md.) was stored at −79°C and, after weighing, was immediately dissolved in absolute ethanol, diluted first in BSS and then in medium to obtain the various stock concentrations. Of each stock concentration, 1.5 ml was immediately placed in treatment flasks containing tumor cells growing in 13.5 ml of complete medium. In all experiments extreme care was taken to maintain the temperature at 35–37°C and the pH at 7.2 to 7.4, since greater variations, even for short time intervals, can drastically affect posttreatment survival (K. T. Wheeler, unpublished observation). After exposure times of 15 min, 30 min, 1 hr, 2 hr, 4 hr, and 24 hr, the BCNU-containing medium was decanted and the cells were washed twice and trypsinized. Single cell suspensions were stored in ice until they were plated in 60-mm plastic Petri dishes (Microbiological Associates) containing 10⁴ heavily irradiated (4000 rads) 9L feeder cells. Although some experiments required cells to be stored in ice for 3 to 4 hr before plating, no change in the survival of treated and untreated cells was observed between those plated as rapidly as possible and those held for up to 6 hr in ice (K. T. Wheeler, unpublished observation). After incubation for 12 days, the medium was removed and the colonies were fixed in absolute methanol, stained with 2% Giemsa (Harleco Chemical, Philadelphia, Pa.), and counted. The factors influencing the choice of culture conditions for colony formation are identical to those previously published for our in vivo to in vitro colony formation assay (9).

For determination of the BCNU exposure dose received by the cells and the amount of BCNU bound to the cells, a gravimetrically determined amount of BCNU labeled with ¹⁴C in the ethylene moieties (supplied by Dr. Robert Engle) (specific activity, 47 μCi/mg) was added to the stock solutions. The purity of the labeled BCNU was determined before treatment by high-pressure liquid chromatography (Model 3510, Chromatronics, Mountain View, Calif., and Bio-Sil A, Bio-Rad, Richmond, Calif.; columns, 50 cm x 2.1 mm). The actual BCNU concentrations (corrected for radiochemical degradation of the labeled BCNU) used to treat the cells were 4.29 and 9.75 μg/ml. Duplicate flasks of cells were treated for 15 min, 30 min, 1 hr, 2 hr, 4 hr, and 24 hr. After each exposure interval, the medium was removed and the cells were washed twice. The cells were then trypsinized, resuspended in BSS, and pelleted in a low-speed refrigerated centrifuge at 4°C. Aliquots of all samples were placed in Aquasol (New England Nuclear, Boston, Mass.) and counted for radioactivity in a Beckman LS-250 scintillation spectrometer. The cpm were corrected for quench by the method of external standardization. More than 95% of the radioactivity was accounted for in each experiment.

To determine the free BCNU remaining after each exposure time, 2-ml samples of medium were extracted 3 times with 5 ml of dichloromethane. This technique recovers more than 99% of the free BCNU present (V. A. Levin, unpublished observation). All the dichloromethane fractions were pooled and evaporated at 37°C in a vacuum. The soluble residue was redissolved in dichloromethane and the final volume was brought to 2 ml. Duplicate 100-μl samples were counted for radioactivity. Portions of the remaining dichloromethane were subjected to high-pressure liquid chromatography to determine the amount of free BCNU remaining. The free BCNU was eluted with isooctane:chloroform (1:1, v/v) at a flow rate of 1.4 ml/min. Under these conditions the BCNU peak occurred 4 min after sample injection. The entire eluent peak was collected and counted for radioactivity. No significant radioactivity above background was observed in the fractions before or after the BCNU peak. To determine the amount of radioactivity bound to the cells, the pellet was digested overnight in 0.3 ml of NCS (Amersham/Searle, Arlington Heights, Ill.) and counted in a mixture of Permablend (Packard Instruments, Chicago, Ill.) and toluene.

To determine whether BCNU breakdown products and serum factors influence survival, tumor cells were treated with medium containing 5 or 10 μg of BCNU per ml. After 30 min incubation, the BCNU-containing medium was transferred to another flask of cells and incubated for an additional 30 min. This procedure was repeated twice more for a total drug utilization time of 2 hr. After treatment each flask was trypsinized and the cells were plated for colony formation analysis. Similarly, BCNU-containing medium without cells and BCNU-containing BME without FCS were incubated at 37°C and removed to treat cells every 30 min for incubation times up to 2 hr. In order to maintain the pH at 7.2 to 7.4, it was sometimes necessary to gas each container with 5% CO₂ + 95% air after the transfer.

All survival values reported represent the mean ± S.E. of 2 or 3 independent experiments composed of 5 plates at each of 2 to 3 different dilutions. During this study the control plating efficiency varied from 60 to 80%.

RESULTS

As the exposure time was increased from 15 min to 2 hr, the shoulder on the BCNU dose-response curves progressively decreased until there was virtually no shoulder remaining at 2 hr (Chart 1). There was also a corresponding increase in the slope of the exponential portion of the curve (Chart 1). Increasing the exposure time from 2 to 24 hr did not result in any further change in the dose-response curve. After a 1-hr exposure, the survival at the highest doses might suggest that a small but possibly significant population of “resistant” cells exists (Chart 1, dashed line). A similar phenomenon has been observed with solid tumors treated with BCNU in vivo, but the resistant cell population obtained in that situation was probably due to impaired drug delivery rather than to any biochemical resistance per se (10). In addition, the lack of a similar plateau in the dose-response curves for the other in vitro exposure times suggests that the plateau observed after a 1-hr exposure (Chart 1) reflects an artifact of the system.

The time-dependent changes in the dose-response curve might be associated with the drug clearance rate. When BCNU was added to BME + FCS and cells at concentra-
Chart 1. Dose-response curves for cultured rat 9L cells exposed to graded doses of BCNU as a function of exposure time. Points, mean ± S.E. of 2 to 3 separate experiments. The linear portion of each curve was fitted by a least-squares regression analysis. The shoulders were fitted by eye. Exposure times: △, 15 min; ■, 30 min; ○, 1 hr; Δ, 2 hr; ▽, 4 hr; ○, 24 hr. ——, population of "resistant" cells existing after a 1-hr exposure (see "Results").

Concentrations of 9.75 and 4.29 µg/ml, the half-times for clearance were ~76 and ~50 min, respectively (Chart 2). The data therefore support a BCNU dose-dependent clearance similar to those observed for drugs in other biological systems (14).

Using the BCNU disappearance curves from Chart 2, a BCNU exposure dose was calculated for concentrations of 4.29 and 9.75 µg/ml with the following formula:

\[
BCNU\ exposure\ dose = \int C(t)dt
\]

where \(C_0\) is the initial concentration of BCNU in µg/ml and \(t\) is the exposure time.

A linear regression analysis of the data in Chart 3 clearly demonstrates a 1st-order relationship (correlation coefficient, 0.99) between the BCNU exposure dose and the log of the percentage of survival. It also demonstrates that an exposure dose of 125 nmoles·min/ml is required to produce a decline in survival from the 100% level (Chart 3). This threshold corresponds to a 5.9- and a 8.5-min exposure at concentrations of 10 and 5 µg/ml, respectively.

Assuming the rate of disappearance of BCNU from medium is not concentration dependent, so that the exposure dose calculated at 1 concentration can be extrapolated proportionally to all concentrations, a similar relationship between the BCNU exposure dose and the log of the percentage of survival was observed (Chart 4). The survival values shown in Chart 4 include all the BCNU concentrations and exposure times shown in Chart 1, and the calculation of the BCNU exposure doses was based on the medium clearance value obtained from the 4.29 µg/ml experiment in Chart 2. Clearly, this is an oversimplification. Based on the exposure dose at 9.75 µg/ml, an error of ~10% results from applying direct proportionality to the doses (Charts 3 and 4). Nevertheless, the data are reasonable approximations, as the linear regression analysis of these data had a correlation coefficient of 0.96 and indicated that an exposure dose of 78 nmoles·min/ml was required before a reduction in survival was observed. This is close to the

Chart 2. BCNU clearance from various incubation mixtures. All incubations were done at 37° and pH 7.2 to 7.4. Data were fit by a least-squares linear regression analysis. ○, BCNU, 4.29 µg/ml, in BME + FCS + 3 to 4 \(\times 10^8\) tumor cells; ln \(Y = 2.263 - 0.0138X\) (correlation coefficient, 0.99). ○, BCNU, 9.75 µg/ml, in BME + FCS + 3 to 4 \(\times 10^8\) tumor cells; ln \(Y = 3.046 - 0.0091X\) (correlation coefficient, 0.98). △, BCNU, 97.5 µg/ml, in BME; ln \(Y = 6.214 - 0.0062X\) (correlation coefficient, 0.96).

Chart 3. Survival of rat 9L cells as a function of BCNU exposure dose obtained at 2 different treatment concentrations. The exposure doses for 5 and 10 µg/ml were calculated from the BCNU disappearance rates determined in Chart 2 for the 4.29- and 9.75-µg/ml concentrations, respectively. Data were fit by a least-squares linear regression analysis: ln \(Y = 5.556 - 0.0076X\) (correlation coefficient, 0.99). Doses at which survival in unlabeled BCNU was determined: ○, 5 µg/ml; O, 10 µg/ml.

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Chart 5. Survival of rat 9L cells as a function of the amount of 14C-labeled bound BCNU/10⁶ cells. The BCNU dose at time 0 was 5 μg/ml. Data were fit by a least-squares linear regression analysis; ln Y = 5.77 — 9.0X (correlation coefficient, 0.97).

value of 125 nmoles·min/ml obtained from the data in Chart 3.

A similar linear relationship existed between the log of the percentage of survival and the amount of bound 14C-labeled BCNU per 10⁶ cells (correlation coefficient, 0.97; Chart 5). Approximately 0.13 nmole of bound BCNU per 10⁶ cells was required to produce a reduction in survival. Similar results were obtained when these experiments were repeated at a concentration of 9.75 μg/ml.

When BCNU-containing medium was serially transferred from flask to flask at 30-min intervals, there was an increase in the survival of cells as a function of the length of the incubation time before transfer for both the 5- and 10-μg/ml doses (Table 1). This result was predicted from the pharmacokinetic data in Chart 2.

When BCNU was incubated for 90 min in BME + FCS without cells and then used to treat cells, there was no decrease in the level of survival observed at a dose of 5 μg/ml, but there was a slight decrease in the level of survival at a dose of 10 μg/ml (Table 1). If it is assumed that the half-time for the disappearance of BCNU from BME + FCS is similar to that determined in Chart 2 for complete medium with cells, the survival levels obtained in Table 1 agree within experimental error with those predicted from the pharmacokinetic data.

When BCNU was incubated in BME alone, the percentage of survival in the 1st 30-min treatment interval was essentially the same as that observed for the other incubation conditions (Table 1). However, at each of the subsequent treatment intervals, the survival was substantially less than that observed when BCNU was incubated with serum or with serum and cells (Table 1). To determine whether this observed decrease in survival after incubation in BME alone was due to a build-up of cytotoxic breakdown products or to a decrease in BCNU clearance from the medium, the rate of disappearance of 14C-labeled BCNU from BME was measured. Since the half-time increased to ~112 min (Chart 2), the observed decrease in survival probably reflects an increase in drug exposure dose.

DISCUSSION

In recent years there has been a tendency to use dose-response curves obtained for cells treated in culture with chemotherapeutic agents to suggest principles of in vivo chemotherapy. Although most investigators working with culture systems caution against overinterpretation of their results, it is often difficult, if not impossible, to prevent this. Our data (Chart 1, Table 1) demonstrate that in vitro dose-response curves with shoulders and slopes of various magnitudes can be obtained after treatment with BCNU, depending on the presence of serum and the length of drug exposure time. We also have indirect evidence that serum quality, cell density, cell type, temperature, and pH influence the in vitro cell response to BCNU (K. T. Wheeler, unpublished results). Therefore, the D₅₀, the D₀, and the extrapolation number obtained from dose-response curves generated in vitro after treatment with chemotherapeutic agents like BCNU for a single exposure time need not reflect inherent cell sensitivity parameters such as those observed for cells exposed to X- or γ-rays.

Our data also demonstrate that cell survival is directly related to the disappearance of free BCNU from medium (Chart 3) and to the binding of BCNU to cells (Chart 5). These parameters are affected by the quantity and quality of the serum and other proteins present in the medium (Table 1). It is thus clear that measurement of pharmacokinetic parameters is important if a meaningful interpretation of survival data is to be made.

Based on the 5- and 10-μg/ml data (Charts 2 and 3), it is probable that it takes 5 to 9 min to transport BCNU from
The experiments shown in Table 1 support this concept. If cause more lethality in the 30- to 60-mm exposure interval without serum or cells, a build-up of by-products would due to drug delivery rather to inherent cell sensitivity and experiments in Table 1 were repeated at 15-mm intervals case (Table 1). To eliminate the possibility that the 30-mm would predict that, when BCNU is incubated in BME would predict that, when BCNU is incubated in BME cellular components (e.g., DNA, RNA, protein) is to occur. Although substantive evidence at the present time indicates that alkylation is the probable biochemical event involved in the cell-killing mechanism (8, 15, 16), the critical intracellular target is still unknown. The delineation of this target is the next logical step in the development of a rationale for the design of BCNU therapy schedules.

**ACKNOWLEDGMENTS**

The technical assistance of M. Freeman-Dove, C. Maroten, and J. D. Linn is appreciated. We thank Dr. M. Rosenblum, Dr. M. Barker, and Dr. C. B. Wilson for their helpful discussions and critical evaluation of this manuscript. We also thank M. Zeiger for editorial assistance.

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*Cancer Res* 1975;35:1464-1469.