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In vitro pharmacokinetics and pharmacodynamics of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)

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Abstract

The relationship between treatment efficacy and the pharmacokinetics (PK) and pharmacodynamics (PD) of anticancer drugs is poorly defined. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is an alkylating agent used in the treatment of brain and other forms of cancer. It is postulated that BCNU kills cells by forming DNA interstrand cross-links. The present study was undertaken to characterize the PK and PD of BCNU in mouse L1210 cells. L1210 cells were exposed to BCNU (0–160 μM) and analyzed for intracellular BCNU concentrations, DNA interstrand cross-links, cell cycle phase, and cytotoxicity. The half-life of BCNU in cells was ≈40 min. The maximum reduction of mitochondrial enzyme activity (maximum cell death) achieved within 24 hr after exposure to BCNU was concentration-dependent and could be described by a Hill equation. At lower concentrations, the area under the DNA interstrand cross-link–time curve linearly correlated with the maximum cell death and the area under the BCNU concentration–time curve. BCNU induced cell accumulation in the G_2/M phase of the cell cycle, which continued even after apparent completion of cross-link repair. Loss of membrane permeability was minimal (≈2%) during the first 24 hr. Thereafter, cells died exponentially over the next 9 days, primarily by necrosis. In conclusion, while cytotoxicity was concentration-dependent, an indirect relationship was found among the time-course of BCNU concentrations, DNA interstrand cross-links, and cell death. Because of the disparity between the time-scale of PK and PD, focusing only on the early events may provide limited information about the process of anticancer drug-induced cell death.

Keywords: BCNU; Pharmacokinetics; Pharmacodynamics; DNA interstrand cross-links; Cell cycle; Cell death

1. Introduction

The response of individual patients to cancer chemotherapy is largely unpredictable. It is recognized that many factors influence the outcome of cancer chemotherapy treatment. Two important components are the PK (absorption and disposition) and the PD (interaction of the drug with the tissues to cause a desirable effect) of the drug. Three- to ten-fold interpatient variability in PK (clearance and area under the serum drug concentration–time curve) has been found for many anticancer drugs [1,2]. Such variability is of therapeutic significance for anticancer drugs because of their narrow therapeutic ranges, i.e. the serum concentration required for therapeutic effect is very close to that producing toxicity. For these drugs, the same standardized dose may serve as a therapeutic dose for some patients but be subtherapeutic or toxic for others. Recognition of intersubject PK variability in anticancer drugs has led to attempts to adjust drug dosage based on PK indicators [3]. The important assumption of these dosage adjustments compensating for interpatient variability in PK is that the PD response is the same for each patient. That is, once the target steady-state drug serum concentration or the area under the drug concentration–time curve (AUC) is reached, response should be uniform. However, this assumption may not always hold. For example, the only significant predictor of response to cisplatin therapy was shown to be binding of cisplatin to DNA in white blood cells [4,5]. No relationship was observed between response and drug AUC. Thus, PK by itself may not predict response reliably when the PD of the drug is as variable as, or even more variable than, the PK. In such situations, there

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD, pharmacodynamics; PI, propidium iodide; PK, pharmacokinetics.

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is no fixed population target blood concentration to achieve for these drugs, and measurement of PD is as important as that of PK.

To adjust anticancer drug regimens for this interpatient PK and PD variability, patients should have individualized dosage regimens based on the PK, PD, or both. Thus, understanding and quantifying both the PK and PD of the anticancer drugs are necessary to dose rationally and optimize chemotherapy in an individual patient.

BCNU is a widely used drug found in treatment regimens for brain tumors and lymphoma [6]. The mechanism of action of BCNU is the formation of DNA interstrand cross-links [7–10]. The drug is a useful substrate to study anticancer PK and PD because it does not produce an active metabolite, and its apparent effect on the target cell, i.e. the formation of DNA interstrand cross-links, can be quantified. This study was therefore undertaken to quantitatively describe the PK and PD of BCNU in mouse L1210 leukemia cells. The PD of BCNU was investigated at two levels: the time-course of the immediate effect of BCNU, i.e. formation and repair of DNA interstrand cross-links, and the time-course of attendant effects, i.e. cell cycle changes and cell death.

2. Materials and methods

2.1. Chemicals

BCNU was purchased from Bristol-Myers Squibb. All other chemicals and enzymes were purchased from the Sigma Chemical Co. Cell culture materials were obtained from Gibco Laboratories.

2.2. Cell culture

Mouse lymphocytic leukemia L1210 cells were grown, as suspension cultures, in RPMI 1640 medium supplemented with 1% (v/v) fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37° under a 5% CO_2 atmosphere. Cell density did not exceed 2×10^6 cells/mL. The cell number was measured using a Coulter Counter.

2.3. BCNU exposure

BCNU was dissolved in 100% ethanol immediately prior to use. The final concentration of ethanol in culture never exceeded 0.1% (v/v), which is non-toxic to the cells. All experiments were initiated in culture flasks at a cell density of 1×10^6/mL, unless otherwise indicated. L1210 cells were exposed to various concentrations of BCNU (0–160 μM) at 37° over different time periods and subsequently processed for HPLC assay, ethidium bromide fluorescence assay, MTT assay, flow cytometric analysis, and agarose gel electrophoresis.

2.4. BCNU assay

To characterize the *in vitro* PK of BCNU, specifically the intracellular BCNU decomposition rate constant (K) and half-life ($T_{1/2}$), BCNU concentrations in both the cell suspension medium and the cells were measured using HPLC. Cells were initially exposed to 20, 40, or 60 μM BCNU and incubated for various time periods (0–120 min).

2.4.1. Extraction procedure for BCNU in cell suspension medium

Cell suspension medium was removed from the culture flasks at the end of the incubation. Calibration standards were prepared in the cell suspension medium with stock solutions of appropriate concentrations of BCNU in 100% ethanol to yield concentrations in the range of 0–60 μM. Each sample and standard were immediately placed into a 12-mL glass centrifuge tube containing 2 mL of ice-cold methyl-*tert*-butyl ether (MTBE), vortexed for 2 min, and centrifuged at 500 *g* for 10 min at 4°. The organic layer (2 mL) was removed from the top of the sample and evaporated to dryness at room temperature under nitrogen. The residue was reconstituted with 200 μL of mobile phase and filtered through a 4-mm syringe filter with a 0.45-μm nylon membrane. A 50-μL aliquot of this solution was injected immediately onto the HPLC column.

2.4.2. Extraction procedure for BCNU in cells

Following exposure to BCNU (40 μM), the cell suspension medium containing 3×10^7 cells was centrifuged in a 15-mL centrifuge tube at 500 *g* for 4 min at 4°. The supernatant was removed, and the cell pellet was washed in 30 mL PBS and centrifuged at 500 *g* for 4 min at 4°. The cell pellet was resuspended in 200 μL PBS, followed by the addition of 5 mL of ice-cold MTBE and 10 μL of internal standard solution (20 μg/mL of methylphenytoin (MPPH) in 70% (v/v) methanol). The mixture was sonicated (Ultrasound Homogenizer) for 3×15 s while the tube was kept on ice to lyse the cells. Following a further centrifugation of the sample (500 *g* for 10 min at 4°), the organic layer was removed and evaporated to dryness at room temperature under nitrogen. The residue was reconstituted with 120 μL of mobile phase, and a 50-μL aliquot of this solution was injected immediately onto the HPLC column. Calibration standards were prepared in PBS with stock solution of appropriate concentrations of BCNU in absolute ethanol to yield concentrations in the range of 0–6 μM. The same extraction and analysis procedures were applied for each standard (200 μL) except that the ultrasound homogenization was omitted.

2.4.3. Chromatography

HPLC analysis was performed on a Shimadzu HPLC system. An Ultrasphere™ C_8, reversed-phase column (15 cm × 4.6 mm i.d., 5 μm) with a guard column (4.5 cm × 4.6 mm i.d.) packed with the same material

was used for all analyses. The mobile phase consisted of 45% (v/v) methanol in water at a flow rate of 1 mL/min for extracellular BCNU and 32% (v/v) acetonitrile in water at a flow rate of 1.5 mL/min for intracellular BCNU. Detection was by UV set at 230 nm. The retention times for the BCNU peak were 8.9 min under the former conditions and 8.7 and 10.6 min, respectively, for the BCNU and MPPH peaks under the latter conditions.

2.5. PK analysis

Individual BCNU concentration–time curves for each set were fitted to a monoexponential model using the nonlinear least squares regression program, PCNONLIN (Statistical Consultants Inc.), and by the following equation:

$$C = C_0 e^{-Kt}$$

where C is the intracellular concentration of BCNU at time t, K is the decomposition rate constant, and C_0 is the initial BCNU concentration in the medium. The area under the intracellular BCNU concentration–time curve (AUC_{BCNU}) was determined using the trapezoidal rule [11].

2.6. Statistical analysis

To examine the linearity of the BCNU decomposition process in the cell suspension medium, the values of K at each of the three initial BCNU concentration levels were compared. Analysis of variance using unweighted means was applied. A P value of 0.05 or less was considered statistically significant.

2.7. DNA interstrand cross-links

The ethidium bromide fluorescence assay described by De Jong *et al.* [12] was used to measure BCNU-induced DNA interstrand cross-links in L1210 cells. Cells were initially exposed to 20, 40, or 60 μM BCNU and incubated for 0.5, 1, 2, 4, 6, 8, 10, 12, and 14 hr. Following incubation with BCNU, 1.0×10^7 cells were collected by centrifugation at 500 *g* for 5 min at room temperature and resuspended in PBS. A 40-μL aliquot of the cell suspension was incubated for 16 hr at 37° with 200 μL of lysing solution (pH 7.2) containing 4 M NaCl, 50 mM KH_2PO_4, 10 mM EDTA, and 1% (w/v) sarcosyl and 20 μL of heat-inactivated pancreatic ribonuclease (2 mg/mL in PBS). After lysing the cells, 25 μL of heparin (500 IU/mL) was added, and incubation at 37° was continued for another 20 min followed by the addition of 3 mL of ethidium bromide solution (10 μg/mL of ethidium bromide, 20 mM K_2HPO_4, and 0.4 mM EDTA, pH 12.1). The mixture was heated at 100° for 5 min to denature the DNA and then cooled on ice for 6 min to renature it. Fluorescence was measured, before and after heating/cooling, in a spectrofluorophotometer (Shimadzu RF5000U) with excitation and emission wavelengths of 525 and 580 nm, respectively. The percentage of interstrand cross-linked DNA in BCNU-treated cells (C_t) was determined using the following equation:

$$C_t = \frac{(f_{after}/f_{before})_t - (f_{after}/f_{before})_u}{1 - (f_{after}/f_{before})_u} \times 100\%$$

where f_{before} and f_{after} are fluorescences before and after heat denaturation for treated (t) and untreated (u) cells.

2.8. Cytotoxicity

Cells (4×10^5/mL) in 100 μL of medium containing 0–160 μM BCNU were pipetted into each well of 96-well plates, and BCNU-induced cytotoxicity was assessed using the MTT assay developed by Hansen *et al.* [13].

2.9. Flow cytometric analysis

Following exposure to 40 μM BCNU, both cytotoxicity and the distribution of cells within the cell cycle were analyzed over a 10-day period using flow cytometric methods.

2.9.1. Cytotoxicity

The flow cytometric method described by Sun *et al.* [14] was used to quantify normal, apoptotic, and necrotic cells. Following BCNU exposure, cells (2×10^6) were incubated with Hoechst 33342 (0.1 μg/mL) for 10 min at 37°, cooled on ice, centrifuged at 500 *g* for 4 min at room temperature, and resuspended in PBS containing PI (5 μg/mL). Flow cytometric analysis was performed using a Becton Dickinson FACStar^{Plus} connected to a Macintosh system. Two figures were created using a Dot plot: forward scatter vs. red fluorescence (PI) and blue fluorescence (Hoechst) vs. forward scatter. The percentage of necrotic cells was estimated by subtracting cells that exclude PI (normal and apoptotic cells) from the total number of cells. Normal and apoptotic cells were differentiated based on their size (forward light scatter) and Hoechst 33342 uptake. Apoptotic cells, which are smaller and more permeable to Hoechst 33342 than normal cells, show lower forward light scatter and higher Hoechst fluorescence.

2.9.2. Cell cycle

DNA content of the cell nuclei was measured using the method of Telford *et al.* [15]. Cells (1×10^6) were centrifuged (500 *g* for 5 min at room temperature), washed with PBS, resuspended, and incubated in 70% ethanol at 4° for 1 hr. The ethanol-fixed cells were pelleted, washed with PBS, resuspended in 1 mL of Telford reagent (0.1% Triton X-100, 0.1 mM EDTA disodium salt, 0.05 mg/mL of RNase A, 50 μg/mL of PI in PBS), and incubated overnight at 4°. Determinations of the distribution of single cells in different phases of the cell cycle were performed using the same instrument described above. The sample was run at a speed of approximately 100 events/s. A percentage value

for the given peaks in a DNA sample was produced by the MODFit LT program (Verity Software House, Inc.) through a series of mathematical models. Apoptotic cells, which contain smaller fragments of DNA, appear at a sub-G_1 peak.

2.10. Agarose gel electrophoresis

To confirm apoptosis, DNA gel electrophoresis was performed on the cells (days 3–10) using a method adapted from Jiang *et al.* [16]. Briefly, after treatment, 4×10^6 cells were collected, washed, resuspended in 700 μL of lysis buffer (pH 8.0) consisting of 10 mM Tris–HCl, 100 mM EDTA, and 0.5% (w/v) sodium dodecyl sulfate, and incubated for 24 hr with 0.5 mg/mL of proteinase K at 50°. Proteins were removed from the lysate with 700 μL of phenol/chloroform/isoamyl alcohol (25:24:1) (PCI). DNA in the aqueous phase was extracted again with an equal volume of PCI and then with an equal volume of chloroform. DNA was precipitated by the addition of 0.04 vol. of 5 M NaCl and 2.5 vol. of 100% ethanol at −70° for 10 min and recovered by centrifugation (18000 *g* for 10 min at 4°). After removing the supernatant, the DNA pellet was resuspended in, and incubated with, 20 μL of 50 μg/mL DNase-free RNase A in Tris–EDTA buffer (TE; pH 8.0) for 1 hr at 37°. The extraction and precipitation steps described above were repeated following the addition of 680 μL of TE to the lysate. The purified DNA, the final pellet, was dissolved in 50 μL of TE and loaded into a 1.6% agarose gel containing 0.1 μg/mL of ethidium bromide. The gel was photographed with Polaroid film using UV light.

3. Results

3.1. BCNU concentration- and time-dependent growth inhibition

The percent of surviving BCNU-treated cells when compared with controls (survival fraction) initially decreased with time of incubation and was a function of BCNU concentration. At higher concentrations (100–160 μM), all cells eventually died after 12 hr of incubation. In contrast, the survival fraction of the cells exposed to lower concentrations (10–60 μM) initially decreased and then appeared to remain constant until at least 24 hr after BCNU administration, indicating that no BCNU-induced cell death occurred during the latter period (Fig. 1). A plot of the percentage of cell death measured at the point of the minimum survival fraction (i.e. maximum cell death) as a function of BCNU concentration is shown in Fig. 2. The data were fit to a sigmoid E_{max} or Hill equation using the nonlinear regression program PCNONLIN:

$$E = \frac{E_{max} \cdot C^n}{(EC_{50})^n + C^n}$$

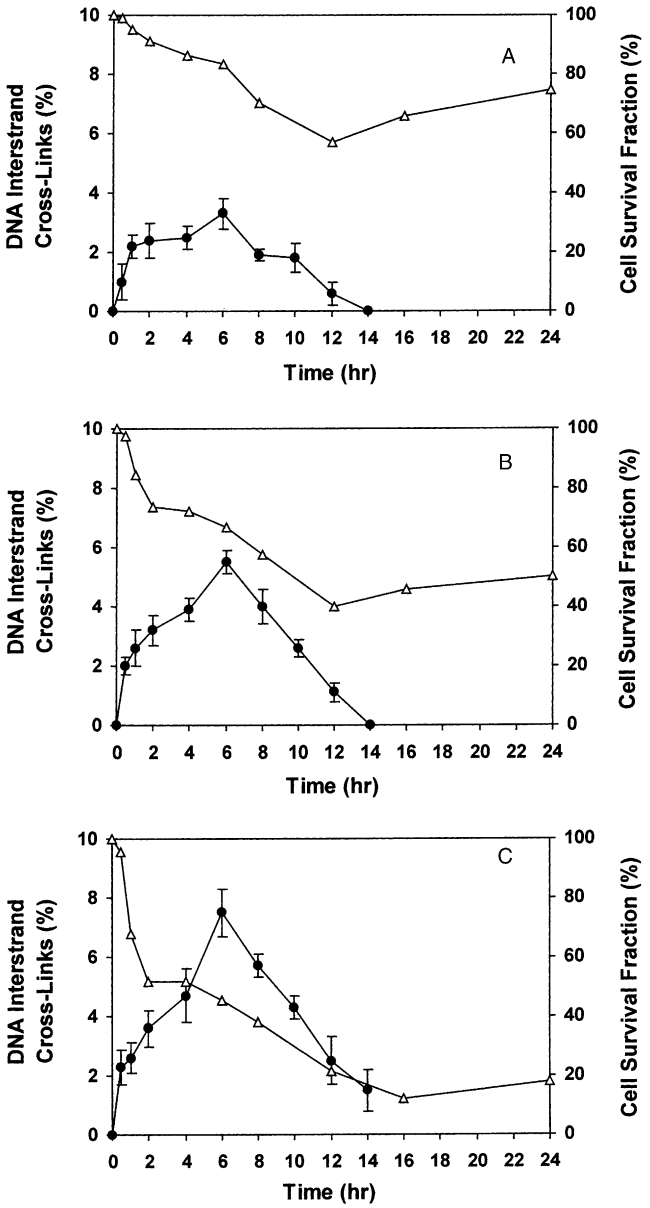

Fig. 1. Combined time-course of BCNU-induced DNA interstrand cross-links (●) and cell survival fraction (△) following exposure to (A) 20 μM, (B) 40 μM, and (C) 60 μM BCNU. Values are the means ± SEM of 5 replicates for % DNA interstrand cross-links and means of duplicates for cell survival fraction.

where E is the percentage of cell death compared with untreated cells, E_{max} is the maximum cell death, EC_{50} is the concentration of BCNU at which 50% maximum cell death occurs, and n is a slope factor. Nonlinear regression yielded values of 107.2% for E_{max}, 27.5 μM for EC_{50}, and 1.8 for n. Based on this characterization of the concentration-dependent effects of BCNU on cell death, BCNU concentrations of 20, 40, and 60 μM were chosen for subsequent studies.

3.2. BCNU decomposition kinetics

BCNU rapidly disappeared from the cell suspension medium during the incubation periods according to a

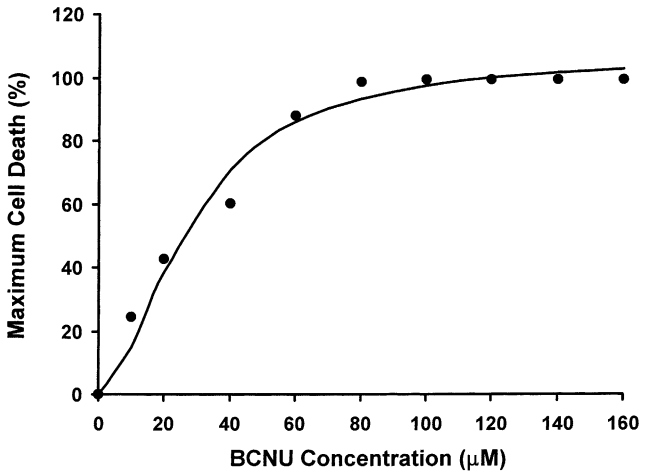

Fig. 2. BCNU concentration-dependent maximum cell death as determined by the MTT assay. Values are means of duplicates.

first-order process. The half-lives of BCNU in cells and in medium were equal as demonstrated by Weinkam and Deen [17] and exhibited mean half-lives of 37.4 ± 0.9, 39.9 ± 2.3, and 41.3 ± 5.8 min following initial exposure to 20, 40, and 60 μM BCNU, respectively. No significant difference in the half-lives was observed among the three initial concentrations tested, indicating the concentration-independent disappearance of BCNU in the cell suspension medium. Table 1 summarizes the values of the PK parameters of BCNU for each of the three initial concentrations to which the L1210 cells were exposed *in vitro*.

3.3. BCNU-induced DNA interstrand cross-links

The time-course of DNA interstrand cross-links in L1210 cells treated with BCNU (20, 40, and 60 μM) was measured using the ethidium bromide fluorescence assay (Fig. 1). Maximum cross-links were observed at 6 hr after BCNU exposure. Cross-links declined presumably due to repair. The decline could not be attributed to dilution since the amount of total cell DNA did not change significantly during the time course of cross-link formation and repair. The area under the cross-link–time curve (AUC_{XL}), the cumulative amount or number of cross-links formed, was estimated using the trapezoidal method [11]. A linear relationship existed between AUC_{XL} and AUC_{BCNU} (Fig. 3) as well as between maximum cell death

Table 1
PK parameters for BCNU in L1210 cell suspension medium

C_0 (μM)	K (min^{-1})	$T_{1/2}$ (min)	AUC_{BCNU} (μM min)
20.02 ± 1.48	0.0185 ± 0.0004	37.36 ± 0.88	1079.48 ± 97.09
38.65 ± 4.42	0.0172 ± 0.0010	39.93 ± 2.30	2218.92 ± 182.59
63.88 ± 3.04	0.0170 ± 0.0024	41.25 ± 5.77	3812.81 ± 650.97

C_0, initial BCNU concentration in the medium; K, decomposition rate constant; $T_{1/2}$, decomposition half-life; and AUC_{BCNU}, area under the concentration–time curve. Values are means ± SD of 3 replicates.

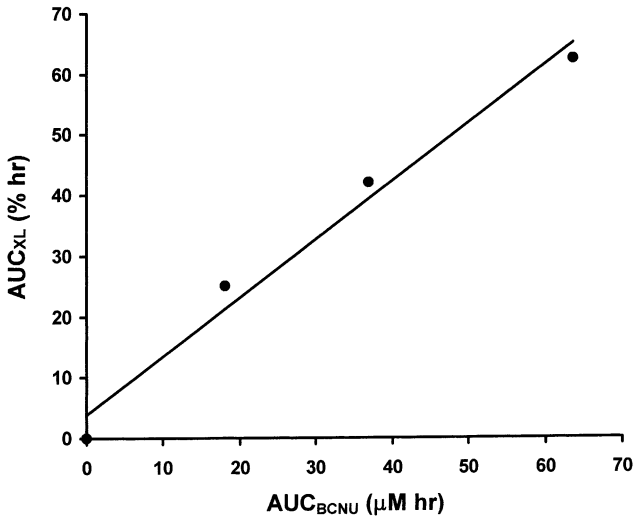

Fig. 3. Relationship between the area under the cross-link–time curve (AUC_{XL}) and the area under the intracellular BCNU concentration–time curve (AUC_{BCNU}). Values are means of 5 and 3 replicates for AUC_{XL} and AUC_{BCNU}, respectively.

and AUC_{XL} (Fig. 4). In each case, a correlation coefficient (r^2) of greater than 0.9 was observed.

3.4. Cell cycle analysis

It is known that alkylating agents, including BCNU, induce accumulation or arrest of cells in the G_2/M phase of the cell cycle [18]. G_2/M arrest has been rationalized as a mechanism for providing time for the repair of any DNA damage before the cells enter mitosis and propagate. The combined time-course of DNA interstrand cross-links and survival fraction (Fig. 1) shows a constant cell survival fraction only after DNA interstrand cross-links were no longer detectable. This is consistent with L1210 cells arresting in G_2/M phase until cross-link repair is complete,

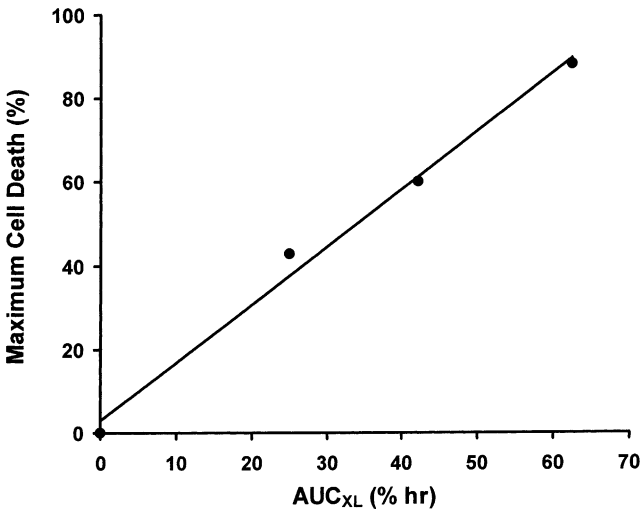

Fig. 4. Relationship between BCNU-induced maximum cell death and the area under the cross-link–time curve (AUC_{XL}). Values are means of 2 and 5 replicates for % maximum cell death and AUC_{XL}, respectively.

and thus seems to support this hypothesis. To verify that L1210 cells are actually arrested in G_2/M phase until BCNU-induced DNA interstrand cross-links were repaired completely, we conducted a flow-cytometric analysis.

L1210 cells were exposed to 40 μM BCNU and incubated for up to 10 days to monitor the fraction of cells in each phase of the cell cycle at various times. The representative histograms for 0.5 hr through day 5 are shown in Fig. 5. The cell

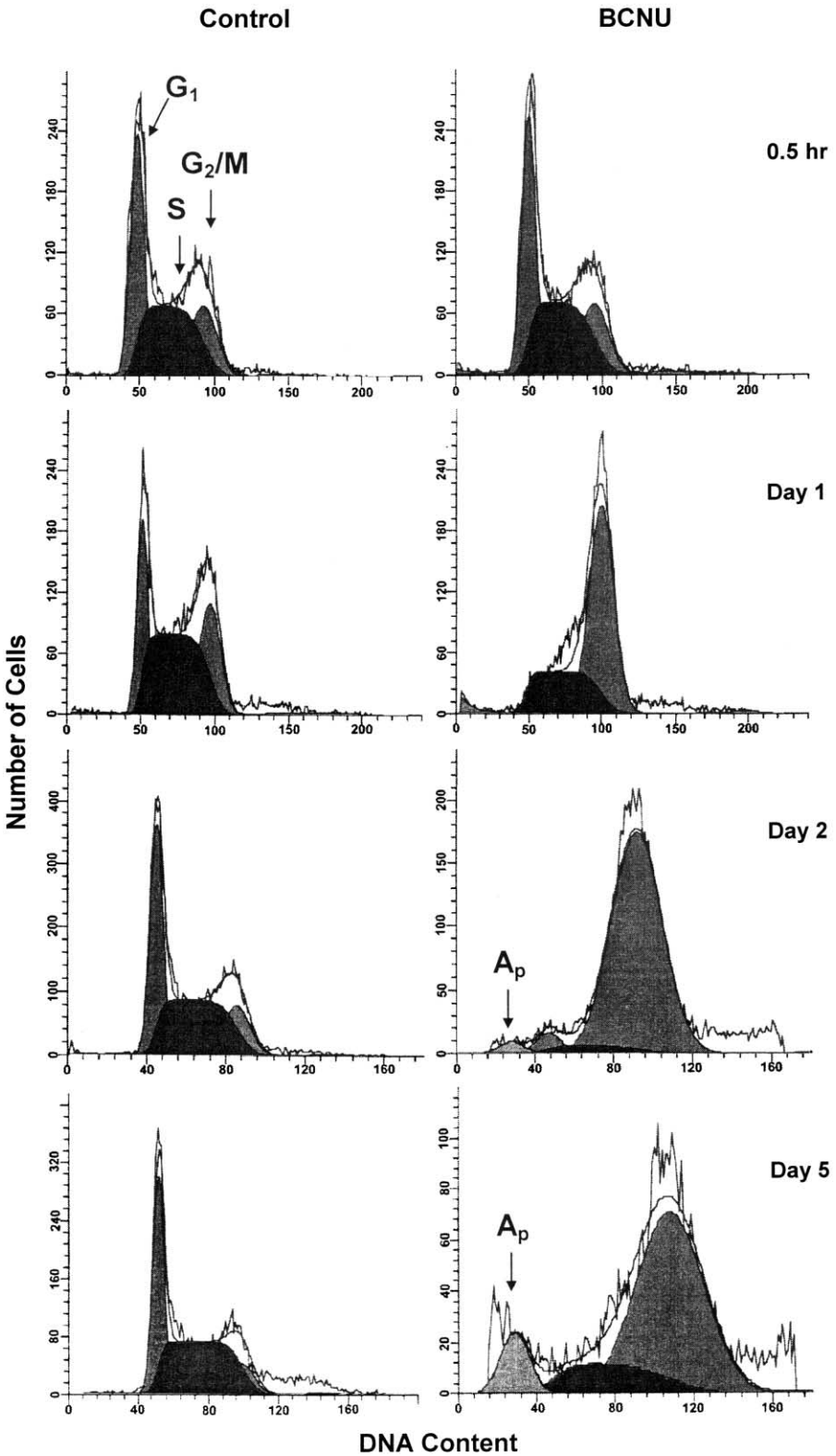

Fig. 5. Representative histograms, obtained by flow cytometric analysis, for L1210 cells following exposure to 0 μM (control) and 40 μM BCNU for 0.5 hr and 1, 2, and 5 days. Ap, apoptosis.

cycle distribution of control L1210 cells was 33 ± 5, 49 ± 10, and $18 \pm 5\%$ in the G_1, S, and G_2/M phases, respectively, after 30 min of incubation. During the first 8 hr of incubation, there was no significant difference in cell cycle distribution between control and BCNU-treated cells. After that time, however, a significant accumulation of treated cells in G_2/M began to occur and reached a maximum (87 ± 8 vs. $12 \pm 5\%$) on day 2 (Fig. 6). Thus, the accumulation in G_2/M unexpectedly continued even after apparent completion of cross-link repair, which occurred by 12 hr. The continuous accumulation of BCNU-treated cells in the G_2/M phase was accompanied by a decline of cells in S and G_1 phases. Thus, the progression of cells through the G_1 and M phases was apparently decreased while the cells accumulated in the G_2/M phase. After the maximum accumulation in G_2/M phase on day 2, the G_2/M fraction gradually declined to $41 \pm 27\%$ by 10 days.

3.5. Cytotoxicity and mechanism of cell death

The MTT assay fails to distinguish between growth arrest and reduction of cell number due to cell death, so an alternative measure to assess the cytotoxicity of BCNU was necessary. Furthermore, in light of the assertions that BCNU-induced cell death is characterized by apoptosis [19], the nature of cell death (i.e. apoptosis or necrosis) was also examined by flow cytometry. These studies were carried out by exposing L1210 cells to 40 μM BCNU and examining them over a 10-day period. Cells were incubated with both Hoechst 33342 and PI prior to analysis.

The percentage of cells that either appeared to be normal (cells that excluded PI), necrotic, or apoptotic, following exposure to 40 μM BCNU as a function of time is shown in Fig. 7. The percentage of apoptotic cells was that determined from the cell cycle analysis, since the flow cytometric method utilizing Hoechst 33342 failed to distinguish apoptotic cells from normal cells. Cell death over the first 24 hr was minimal (approximately 2% of the cells were necrotic). During this time, the cells that excluded PI could represent normal cells since less than 1% of the cells were found to be apoptotic. After 24 hr, the

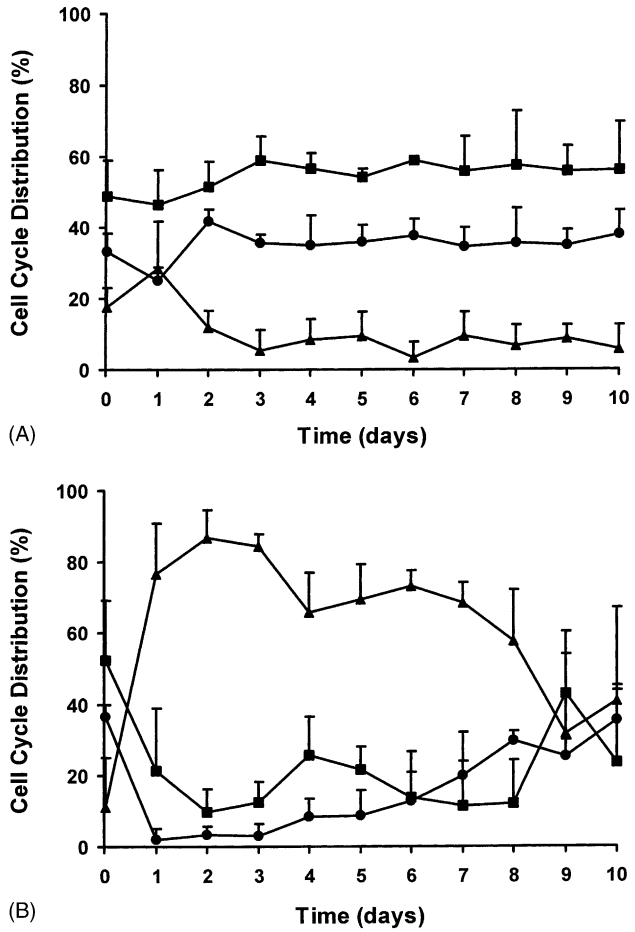

Fig. 6. Cell cycle distribution of L1210 cells during 10 days following exposure to (A) 0 μM and (B) 40 μM BCNU. Each datum represents the percentage of cells in the G_1 (●), S (■), and G_2/M (▲) phase. Values are means ± SD of 3–5 replicates.

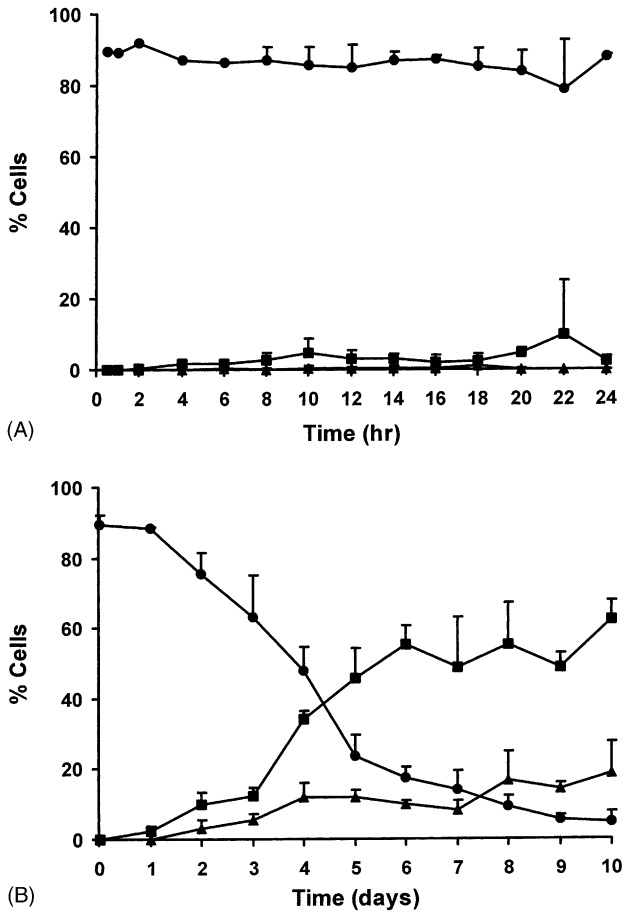

Fig. 7. Percentage of normal (cells that excluded PI, ●), necrotic (■), and apoptotic (▲) cells during (A) 0.5–24 hr and (B) 0–10 days following exposure to 40 μM BCNU. The percentages of normal and necrotic cells were determined by the PI exclusion method using flow cytometry, and those of apoptotic cells were determined from the sub-G_1 peak in the DNA histogram obtained by the cell cycle analysis. Values are means ± SD of 3–5 replicates, except for the 0.5–6 hr data, which are means of duplicates.

cells lost the ability to exclude PI, i.e. the loss of cell membrane integrity, in an exponential fashion over the next 9 days. On day 10, only $4.72 \pm 3.27\%$ of the cells excluded PI, whereas necrotic and apoptotic cells accumulated as 62.35 ± 5.41 and $18.90 \pm 8.92\%$, respectively, indicating that cell death was primarily by necrosis, not apoptosis. This was confirmed by DNA gel electrophoresis, which was carried out on days 3 through 10. Only faint ladders accompanied by smears in gels were observed, indicating some apoptosis accompanied by necrosis (data not shown).

4. Discussion

To gain a better understanding of the time-course of action of BCNU, these studies investigated the kinetics of the drug in L1210 cells (PK) and the time-course of the subsequent effects of the drug on DNA interstrand cross-link formation and repair, cell cycle changes, and the time-course of cell death (PD). The PK aspects of the study demonstrated that the intracellular half-life of BCNU was approximately 40 min, which is consistent with previously reported values [17]. The time-course of BCNU-induced DNA interstrand cross-links reflected the formation and repair of cross-links. For each of the three initial BCNU concentrations examined, DNA interstrand cross-links were detected as early as 30 min and approached a maximum at 6 hr after drug exposure. Greater than 99% of BCNU would have been eliminated by 6 hr based on the *in vitro* BCNU half-life determined in this study. The repair of DNA interstrand cross-links was apparently complete 14 hr after the exposure of cells to 20 and 40 μM BCNU.

Formation of BCNU-induced DNA interstrand cross-links involves (i) chemical decomposition of BCNU to the reactive intermediate carbonium ion [7], (ii) binding of the carbonium ion to guanine at the O^6-position of one DNA strand to produce the monoadduct, O^6-chloroethylguanine [20,21], (iii) rearranging of the monoadduct to a cyclized intermediate, O^6,N^1-ethanoguanine, which is then cleaved at the N^1-position [22], and (iv) formation of an ethyl bridge between the N^1-position of guanine and the N^3-position of cytosine on the opposite DNA strand to complete an interstrand cross-link, 1-(N^3-deoxycytidyl)-2-(N^1-deoxyguanosinyl)-ethane [23,24]. Interstrand cross-links are repaired by the combined action of nucleotide excision and recombinational repair pathways [25,26]. O^6-Chloroethylguanine can be repaired by O^6-alkylguanine-DNA alkyltransferase (O^6-AGT), which specifically catalyzes the transfer of the alkyl group from the O^6-position of guanine in DNA to the sulfhydryl group of a cysteine residue in the enzyme, yielding *S*-alkylcysteine and normal guanine in the DNA substrate [22,27]. O^6-AGT also binds to O^6,N^1-ethanoguanine to yield a DNA-protein cross-link to prevent the formation of DNA interstrand cross-links [22,28]. It has been shown that O^6-AGT activity can be a determinant of BCNU sensitivity in a variety of tumor cells [29–32], including human gliomas and colon carcinomas. In contrast, no correlation was found between the O^6-AGT levels and BCNU sensitivity in medulloblastoma cell lines [33], suggesting that BCNU-induced monoadducts could also be repaired by a mechanism other than O^6-AGT, such as base excision and nucleotide excision repair.

Interestingly, the time-course of BCNU-induced cytotoxicity, as determined by the MTT assay over a 24-hr period, indicated that BCNU no longer caused cell death after DNA interstrand cross-links were repaired. This observation supports the hypothesis that the cells are arrested in the G_2/M phase of the cell cycle to complete cross-link repair before entry into mitosis. The subsequent cell cycle study confirmed that BCNU induced the cell cycle arrest at the G_2/M phase and that the BCNU-induced interstrand cross-links were repaired during the G_2/M arrest. It was expected that the cells accumulated in the G_2/M phase would continue their progression through the cell cycle after the DNA interstrand cross-links were completely repaired. On the contrary, the G_2/M arrest continued even after the cross-link repair was apparently complete, suggesting that, at least, the cyclin-dependent kinase (cdk) Cdc2 was still inactivated beyond the time of apparent completion of cross-link repair [34–36].

In the present study, BCNU cytotoxicity was assessed by two different methods: the MTT assay and the PI exclusion method. The MTT assay measures UV absorbance of formazan, a product of MTT metabolism catalyzed by a dehydrogenase in the mitochondria. Thus, MTT, in a sense, measures mitochondrial viability. Since the number of both treated and untreated cells does not change significantly for at least 8 hr (data not shown), the initial MTT reduction represents a reduction of mitochondrial enzyme activity, specifically dehydrogenase, not a decreased fraction of the number of cells arrested at a cell cycle. The PI exclusion method, on the other hand, determines the integrity of the cell membrane. This integrity is lost in necrotic cells. The cell is considered to be dead if the plasma membrane becomes permeable to PI. Cells treated with 40 μM BCNU appeared to be viable during the first 24 hr post-incubation period using the PI exclusion method. Mitochondrial damage assessed by the loss of rhodamine-123 uptake and MTT reduction to formazan has also been observed to occur before changes in plasma membrane integrity were detected by trypan blue staining [37] and lactate dehydrogenase release [38]. These findings are consistent with the present observation that the cells became permeable to PI only after the changes that the MTT assay detected had occurred. It also suggests that an early toxic effect of BCNU in the cells is diminished mitochondrial function. The effect of this on the subsequent ability of the cell to enter apoptosis is of interest.

After 24 hr of exposure to BCNU, the cells continued to die in an exponential fashion over the next 9 days. Cell death was due primarily to necrosis, rather than apoptosis. The apparent induction of apoptosis as seen in L1210 cells

on day 2 appeared to be associated with the exit from BCNU-induced G_2/M arrest. It would be interesting to examine the effect of BCNU concentration on both the mechanism of cell death, i.e. necrosis or apoptosis, and the length of G_2/M arrest. In addition, the interrelationships between the time-course of cross-link formation and repair and both G_2/M arrest and the mechanism of cell death would be instructive. Furthermore, it would be beneficial to determine whether the BCNU-induced G_2/M arrest or apoptosis are p53-dependent. BCNU-induced G_2/M arrest was reported to be p53-dependent in ADF glioblastoma cells [39] and p53-independent in mouse astrocytes [40]. The involvement of p53 in the G_2 checkpoint could depend on, at least, cell type and treatment. This additional information on the mechanism of action of BCNU would provide ideas for future rational strategies to improve BCNU efficacy.

In conclusion, the present study describes the sequence of events in L1210 cells treated with BCNU. Maximum cell death (maximum reduction of mitochondrial enzyme activity) achieved within 24 hr of BCNU exposure was concentration-dependent and could be described by a Hill equation. At BCNU concentrations up to 60 μM, a linear relationship was found between the AUC_{XL} and AUC_{BCNU} as well as between BCNU-induced maximum cell death and AUC_{XL}. While cytotoxicity was concentration-dependent, an indirect relationship was found among the time-course of intracellular BCNU concentrations, DNA interstrand cross-links, and cell death. Because of the disparity between the time-scale of PK and PD (PK in minutes, DNA damage and repair in hours, and cell death in days), focusing only on the early events (i.e. PK) may provide limited information about the process of anticancer drug-induced cell death. The present study demonstrated further that a study of the PK and PD of anticancer drugs can serve as a useful method of probing the mechanism of action of these drugs.

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