Cancer Chemother Pharmacol (1983) 11:159–161

Cancer Chemotherapy and Pharmacology

Comparison of the transport of chlorozotocin and CCNU in L1210 leukemia and murine bone marrow cells in vitro

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Summary. *The uptake of radiolabeled CLZ and CCNU by L1210 leukemia and murine bone marrow cells was investigated to determine whether the preferential ratio of alkylation of L1210 DNA to murine bone marrow DNA of 1.3 by 0.1 mM CLZ, as against a ratio of 0.6 by equimolar CCNU, is secondary to differences in uptake.*

The concentration of intact CLZ was determined in the medium and the intracellular water space. The cell : medium ratio (intracellular concentration/medium concentration) of CLZ in bone marrow cells was greater than that seen for L1210 cells. However, the intracellular CLZ concentration generally remained constant in both cell types at 37° C, between 7.0 and 10.0 pmole/μl. The L1210 : murine bone marrow cell ratio of intracellular CLZ concentrations was approximately 1.0 from 10 to 60 min.

The intracellular CCNU concentration during the uptake of 0.1 mM (chloroethyl-U-^{14}C) CCNU at 37° C was constant at 85 pmol/μl from 10 to 60 min in L1210 cells, but slowly decreased from 66 pmole/μl at 20 min to 43 pmole/μl at 60 min in bone marrow cells. The L1210 : murine bone marrow cell ratio of intracellular CCNU concentrations ranged from 1.45 to 1.98 from 20 to 60 min. Thus, it appears that the preferential ratio of alkylation of L1210 DNA to murine bone marrow DNA by CLZ compared with equimolar CCNU cannot be explained by differences in uptake of the two agents by the two cell types.

The uptake of 0.1 mM CLZ at 37° C by L1210 cells in McCoy's 5A medium containing 300 mg% glucose was not affected by the addition of 5 mM cold drug, nor was it affected by the absence of glucose in the medium, with or without cold drug. This suggests that CLZ uptake into L1210 cells is via passive diffusion and that CLZ does not enter these cells via the glucose transport mechanism.

Introduction

The chloroethylnitrosoureas are an important class of antitumor agents with a broad spectrum of activity in human cancers [2]. However, all the agents in active clinical use, like the lipid-soluble CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea], produce delayed and cumulative bone marrow depression, which significantly limits their clinical usefulness [5].

Chlorozotocin (CLZ) is a water-soluble chloroethylnitrosourea with a glucose carrier, which like CCNU has curative activity for murine L1210 leukemia but with relative sparing of bone marrow toxicity in mice compared with CCNU. At optimal L1210 leukemia antitumor doses CLZ produces no myelosuppression, while CCNU results in significant leukopenia in mice. This has been correlated with a preferential ratio of alkylation of L1210 DNA to murine bone marrow DNA by 0.1 m*M* CLZ as against equimolar CCNU in vitro (L1210 : bone marrow DNA ratio was 1.3 for CLZ and 0.6 for CCNU) [6]. In this study we determined the uptake of CLZ and CCNU into L1210 leukemia and murine bone marrow cells to ascertain whether this favorable ratio is secondary to differences in uptake of the two agents by the two different cell types. In addition, we examined the mechanism of transport of CLZ into L1210 leukemia cells in detail.

Materials and methods

Male BALB/C × DBA/2F mice, weighing 17–25 g and maintained on LAB-BLOX laboratory chow pellets and water ad libitum were used throughout. The chloroethylnitrosoureas investigated in the study were CCNU (NSC 79037), and 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glycopyranose or chlorozotocin (NSC 78248). We utilized [chloroethyl-U-^{14}C] chlorozotocin (specific activity: 8.97 mCi/mmole), [glucose-1-^{14}C]chlorozotocin (specific activity 9.20 mCi/mmole), and [chloroethyl-U-^{14}C] CCNU (specific activity 15.70 mCi/mmole). The purity of these compounds was 95% as determined by the thin-layer chromatography (TLC) test described below. All nitrosoureas were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA. Chlorozotocin was dissolved in 0.01 *M* citrate buffer (pH 4.0) and CCNU was suspended in absolute ethanol.

Murine L1210 leukemia cells were prepared in a similar manner for all experiments. Mice were sacrificed by cervical traction. L1210 cells were aspirated from the peritoneum and suspended in 1 *N* saline (pH 7.4). After centrifugation, cell pellets were resuspended in distilled H_2O for 20 s to lyse any red blood cells in solution, followed by addition of an equal volume of 2 *N* saline. The solution was again centrifuged (500 rpm for 10 min) and the supernatant discarded. Cells were suspended in the appropriate medium and diluted to a concentration of 4×10^7 cells/ml, then pre-incubated for 15 min prior to initiation of the experiment to duplicate the experimental conditions utilized in the DNA alkylation studies [6]. Murine bone marrow cells were aspirated from the tibia and femur bones of mices hind legs, and were then prepared in

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a similar manner to the L1210 cells. The medium used throughout was McCoy's 5A (modified) without serum and, unless indicated otherwise, with 300 mg% glucose.

Uptake was initiated by the addition of labeled drug to either L1210 or murine bone marrow cells after the 15-min pre-incubation. For CLZ, 200-µl aliquots of the incubation mixture were layered onto Versilube F-50 silicone oil in a microcentrifuge tube in triplicate at specific time points, and were then centrifuged at 12,000 *g* for 1 min in a Brinkman's Eppendorf microcentrifuge to separate medium from cells as described previously [8]. Experiments for CCNU were conducted by centrifugation of 200 µl aliquots in duplicate through 8 ml of 0.25 *M* sucrose in a Sorvall swinging bucket at 12,000 *g* for 15 min at 4° C at specific time points to separate medium from cells [4]. All samples were stored in a solution of ethanol : acetate buffer at pH 4 (7 : 3) at −70° C prior to TLC.

The intracellular water space was determined with tritiated H_2O plus ^{14}C-inulin for all uptake studies by centrifugation through Versilube F-50 silicone oil to separate medium from cells, as described previously [7]. The results were later modified to allow for trapped extracellular label in the cell pellets.

Intact CLZ or CCNU in the cellular suspensions and medium samples were separated from their by-products by TLC on silica gel in *n*-butanol : acetate buffer (pH 4) : water (4 : 2 : 1). The sheets were cut into 1-cm × 2.54-cm strips and radioactivity was measured by liquid scintillation spectrometry. CLZ has an R_f of approximately 0.55, and CCNU an R_f of approximately 0.9 in this system.

All results were expressed as cell : medium ratios, i.e., the intracellular concentration divided by the extracellular concentration of drug. Trypan blue tests showed a cell viability of > 90% during cell uptake experiments. All experiments were done in duplicate or triplicate.

Results

The uptake of 0.1 m*M* CLZ, radiolabeled on either the glucose or chloroethyl moieties, by L1210 cells is shown at both 4° C and 37° C in Fig. 1. Uptake is expressed as the cell-to-medium ratio of radioactive drug over time (i.e., intracellular concentration/extracellular concentration = c/m). At 4° C, the uptakes of the two labels did not significantly differ, as their c/m ratios remained relatively constant, at about 0.05, over time. At 37° C, the c/m ratios slowly increased and the CLZ uptake was similar for both labels.

Figure 1 also shows the uptake of 0.1 m*M* CLZ with both labels by murine bone marrow cells at 37° C. The c/m ratios increased with time, but more dramatically than seen for L1210 cells.

The intracellular concentration of intact drug remained generally constant with time in both cell types at 37° C, between 7.0 and 10.0 pmole/µl. The cellular concentration in L1210 cells at 4° C also remained relatively constant, ranging between 3.0 and 5.0 pmole/µl. The medium intact drug concentration remained constant at 4° C, but decreased with time at 37° C. The CLZ half-life in the extracellular fluid at 37° C was 35–40 min for L1210 cells and 25–30 min for murine bone marrow cells.

To investigate the mechanism of transport of CLZ into L1210 cells, uptake experiments using 0.1 m*M* and 0.033 m*M* (chloroethyl-U-^{14}C) CLZ were conducted. The c/m ratio of

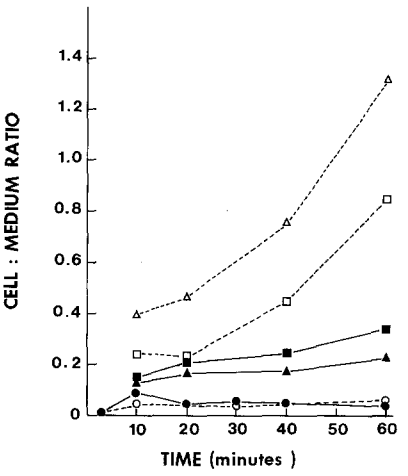

Fig. 1. Uptake of 0.1 m*M* (^{14}C-chloroethyl) or (^{14}C-glucose) chlorozotocin by L1210 murine leukemia and murine bone marrow cells at 37° C, and by L1210 cells at 4° C in McCoy's 5A media (+ glucose, − serum). (●) (^{14}C-glucose)-chlorozotocin in L1210 cells at 4° C; O-(^{14}C-chloroethyl)-chlorozotocin in L1210 cells at 4° C; (▲) (^{14}C-glucose)-chlorozotocin in L1210 cells at 37° C; (■) (^{14}C-chloroethyl)-chlorozotocin in L1210 cells at 37° C; □ (^{14}C-chloroethyl)-chlorozotocin in bone marrow cells at 37° C; △, (^{14}C-glucose)-chlorozotocin in bone marrow cells at 37° C

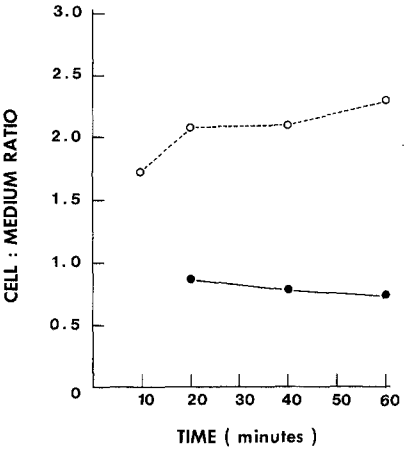

Fig. 2. Uptake of 0.1 m*M* (^{14}C-chloroethyl)-CCNU by L1210 murine leukemia and murine bone marrow cells at 37° C in McCoy's 5A media (+ glucose, − serum). (●) Bone marrow cells; (O) L1210 cells

CLZ was similar at both concentrations. The uptake of drug at 0.1 m*M* was not affected by the addition of 5 m*M* cold drug in McCoy's medium containing 300 mg% glucose. There was no significant difference in the transport of 0.1 m*M* (chloroethyl-U-^{14}C) CLZ in McCoy's medium without glucose, in McCoy's medium with 300 mg% glucose, or in McCoy's medium without glucose but with 5 m*M* cold CLZ.

The uptake of (chloroethyl-U-^{14}C) CCNU into L1210 and murine bone marrow cells at 37° C is presented in Fig. 2. The c/m ratios for L1210 cells varied from 1.72 at 10 min to 2.30 at 60 min. The intracellular CCNU concentration remained generally constant at about 85 pmole/µl throughout this time period. The c/m ratios for murine bone marrow cells remained at approximately 0.80 from 20 to 60 min. The intracellular CCNU concentration in bone marrow slowly decreased with time, from 66 pmol/µl at 20 min to 43 pmole/µl at 60 min. The

Table 1. Intracellular concentrations of (^{14}C-chloroethyl)-chlorozotocin in L1210 and murine bone marrow cells following a 2-h in vitro incubation with a 0.1 m*M* drug concentration

Time	L1210 (pmole/μl)	Bone marrow (pmole/μl)	L1210 : bone marrow ratio
10 min	8.27 ± 2.30[a]	9.66 ± 0.90	0.86
20 min	9.25 ± 0.97	7.14 ± 0.93	1.30
40 min	8.25 ± 0.65	8.09 ± 0.66	1.02
60 min	8.28 ± 0.50	10.5 ± 1.10	0.79

[a] Mean ± SEM

Table 2. Intracellular concentrations of (^{14}C-chloroethyl)-CCNU in L1210 and murine bone marrow cells following a 2-h in vitro incubation with a 0.1 m*M* drug concentration

Time	L1210 (pmole/μl)	Bone marrow (pmole/μl)	L1210 : bone marrow ratio
10 min	79.3 ± 4.7[a]	–	–
20 min	95.7 ± 7.2	66.1 ± 9.9	1.45
40 min	81.9 ± 14.5	55.8 ± 6.3	1.47
60 min	85.0 ± 10.3	43.0 ± 6.5	1.98

[a] Mean ± SEM

extracellular CCNU concentration slowly decreased for both L1210 and murine bone marrow cells. The CCNU half-life in the extracellular fluid at 37° C was approximately 110 min in both cell types.

The L1210 : murine bone marrow cell ratio of intracellular CLZ concentrations was approximately 1.00 from 10 to 60 min (Table 1). This contrasts with the ratio for intracellular CCNU concentrations, which ranged from 1.45 to 1.98 from 20 to 60 min (Table 2).

Discussion

The uptake of CLZ into L1210 cells at 0.1 m*M* and at 0.033 m*M* concentrations at 37° C was similar. The uptake of 0.1 m*M* CLZ was not affected by the addition of 5 m*M* cold drug in medium with or without 300 mg% glucose. This shows that CLZ uptake was not saturable, suggesting a passive mechanism of uptake. Also, the uptake of CLZ was not different with or without 300 mg% glucose in the medium, suggesting that CLZ does not enter L1210 cells via the glucose transport system despite the presence of a glucose moiety within the CLZ molecule. This is similar to the findings of Lam et al., who studied the mechanism of transport of CLZ into L5178Y cells [4]. Also, the c/m ratios obtained for CCNU and CLZ in L1210 cells are in agreement with those obtained by Lam's group in L5178Y cells [1, 4].

At 37° C, the intracellular concentration of CLZ remained generally constant over time during uptake into L1210 cells. The medium concentration decreased over the same period. This interesting aspect of CLZ transport is currently being investigated by means of efflux studies.

The uptake of CLZ in murine bone marrow cells at 37° C differs from that seen in L1210 leukemia cells at 37° C. This appears to be due to different rates of metabolism of CLZ in the medium for the two cell types. Different growth rates and/or nutrient requirements causing changes in medium pH alter the half-life of CLZ in the respective medium, i.e., the L1210 cells divide more actively and the medium is more acidic, resulting in a slightly longer half-life of CLZ. The half-life of CLZ in the extracellular fluid at 37° C was between 35 and 40 min for L1210 cells and between 25 and 30 min for murine bone marrow cells. A difference in half-life in the extracellular fluids for the two cells was not observed with CCNU, which was therefore not affected by these pH variations.

CLZ was found not to produce myelosuppression in mice at optimal L1210 leukemia antitumor doses, unlike the significant leukopenia seen in mice with CCNU. This correlated with a ratio of alkylation of L1210 : murine bone marrow DNA of 1.3 by 0.1 m*M* CLZ, as against 0.6 by equimolar CCNU in vitro [6]. Our study shows that this favorable ratio is not secondary to differences in uptake of the two agents by L1210 leukemia and murine bone marrow cells. The L1210 : murine bone marrow cell ratio of intracellular CLZ concentrations of approximately 1.00 from 10 to 60 min was lower than the ratio of 1.45–1.98 from 20 to 60 min for CCNU. Bone marrow cells thus had relatively higher intracellular CLZ concentrations than intracellular CCNU concentrations in comparison with their relative concentration in L1210 cells. This is in contrast with the favorable ratio of alkylation of DNA in the two cell lines by the two nitrosoureas.

It has recently been reported that CLZ and CCNU preferentially alkylate at different sites within murine bone marrow cell chromatin but at the same site in L1210 leukemia cell chromatin [3]. This may contribute to both the favorable ratio of alkylation of DNA in the two cells and the reduced myelotoxicity of CLZ compared with CCNU.

Acknowledgements. This work was supported in part by a grant from the Cancer Society of Quebec; by a Public Health Service grant (CA-28984) from the National Cancer Institute, National Institute of Health, Department of Health and Human Services; by a research grant from the National Cancer Institute of Canada; and by a contribution from Mr Tibor Schiff.

PL is a pre-doctoral Fellow of the Cancer Society of Quebec.

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Received September, 1982/Accepted May 4, 1983