Chlorozotocin

MECHANISM OF REDUCED BONE MARROW TOXICITY IN MICE

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ABSTRACT Chlorozotocin is a chloroethyl nitrosourea with a glucose carrier that has curative activity for the murine L1210 leukemia, but is nonmyelosuppressive in mice. To determine the mechanism for this unique property of reduced bone marrow toxicity, comparative studies were conducted with chlorozotocin and CCNU, a myelotoxic chloroethyl nitrosourea.

Suspensions of L1210 leukemia and murine bone marrow cells were incubated for 2 h with 0.1 mM [^{14}C]-chloroethyl chlorozotocin or CCNU. Chlorozotocin demonstrated a fourfold increased covalent binding of the chloroethyl group to L1210 nuclei when compared to equimolar CCNU. Chlorozotocin alkylation of L1210 cells resulted in the binding of 57 pmol of [^{14}C]ethyl group/mg of DNA, which represented a 2.3-fold increased alkylation when compared to CCNU. In marked contrast, the binding of the chloroethyl group to bone marrow nuclei was equivalent for both drugs. In addition, chlorozotocin alkylation of murine bone marrow DNA, 45 pmol of [^{14}C]ethyl group/mg of DNA, was equivalent to that of CCNU. The ratio of L1210:bone marrow DNA alkylation was 1.3 for chlorozotocin compared to 0.6 for CCNU. The intracellular carbamoylation of L1210 and bone marrow protein by CCNU was 400- to 600-fold greater than that produced by chlorozotocin.

After a 2-h exposure to 0.1, 0.05, or 0.01 mM drug, both chlorozotocin and CCNU produced a reduction in the cloning efficiency of L1210 cells that was dose dependent. However, chlorozotocin was significantly more cytotoxic than CCNU at all three molar concentrations ($P < 0.01$). Chlorozotocin, 0.1 mM, reduced L1210 DNA synthesis to 1% of control by 48 h, in contrast to 16% with equimolar CCNU ($P < 0.01$).

In mice bearing 10^5 L1210 cells, chlorozotocin produced its optimal antitumor activity (332% increased life span [ILS]) at doses of 48–64 μmol/kg, with >50% indefinite survivors. In contrast, CCNU at the same molar doses resulted in only a 191% ILS; a CCNU dose of 128 μmol/kg was required for comparable optimal L1210 antitumor activity, 413% ILS.

On a molar basis, the dose of chlorozotocin that produced optimal in vivo L1210 antitumor activity was one-third to one-half that of CCNU. Chlorozotocin, unlike CCNU, produced no murine bone marrow toxicity at its optimal therapeutic dose. This unique combination of antitumor activity without myelosuppression can be correlated with the advantageous ratio of L1210: bone marrow in vitro DNA alkylation by chlorozotocin (1.3) as compared to equimolar CCNU (0.6).

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INTRODUCTION

The chloroethyl nitrosoureas are an important class of antitumor agents with a broad spectrum of activity in human cancer (1). However, all of the agents in active clinical use, 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU),[1] and methyl CCNU, produce delayed and cumulative bone marrow depression, which significantly limits their clinical usefulness (2). Structure-activity studies with the nonmyelosuppressive methyl nitrosourea, streptozotocin, and its cytotoxic group, 1-methyl-1-nitrosourea, suggested that this bone marrow toxicity could be reduced by the attachment of the cytotoxic group to the carbon-2 position of glucose (3). To evaluate the influence of the glucose carrier for the more active chloroethyl nitrosourea class of compounds, a new compound, 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose, chlorozotocin, was synthesized for our studies by Johnston et al. (4). Chlorozotocin has curative antitumor activity for the murine L1210 leukemia and is nonmyelosuppressive in mice (5).

[1] *Abbreviations used in this paper*: CDF_1 mice, male BALB/C × DBA/2F mice; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; GANU, 1-(2-chloroethyl)-3-(B-D-glucopyranosyl)-1-nitrosourea; HU, hydroxyurea; ILS, increased life span; LD_{10}, one-tenth lethal dose; WBC, leukocyte(s).

The mechanism by which the glucose carrier on the nitrosourea cytotoxic group confers the specific protective effect for murine bone marrow remains undetermined. In this report, we compare the quantitative in vitro alkylation and carbamoylation of bone marrow and L1210 leukemia DNA, cytoplasmic RNA, and protein by chlorozotocin and CCNU. These results are correlated with the relative antitumor and myelosuppressive activities of the two nitrosoureas in mice.

METHODS

Animal studies

Male BALB/C × DBA/2F (CDF_1) mice, weighing 17–25 g and maintained on Lab-Blox laboratory chow pellets and water, ad libitum, were used throughout. The chloroethyl nitrosoureas investigated in the study are 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glycopyranose or chlorozotocin (NSC 178248) and CCNU (NSC 79037); the structures of these two agents are presented in Table I. Both nitrosoureas were supplied by the Drug Development Branch, Div. of Cancer Treatment, National Cancer Institute, Bethesda, Md. Chlorozotocin was dissolved in 0.01 M citrate buffer, pH 4, and CCNU was suspended in 5% ethanol, 5% polyoxyethylated vegetable oil, and 90% saline for all the animal studies; each drug was administered intraperitoneally as a single dose in a volume of 0.1 ml/10 g body wt. Radiolabeled chlorozotocin and CCNU were dissolved in these vehicles, respectively, for the in vivo murine bone marrow distribution study. All experiments were performed three times.

The murine L1210 leukemia system was used to assess antitumor activity. Treatment was administered to groups of 10 mice as a single dose on the second day after implantation of 1×10^5 L1210 leukemia cells i.p. The percent increased life span (ILS) was calculated from the survival of control tumor-bearing animals that received appropriate volumes of the respective diluents (6); long-term survival was calculated to 45 d. Measurement of peripheral leukocyte (WBC) count was performed using a 20-μl sample of retroorbital sinus blood from normal CDF_1 mice; this was placed in 9.98 ml of Isoton (Coulter Diagnostics, Inc., Hialeah, Fla.) and counted in a model ZBI Coulter counter after lysis of erythrocytes with Zap-oglobin (Coulter Diagnostics, Inc.). WBC differential counts were performed on Wright-stained smears of blood taken on the third day after treatment; this time period was chosen to correspond with the nadir of the WBC count after nitrosourea treatment in this species (5). The WBC and absolute neutrophil counts are presented as a percentage of control values.

In vivo studies of drug distribution to the murine bone marrow cells were carried out using 2-[1-^{14}C]chloroethyl chlorozotocin, 16.1 mCi/mmol sp act, and 2-[U-^{14}C]chloroethyl CCNU, 12.4 mCi/mmol sp act (Monsanto Research Corp., Dayton, Ohio). Radiochemical purity, as determined by thin-layer chromatography, was >99% for all labeled nitrosoureas. Either chlorozotocin or CCNU was administered at a dose of 64 μmol/kg i.p., one-tenth the lethal dose (LD_{10}) of chlorozotocin, to three normal mice; CCNU was also administered at its LD_{10} of 128 μmol/kg i.p. Each mouse was sacrificed by cervical traction at 60 min after drug injection and bone marrow cells from both femurs and tibiae were expressed using phosphate-buffered saline (144 mM NaCl and 10 mM Na phosphate buffer, pH 7.4). The amount of [^{14}C]ethyl group covalently bound to the marrow cells from each mouse was determined as follows. After contaminating erythrocytes were osmotically lysed, the nucleated cells were suspended in 0.5 ml of phosphate-buffered saline and a cell count was done. 1 ml of 5% TCA was added to each sample to precipitate proteins and nucleic acids; after 30 min at 0°C, the samples were centrifuged in an Eppendorf Brinkmann centrifuge 3200 (Brinkmann Instruments, Inc., Westbury, N. Y.) and the precipitates were washed twice with 5% TCA. Residual acid soluble radioactivity was counted after each wash, and a steady state was achieved after the second wash. Each resultant precipitate was suspended in 0.5 ml of phosphate-buffered saline and added to a scintillation counting vial containing 0.5 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.). In all the studies reported, radioactivity was assayed by adding 10 ml of aqueous counting scintillant (Amersham Corp.) to each sample-containing vial. Samples were counted in a Searle Mark III scintillation spectrometer (Searle Radiographics, Inc., Des Plaines, Ill.) with an automatic quench correction and a counting efficiency of 90% for ^{14}C and 35% for ^{3}H. Results of the marrow distribution studies are expressed as picomoles of [^{14}C]ethyl group covalently bound to protein and nucleic acids per 10^7 nucleated cells (mean±SD of three experimental values).

The peak concentration of intact *N*-nitroso chlorozotocin in mouse plasma was determined after administration of an LD_{10} (20 mg/kg i.p.). The plasma disappearance of chlorozotocin was followed by measurement of the intact *N*-nitroso group of the drug using the Forist (7) modification of the Bratton-Marshall reaction. Plasma clearance of intact chlorozotocin was measured in three mice for each of the following time points: 2.5, 5, 7.5, 10, and 15 min after administration. At the appropriate time point, each mouse was anesthetized with ether and a 1-ml sample of blood was obtained by cardiac puncture. Immediately after withdrawal, the 1-ml sample was centrifuged at 8,000 *g* for 2 min. A 0.4-ml aliquot of the plasma was then added to 0.1 ml of 3 N perchloric acid, vortexed for 10 s, and recentrifuged. In this procedure, the intact *N*-nitroso group is cleaved to yield nitrous acid in the resulting supernate. A 0.25-ml aliquot of this supernate was added to 0.25 ml of 0.1 mM acetate buffer (pH 4), 2.5 ml of color reagent (0.5% sulfanilic acid and 0.5% *N*-(1-napthyl)ethylenediamine dihydrochloride in 30% acetic acid), and 0.5 ml of 6 N HCl. This reaction mixture was incubated for 45 min at 60°C; the nitrous acid diazotizes the sulfanilic acid, which then couples to the napthylethylenediamine to form a colored product with an absorbance maximum at 550 nm. A pretreatment blood sample served as a control. The plasma concentration of *N*-nitroso intact chlorozotocin was calculated from a standard curve that followed Beer's law over a concentration range of 5–250 μM. The results in Fig. 1 are presented as the mean±SD of the three values obtained per time point.

Chemical and in vitro cell suspension studies

Chemical carbamoylating activity. To measure the chemical carbamoylating activity of each nitrosourea, the following procedure was used: 4.2 μmol of cold lysine, 0.4 μCi of [U-^{14}C]lysine (Amersham Corp., 300 mCi/mmol sp act), 120 μl of 0.1 M Na phosphate buffer, pH 7.4, 160 μl of ethanol, and 4.2 μmol of each nitrosourea were incubated together for 6 h at 37°C. The reaction mixture without nitrosourea served as a control. 10 μl of each reaction mixture were spotted on Whatman No. 3 chromatography paper (Whatman, Inc., Clifton, N. J.) that had been prewetted with 0.1 M Na phosphate buffer, pH 6. Separation was accomplished using a Savant high-voltage flat plate electrophoresis tank (Savant Instruments, Inc., Hicksville, N. Y.) at 3,000 V for 75 min. Radioactivity in 1-inch squares was counted. A distinct peak

of radioactivity not associated with the parent [U-^{14}C]lysine represented products of carbamoylation. The carbamoylated products have previously been identified as a mixture of an α-carbamoyl derivative of lysine, an ϵ-carbamoyl derivative of lysine, and a combined α-carbamoyl and ϵ-carbamoyl derivative of lysine (8, 9). Carbamoylating activity is expressed as the percentage of the total radioactivity associated with the carbamoylated product (10).

Chemical alkylating activity. An aliquot with 0.20–4.0 μmol of each nitrosourea dissolved in acetone was added to 1.5 ml of 5% (wt/vol) 4-(*p*-nitrobenzyl)pyridine in acetone. 4 ml of 0.025 M acetate buffer, pH 6, were added and the solution was incubated at 37°C for 2 h. 2 ml of acetone and 3 ml of ethyl acetate were then added. The mixture was made alkaline by the addition of 1.5 ml of 0.25 N NaOH, shaken vigorously, and centrifuged at 3,000 rpm for 15 s. The absorbance of the ethyl acetate layer was determined at 540 nm. The relative alkylating activity of CCNU is expressed as a percentage of the activity of chlorozotocin (11).

In vitro alkylation and carbamoylation of L1210 leukemia and murine bone marrow cells. Femurs and tibiae were removed from normal CDF_1 mice as described previously and the bone marrow cells were expressed using McCoy's 5A modified media. L1210 leukemia cells were harvested from the peritoneal cavities of CDF_1 mice 7 d after implantation with 1×10^5 cells. Erythrocytes were removed by osmotic lysis. The nucleated cells were then suspended at a concentration of 4×10^7 cells/ml in McCoy's with 10% fetal calf serum. Hepes buffer (Sigma Chemical Co., St. Louis, Mo.) was added to result in a final concentration of 10 mM. For studies involving alkylation, a 0.1-mM concentration of 2-[1-^{14}C]chloroethyl chlorozotocin or 2-[U-^{14}C]chloroethyl CCNU was used. For studies of carbamoylation, a 0.1-mM concentration of [1-^{14}C]cyclohexyl CCNU, 8.4 mCi/mmol sp act or [1-^{14}C]glucose chlorozotocin, 17.1 mCi/mmol sp act (Monsanto Research Corp.) was used. For the in vitro studies, radiolabeled chlorozotocin and CCNU were dissolved in 0.01 M citrate buffer, pH 4, and ethanol, respectively. The cell suspensions were incubated at 37°C under 5% CO_2 for 2 h. Equal amounts of the citrate and ethanol vehicles were added to each in vitro culture regardless of the nitrosourea. Cell viability was confirmed by trypan blue exclusion at the end of each incubation.

Previous studies with [^{14}C]ethyl nitrosourea have demonstrated that a significant proportion of the radioactivity covalently bound to DNA and RNA represented secondary incorporation of the [^{14}C]ethyl decomposition products. Radiolabel was incorporated by *de novo* synthesis of nucleotides rather than direct alkylation of nucleic acids (12). To determine the importance of such nonspecific reuse for these studies, [1-^{14}C]chloroethyl chlorozotocin was maintained in phosphate-buffered saline, pH 7.4, in a closed system at 37°C for 5 h. The half-life of chlorozotocin under these conditions is 48 min and at the end of the incubation, there was no intact *N*-nitroso chlorozotocin as measured by the Forist (7) modification of the Bratton-Marshall determination. After cooling to 22°C, a 0.1-mM concentration of the decomposed drug was incubated with L1210 cells as described above.

Studies with hydroxyurea (HU), an inhibitor of semiconservative DNA synthesis, were performed to determine whether drug decomposition products were nonspecifically incorporated into newly synthesized DNA. L1210 cells, at a concentration of 4×10^7 cells/ml, were incubated (37°C) under the following four experimental conditions: (*a*) 2.5 mM HU for 25 min, then [^{3}H]thymidine and 0.1 mM [1-^{14}C]chloroethyl chlorozotocin for 2 h, (*b*) 2.5 mM HU for 25 min, then [^{3}H]-thymidine for 2 h, (*c*) [^{3}H]thymidine for 2 h, and (*d*) [^{3}H]-thymidine and 0.1 mM [1-^{14}C]chloroethyl chlorozotocin for 2 h. DNA synthesis in the presence of 2.5 mM HU, as measured by the incorporation of [^{3}H]thymidine into phenol-extracted DNA, was <1% of DNA synthesis in control or chlorozotocin-treated L1210 cells. The incorporation of the [^{14}C]-ethyl group of chlorozotocin into DNA of the HU-treated cells was compared to the results in cells without HU.

Isolation of alkylated DNA, RNA, and protein

The extraction of cytoplasmic DNA, RNA, and protein from a single tissue sample was performed by a procedure developed in our laboratory, which is a modification of published methods (13). This method used whole cells that had been stored at −70°C after the drug incubations as described above. Cytoplasmic RNA was extracted with Na deoxycholate and 154 mM NaCl in the presence of phenol. Na deoxycholate, *p*-aminosalicylate, and 1 M NaCl with phenol extracted the DNA. The DNA was then freed of RNA with RNase T1 and T2. Cytoplasmic RNA with no detectable DNA and <0.2% protein contamination was obtained. The DNA isolated had <3% uracil compared to thymine and no more than 0.1% protein. As compared to nucleic acids extracted by the method of Schmidt-Thannhauser, the yields of DNA and RNA by this method were 60–70% and 50–60%, respectively, for both L1210 leukemia and murine bone marrow cells (13). Approximately 3% of added radiolabeled drug was covalently incorporated into cells.

The concentrations of DNA and RNA were determined by the diphenylamine and orcinol methods, respectively (14, 15). The results obtained by these colorimetric methods were in good agreement with the results obtained by ultraviolet absorbance at 260 nm. Calf thymus DNA and yeast RNA (Sigma Chemical Co.) were used as standards for the colorimetric methods and ultraviolet absorbance after standardization by phosphate determination (16). The protein concentrations were determined by the method of Lowry et al. (17). Radioactivity in the isolated DNA, RNA, and protein fractions was measured.

Cytoplasmic and nuclear binding with [^{14}C]-chloroethyl chlorozotocin or CCNU

After a 2-h incubation with 0.1 mM ethyl-labeled drug, bone marrow or L1210 cells were washed with phosphate-buffered saline and fractionated into cytoplasmic and nuclear fractions by the method of Snyder et al. (18). Nuclei were released by agitation of the cellular pellet in 1 ml of a solution containing 0.2% Triton X100 (Rohm and Haas Co., Philadelphia, Pa.), 0.15 M NaCl, 2 mM $MgCl_2$, and 5 mM potassium phosphate, pH 7.4. The homogenate was layered on 50% sucrose with 2 mM $MgCl_2$, and centrifuged for 25 min at 16,500 *g*. The supernate, or cytoplasmic fraction, did not contain significant nuclear debris as measured by [^{3}H]thymidine contamination. The nuclear pellet was subjected to phase microscopy to ensure that it was free of significant cytoplasmic strands. Once isolated, the cytoplasmic fraction was precipitated with 3 vol of absolute ethanol. Both fractions were washed twice with 70% ethanol and 0.1 M Na acetate. Residual radioactivity was counted after each wash and a steady state was achieved after the second wash. Each fraction was then dissolved in 1 ml of 1 N NaOH over a period of 3 d. The protein concentration was determined by the method of Lowry et al. (17). The radioactivity in a 0.5-ml aliquot of each subcellular fraction was determined, and the results are expressed as picomoles of [^{14}C]ethyl group per milligram of protein.

Studies employing cycloheximide were performed to determine the contribution of nonspecific incorporation of drug

decomposition products into newly synthesized amino acids. Cycloheximide inhibits protein synthesis in the 80S ribosomes of eucaryotic cells (19). L1210 cells, at a concentration of 4 $\times 10^7$ cells/ml, were incubated (37°C) with 200 μg/ml of cycloheximide for 15 min; either 0.1 mM [U-^{14}C]chloroethyl CCNU or [1-^{14}C]chloroethyl chlorozotocin was then added as described above. Under these conditions, protein synthesis as measured by [4,5-^{3}H]leucine incorporation into ethanol precipitable counts was inhibited to <5% of control over the 2-h incubation period without affecting cell viability. The results with cycloheximide were compared to those without the inhibitor.

In vitro L1210 DNA synthesis studies

To compare alkylation of DNA with suppression of DNA synthesis, in vitro L1210 studies were carried out with equimolar (0.1 mM) concentrations of chlorozotocin and CCNU. Murine L1210 leukemia cells were kindly supplied by Dr. Kurt Kohn of the National Cancer Institute and maintained in RPMI 1630 supplemented with 20% fetal calf serum. Cell suspensions of 4 $\times 10^7$ cells/ml were incubated for 2 h in the above medium minus calf serum with 0.1 mM nitrosourea. The cells were then washed and resuspended at a concentration of 4 $\times 10^5$ cells/ml in the medium supplemented with calf serum (without nitrosourea). At 23 and 47 h, cells were pulsed with 1 μCi/ml of [^{3}H]thymidine. At both experimental time points, cell aliquots were assayed for viability with trypan blue. After a 1-h pulsing period, the cells were centrifuged at 1,200 rpm for 20 min and the cell pellet was washed with phosphate-buffered saline. The DNA content of each pellet was extracted by the method of Schneider (20). Radioactivity in a 0.5-ml aliquot of the supernate was measured. An additional 0.5-ml aliquot of the supernate was used for the measurement of DNA concentration by the diphenylamine method (14); total DNA content, including the viable and nonviable cells, was determined and the percentage of viable cells was then used to calculate the DNA content of the viable cells. The results (expressed as disintegrations per min/microgram DNA) were compared to vehicle-treated controls.

Colony-forming ability of L1210 cells

To compare the viability of cultured L1210 leukemia cells after exposure to equimolar concentrations of chlorozotocin and CCNU, cloning assays were carried out using the soft agar technique of Chu and Fisher (21). L1210 cells, at a concentration of 4 $\times 10^7$ cells/ml, were treated with either 0.1, 0.05, or 0.01 mM nitrosourea. After a 2-h incubation at 37°C, the cells were washed and resuspended in fresh medium without nitrosourea. Various cell dilutions were added to agar tubes and maintained at 37°C. For both chlorozotocin and CCNU, four tubes were analyzed at each cell dilution. On day 11, the number of colonies formed per tube was determined and compared to the vehicle-treated controls.

Statistical analysis

A Monroe 344 calculator (Monroe, The Calculator Co., Litton Industries, Morristown, N. J.) was used to determine the analysis of variance, the Tukey test, and the Student's *t* test (22). Statistical significance of differences in absolute neutrophil count was determined by the analysis of variance. The analyses of antitumor activity were done by the Tukey test. All of the other statistical analyses employed the Student's *t* test.

RESULTS

Table I presents the structures, chemical carbamoylating activities, and chemical alkylating activities of chlorozotocin and CCNU. The in vitro alkylating activity of chlorozotocin is sixfold greater than that of CCNU as measured by the 4-(*p*-nitrobenzyl)pyridine reaction. The chemical carbamoylating activity of CCNU, as measured by the reaction of drug with [U-^{14}C]-lysine, is 45% of the total radioactivity, in contrast to the negligible carbamoylating activity of chlorozotocin.

After administration of an LD_{10}, 20 mg/kg i.p., the

TABLE I
Chemical Parameters of Chlorozotocin and CCNU

Nitrosourea	Structure	Carbamoylating activity	Alkylating activity
		% carbamoylated [^{14}C]lysine	*% chlorozotocin*
Chlorozotocin	(pyranose ring: CH_2OH, O, OH, OH, OH; HN–C(=O)–N(NO)–CH_2CH_2Cl)	2	100
CCNU	(cyclohexyl–NH–C(=O)–N(NO)–CH_2CH_2Cl)	45	15

initial peak plasma concentration of intact N-nitroso chlorozotocin in the mouse was attained at 5 min after injection and exceeded 0.1 mM (Fig. 1). By 15 min, the plasma concentration of intact drug had decreased to 0.04 mM. This peak plasma concentration of 0.1 mM was employed in the in vitro cell culture studies, and an equimolar concentration of CCNU was used for comparison.

Table II presents the results of in vitro short-term cell cultures with 2-[U-^{14}C]chloroethyl CCNU and 2-[1-^{14}C]chloroethyl chlorozotocin. In L1210 leukemia cells, 0.1 mM chlorozotocin produced a 2.3-fold greater alkylation of DNA and RNA and a 1.4-fold greater alkylation of protein when compared to equimolar CCNU. With murine bone marrow cells, chlorozotocin alkylation was equivalent for DNA, 1.6-fold greater for RNA, and 1.7-fold greater for protein when compared to equimolar CCNU. The ratio of alkylation of L1210:bone marrow DNA was 1.3 for chlorozotocin compared to 0.6 for CCNU.

It was considered possible that a proportion of the radioactivity covalently bound to DNA and RNA represented secondary incorporation of the [^{14}C]chloroethyl nitrosourea decomposition products into nucleotides via *de novo* synthesis. To determine the contribution of such nonspecific reuse, chlorozotocin previously decomposed for 5 h in phosphate-buffered saline was incubated under identical conditions as previously described for intact drug. The decomposed chlorozotocin resulted in 1 pmol of [^{14}C]ethyl group incorporated per milligram of DNA and 17 pmol/mg of RNA, which represented 2 and 7%, respectively, of the values obtained with the intact drug.

In studies employing hydroxyurea as an inhibitor of semiconservative DNA synthesis, the quantitative incorporation of the [^{14}C]ethyl group of chlorozotocin into L1210 DNA in HU-treated cells did not differ from the results in cells without HU. These experiments demonstrate that the nonspecific reuse of [^{14}C]chloroethyl nitrosourea decomposition products via semiconservative DNA synthesis was negligible.

Table III presents the results of cell culture studies using equimolar [1-^{14}C]cyclohexyl CCNU and [1-^{14}C]-glucose chlorozotocin to measure intracellular carbamoylation. In the in vitro 2 h L1210 cell incubation, a minor (<7 pmol/mg) amount of either the [^{14}C]glucose or [^{14}C]cyclohexyl group was bound to nucleic acids. However, CCNU resulted in the binding of 6,600 pmol of [^{14}C]cyclohexyl group/mg of L1210 protein; with

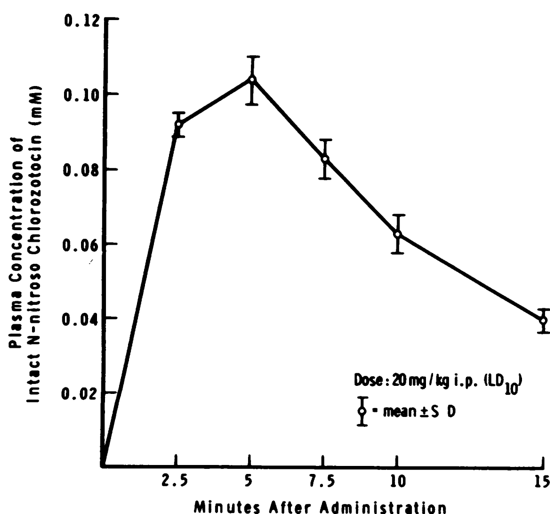

FIGURE 1 Concentration of N-nitroso intact chlorozotocin in mouse plasma after administration of an LD_{10} (20 mg/kg i.p.).

TABLE II
In Vitro Alkylation of Cellular DNA, RNA, and Protein after a 2-h Incubation with 0.1 mM [^{14}C]Chloroethyl Nitrosourea

	CCNU [U-^{14}C]ethyl*	Chlorozotocin [1-^{14}C]ethyl*	Significance Student's *t* test
L1210 leukemia cells			
DNA	24.4±5.3	56.5±1.7	$P < 0.005$
RNA	103±34.3	239±34.3	$P < 0.01$
Protein	98±9.2	139±32	$P > 0.10$
Murine bone marrow cells			
DNA	41.5±2.3	44.9±4.7	$P > 0.10$
RNA	88±12.5	140±17.8	$P < 0.05$
Protein	118±12.7	198±65.8	$P > 0.05$

* Data expressed as picomoles of [^{14}C]ethyl group/milligram DNA, RNA, or protein ±SD.

TABLE III
In Vitro Carbamoylation of Cellular DNA, RNA and Protein after a 2-h Incubation with 0.1 mM [1-^{14}C]Cyclohexyl CCNU or [1-^{14}C]Glucose Chlorozotocin

	CCNU [1-^{14}C]cyclohexyl*	Chlorozotocin [1-^{14}C]glucose*	Significance Student's *t* test
L1210 leukemia cells			
DNA	6.6±3.3	4.8±2	$P > 0.10$
RNA	1.3±0.7	5.8±0.3	$P < 0.01$
Protein	6,600±1,580	17.5±3.5	$P < 0.01$
Murine bone marrow cells			
DNA	4.2±2.5	15.5±1.8	$P < 0.01$
RNA	5.3±2.7	17.8±6.2	$P < 0.05$
Protein	6,460±830	10.5±1.3	$P < 0.01$

* Data expressed as picomoles of [1-^{14}C]cyclohexyl or [1-^{14}C]glucose group/milligram DNA, RNA, or protein ±SD.

chlorozotocin, there were 17.5 pmol of [^{14}C]glucose group bound/mg of protein. These widely divergent results were accurately predicted by the in vitro chemical carbamoylating activities of the two nitrosoureas. The results with murine bone marrow cells were comparable.

The binding of the [^{14}C]ethyl group of chlorozotocin or CCNU to the total cellular and nuclear fractions of L1210 and murine bone marrow cells was measured (Table IV). Compared to equimolar CCNU, there was an eightfold increased covalent binding of the ethyl group of chlorozotocin to the cytoplasmic fraction of L1210 cells and a fourfold increased binding to the nuclear fraction. In contrast, in bone marrow cells there was a threefold increased covalent binding of chlorozotocin to the cytoplasmic fraction, but the binding to the nuclear fraction was equivalent for the two drugs.

Comparable studies were conducted with L1210 cells in the presence of the protein synthesis inhibitor cycloheximide, to prevent the nonspecific reuse of nitrosourea decomposition products. With chlorozotocin, 914±29 pmol of [^{14}C]ethyl group/mg of protein were bound to the cytoplasmic fraction and 466±47 pmol/mg of protein to the nuclear fraction. In contrast, CCNU resulted in the binding of 141±6 pmol of [^{14}C]ethyl group/mg of protein in the cytoplasmic fraction and 140±0.7 pmol/mg of protein in the nuclear fraction. These results with chlorozotocin were sevenfold greater in the cytoplasmic fraction and threefold greater in the nuclear fraction of L1210 cells when compared to equimolar CCNU.

The degree of DNA synthesis inhibition by each nitrosourea was compared in studies conducted under

TABLE IV
Alkylation of Cytoplasmic and Nuclear Fractions of L1210 and Bone Marrow Cells after a 2-h Incubation with [^{14}C]Chloroethyl Nitrosourea

	CCNU [U-^{14}C]ethyl*	Chlorozotocin [1-^{14}C]ethyl*	Significance Student's *t* test
L1210 leukemia cells			
Cytoplasmic fraction	123±22	1024±101	$P < 0.005$
Nuclear fraction	135±19	553±94	$P < 0.005$
Murine bone marrow cells			
Cytoplasmic fraction	130±30	391±68	$P < 0.005$
Nuclear fraction	253±28	233±24	$P > 0.10$

*Data expressed as picomoles of ethyl-^{14}C group/milligram of protein ±SD.

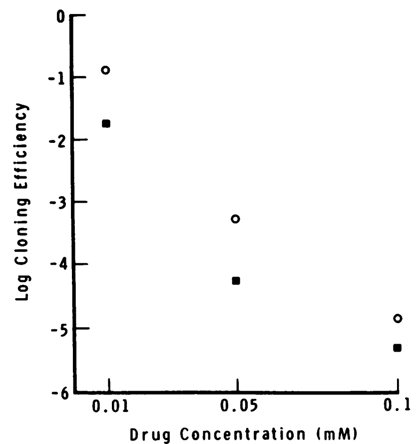

FIGURE 2 Effects of chlorozotocin and CCNU on L1210 survival. Each point represents the mean value of three independent experiments. ■, chlorozotocin; ○, CCNU.

conditions similar to the in vitro alkylation experiments. Chlorozotocin at a concentration of 0.1 mM suppressed DNA synthesis to 7% of control at 24 h and 1% of control at 48 h. CCNU suppressed DNA synthesis to 23% of control at 24 h and 16% of control at 48 h. Chlorozotocin is significantly more cytotoxic to L1210 cells than equimolar CCNU at both time points ($P < 0.01$).

The colony-forming ability of L1210 cells was determined after a 2-h exposure to 0.1, 0.05, or 0.01 mM chlorozotocin or CCNU, and a concentration dependent cytotoxicity was found for both drugs (Fig. 2). At the maximum drug concentration employed, 0.1 mM, chlorozotocin reduced the cloning efficiency by 5.3 logs compared to a reduction by 4.8 logs for equimolar CCNU. At all three molar concentrations, the chlorozotocin-treated cells showed a statistically significant increased cytotoxicity when compared to the CCNU treated cells ($P < 0.01$).

To correlate in vitro L1210 alkylation with in vivo L1210 antitumor activity, the two nitrosoureas were administered as a single intraperitoneal dose on day 2 to mice bearing intraperitoneally implanted L1210 leukemia (Table V). Chlorozotocin, at its LD_{10} of 64 μmol/kg, resulted in a 332% ILS compared to untreated tumor-bearing mice, with 60% indefinite survivors. CCNU administered at this same molar dose resulted in only a 191% ILS, with 20% indefinite survivors ($P < 0.05$). The optimal therapeutic dose of chlorozotocin, 48–64 μmol/kg, was one-third to one-half the molar dose of CCNU, 128 μmol/kg, required for comparable antileukemic activity. There was no statistically significant difference in the maximal antitumor activity of these two nitrosoureas when administered at their respective optimal molar doses.

To correlate in vitro alkylation of murine bone marrow with in vivo myelotoxicity, the total WBC count

Table V
Comparison of the In Vivo Antitumor Activity and Bone Marrow Toxicity of Chlorozotocin and CCNU

	Dose	Lethality of dose for normal CDF_1 mice	Antitumor activity		Bone marrow toxicity	
			L1210 activity	45-d survivors	Nadir WBC, day 3	Neutrophil count
	μmol/kg	*%*	*% ILS*		*% control*	*% control*
Chlorozotocin	48	0	306	6/10	89	129
	64	10	332	6/10	72	110
	96	90	52	1/10		
CCNU	48	0	83	0/10		
	64	0	191	2/10	57	55
	128	0	413	9/10	49	37
	171	10	364	7/10	38	25

For studies of in vivo antitumor activity, CDF_1 mice were treated with a single intraperitoneal dose of the drug on day 2 after the intraperitoneal implantation of 1×10^5 L1210 cells. Survival of treated mice was compared to control tumor-bearing animals.
For studies of in vivo bone marrow toxicity, normal CDF_1 mice were administered a single intraperitoneal dose of the drug and total WBC and neutrophil counts were serially measured. The blood counts on day 3 after injection, the time of leukocyte nadir, are expressed as a percentage of control values.

and absolute neutrophil count were measured. The nadir counts occurred 3 d after an intraperitoneal dose of the nitrosourea (Table V). CCNU, administered at a maximally effective dose of 128 μmol/kg, depressed the absolute neutrophil count to 37% of control. In contrast, chlorozotocin at its maximally effective antitumor dose of 64 μmol/kg did not produce a reduction in the absolute neutrophil count.

In vivo binding of [1-^{14}C]chloroethyl chlorozotocin and [U-^{14}C]chloroethyl CCNU to the murine bone marrow was determined at 60 min after a dose of 64 μmol/kg i.p., the optimal L1210 antitumor dose of chlorozotocin. The amount of [^{14}C]ethyl group covalently bound to protein and nucleic acids per 10^7 nucleated marrow cells was 6.6 ± 1.2 pmol for chlorozotocin and 5.6 ± 1.8 pmol for CCNU. At this equimolar dose, there was no statistically significant difference in the pmol of [^{14}C]-ethyl group bound to the nucleated bone marrow cells. In comparison, CCNU administered at its optimal L1210 antitumor dose of 128 μmol/kg, resulted in the covalent binding of 9.5 ± 0.8 pmol of [^{14}C]ethyl group/10^7 nucleated marrow cells.

DISCUSSION

Many anticancer agents are characterized by a narrow therapeutic index, reflecting their failure to effectively discriminate between target and normal tissues. Bone marrow suppression is often the most important dose-limiting toxicity. The chloroethyl nitrosoureas, in particular, produce profound, delayed and cumulative myelotoxicity (2). Chlorozotocin, a new glucose-containing nitrosourea, was designed to selectively reduce this toxicity for the bone marrow (4, 5). This agent demonstrates curative antitumor activity for the murine L1210 leukemia. At optimal therapeutic doses, however, there was no evidence of myelosuppression as indicated by normal neutrophil counts, bone marrow histology, and DNA synthesis, and survival of murine granulocyte precursor cells (CFU-C) (23, 24). In our recently completed phase I clinical trial of the drug, a single dose of 120 mg/m^2 resulted in peak plasma drug concentrations of 0.1 mM (24, 25). It is of importance to the present study that the optimal L1210 antitumor dose of chlorozotocin in mice, 64 μmol/kg, also produced a peak plasma concentration of 0.1 mM. At the 120 mg/m^2 dose of chlorozotocin in man, objective responses were observed in patients with melanoma, colon cancer, and lymphoma (24, 25). Moreover, there was no leukopenia or thrombocytopenia in patients who had not received extensive prior chemotherapy, and there has been no evidence of cumulative bone marrow toxicity (25).

Under physiological conditions, the chloroethyl nitrosoureas decompose to yield chloroethyl carbonium ions, or diazonium precursors, which are the principal alkylating products (26). In addition, organic isocyanates are generated which carbamoylate the lysine moieties and the terminal amino groups of peptides and proteins (8, 27). In the present study, CCNU was found to have significant carbamoylating activity as measured by the [^{14}C]lysine assay. In the murine cell culture

systems, CCNU carbamoylated protein 400- to 600-fold greater than chlorozotocin. However, the latter compound's excellent antitumor activity is not adversely affected by its negligible carbamoylating activity.

In our previous structure-activity studies using linear regression analyses, we have failed to demonstrate a significant correlation for in vitro carbamoylating activity with either antitumor activity, myelosuppression, or lethal toxicity. In contrast, the same analyses have demonstrated that a glucose carrier for the cytotoxic group is a specific parameter that correlates with reduced murine bone marrow toxicity (9, 10, 28–30). As an important example, ACNU is a pyrimidine analogue that has chemical properties similar to chlorozotocin, including water solubility and negligible carbamoylating activity. In contrast to the latter glucose analogue, ACNU is a potent myelotoxin in both mouse and man (29). 1-(2-chloroethyl)-3-(B-D-glucopyranosyl)-1-nitrosourea (GANU) is a second glucose chloroethyl nitrosourea that has reduced murine myelotoxicity (10, 30). With chlorozotocin, the cytotoxic group is attached to the carbon-2 of glucose, whereas attachment is at carbon-1 with GANU. GANU, unlike chlorozotocin, has significant chemical carbamoylating activity equivalent to the myelosuppressive nitrosourea BCNU, but produced no bone marrow toxicity in mice when administered at a comparable LD_{10} (10, 30). Collectively, these data strongly suggest that nitrosourea carbamoylating activity makes little if any contribution to the myelotoxicity of this class of alkylating agents.

The covalent binding of the [^{14}C]ethyl group of chlorozotocin to L1210 DNA, after a 2-h in vitro incubation, was 2.3-fold greater than that of CCNU at an equimolar dose of 0.1 mM. This concentration of chlorozotocin produced a fourfold increased covalent binding to the nuclear fraction of L1210 cells in vitro as compared to equimolar CCNU. In marked contrast to the results with L1210 cells, the in vitro alkylation of murine bone marrow DNA was equivalent for the two drugs at this 0.1 mM concentration. The in vitro covalent binding to the nuclear fraction of the marrow cells was also equivalent. In these experiments, the ratio of alkylation of L1210:bone marrow DNA was 1.3 for chlorozotocin compared to 0.6 for CCNU. It must be acknowledged that these results were obtained using our phenol method of extraction, which recovers 60–70% of the DNA. While the results are consistent in all experiments, it is possible that a more efficient extraction procedure might alter these results.

The greater in vitro alkylation of L1210 DNA by chlorozotocin as compared to CCNU has biological significance. The optimal in vivo antitumor activity of chlorozotocin against the L1210 leukemia, 332% ILS, was achieved at doses of 48–64 μmol/kg. In comparison, CCNU administered at these same molar doses produced only a 191% ILS, and the dose of CCNU required for comparable optimal L1210 antitumor activity was 128 μmol/kg. As a correlate, in the in vitro cloning assay, L1210 cells treated with 0.1, 0.05, or 0.01 mM chlorozotocin for 2 h showed significantly increased cytotoxicity when compared to cells treated with equimolar CCNU ($P < 0.01$). In similar experiments measuring the cloning efficiency of L1210 cells after a 1-h exposure to drug, Kann (31) has reported that chlorozotocin was significantly more cytotoxic than CCNU at comparable drug concentrations.

To measure differences in the distribution of the two drugs in vivo, the quantitative covalent binding of the ethyl-labeled drugs to nucleated bone marrow cells was determined at 60 min after intraperitoneal injection. Chlorozotocin was administered at its optimal L1210 antitumor dose of 64 μmol/kg. For comparison, CCNU was administered at its optimal L1210 antitumor dose of 128 μmol/kg and at the 64 μmol/kg dose. At the equimolar dose (64 μmol/kg), there was no significant difference in the picomoles of [^{14}C]ethyl group covalently bound per 10^7 nucleated marrow cells, 6.6 pmol for chlorozotocin and 5.6 pmol for CCNU ($P > 0.10$). In comparison, the covalent binding of the [^{14}C]ethyl group of CCNU per 10^7 marrow cells was significantly greater, 9.5 pmol, at its optimal L1210 antitumor dose of 128 μmol/kg ($P < 0.01$).

In conclusion, chlorozotocin has curative antitumor activity for the L1210 leukemia comparable to CCNU, but at one-third to one-half the molar dose of the latter compound. Chlorozotocin, unlike CCNU, produces no murine bone marrow toxicity at its optimal therapeutic dose. This unique combination of antitumor activity without myelosuppression can be correlated with the advantageous ratio of L1210:bone marrow DNA alkylation by chlorozotocin as compared to equimolar CCNU, in vitro.

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