Chlorambucil Pharmacokinetics and DNA Binding in Chronic Lymphocytic Leukemia Lymphocytes

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ABSTRACT

Chlorambucil (CLB) uptake by chronic lymphocytic leukemia lymphocytes was studied using a radiometric and a newly developed high-performance liquid chromatography assay. CLB labeled with $^{14}$C in either the chloroethyl group or phenyl ring was used with identical results. Drug accumulation by the cells was found to peak at 30 s, was independent of temperature, and was proportional to medium CLB concentration over a wide range. Efflux from cells loaded with CLB and resuspended in drug-free medium was nearly complete at 30 s. The metabolite inhibitors 2-deoxyglucose and NaN$_3$, the nitrogen mustard transport inhibitor hemicholinium-3, and another alkylating agent, melphalan, had no effect on drug uptake. We conclude that CLB enters and exits chronic lymphocytic leukemia lymphocytes by simple diffusion. Cells from 17 patients with all stages of chronic lymphocytic leukemia were studied including three with CLB-resistant disease, and no heterogeneity was found in the peak cell-associated CLB content or in metabolite pattern on high-performance liquid chromatography. These findings make it unlikely that transport or cellular drug metabolism are factors in drug resistance.

Drug-DNA binding was found to be temperature-sensitive and increased with time of incubation. Gel filtration of DNA before and after enzymatic digestion indicated the presence of drug-DNA adducts. High-performance liquid chromatography analysis of digested DNA and DNA treated by neutral thermal hydrolysis suggested the presence of multiple adducts. Most of the radioactivity was found as purine adducts. Studies with CLB labeled at two different sites revealed the presence of the phenyl group and ethyl chains in the adducts. A survey of patients showed increased drug-DNA binding in cells from patients with clinical CLB resistance.

INTRODUCTION

CLB is an alkylating agent widely used in the treatment of patients with CLL. The drug has been shown to cause a reduction in the circulating lymphocyte count and total mass of lymphoid tissue, while ameliorating symptoms in a majority of previously untreated patients (1-4). Although CLB has been in use for over 30 years, factors underlying drug action and resistance are incompletely understood.

Previous reports by Begleiter and Goldenberg using L5178Y (5) and by Hill, Harrap, and coworkers who investigated Yoshida ascites sarcoma cell lines and CLL lymphocytes (6-9) indicated that CLB uptake occurs by simple passive diffusion. The present study combines a radiometric with a newly developed HPLC technique, to further investigate this process in CLL lymphocytes.

Many authors attribute the chemotherapeutic action of alkylating agents to their ability to bind to DNA in target cells, damaging these macromolecules (10). Hill, using tritiated CLB, demonstrated the presence of radioactivity in DNA from Yoshida ascites sarcoma cells exposed to the labeled drug (9). Data supporting this concept are also available for cisplatinum, melphalan, the nitrosoureas, mitomycin C, and busulfan (11-15). We used CLB labeled either in the phenyl ring or chloroethyl groups to investigate drug-DNA binding in CLL lymphocytes. Gel filtration and HPLC analysis of DNA modified by enzyme digestion or neutral thermal hydrolysis was carried out to investigate the nature of drug-DNA binding.

MATERIALS AND METHODS

Lymphocyte Isolation

Blood from 20 patients who gave informed consent was collected into heparinized syringes. All but three of these patients were untreated for at least 1 year or had never received therapy. The three treated patients were included because they had clinically resistant disease. Lymphocytes were isolated using Ficoll-Hypaque (Pharmacia, Piscataway, NJ) centrifugation (16). Cell counts and sizing were done on a Coulter ZBI Counter and Channelizer (Coulter Electronics, Hialeah, FL). All patients had monoclonal B-cells as determined by a panel of markers including B-1, Leu-1, and T-11 antisera.

Cell Incubation. Cells (10$^5$/ml) were incubated in RPMI 1640 medium (GIBCO, Grand Island, NY) at pH 7.4 containing human serum albumin (Pentex, Miles Laboratories, Kankakee, IL) at a concentration of 0.4 mg/ml. Cells were counted and their viability tested by erythrosin B exclusion before incubation and at each time point. Cell viability exceeded 90% in all experiments.

Drug Uptake and Efflux. CLB, melphalan, sodium azide, and hemicholinium-3 were purchased from Sigma, St. Louis, MO. [14C]CLB, labeled in the phenyl ring, or in the chloroethyl group, was obtained from Amersham, Arlington Heights, IL, and from the National Cancer Institute. The drug was purified before use by HPLC with an isocratic elution using solvent B (see below). Radiochemical purity after this procedure was found to be 90-95%. CLB was dissolved in dimethyl sulfoxide, and was added to the cell suspension avoiding an organic solvent concentration of more than 0.1%. Uptake was usually studied at a 330 fM drug concentration (100 ug/ml). Most experiments were performed at this high concentration to optimize the HPLC signal, but experiments performed with 33 pM CLB showed similar results. Sodium azide was used at 10 mM, and 2-deoxyglucose at a 5 mM concentration. Melphalan stock was made as described (17). Hemicholinium-3 was dissolved directly in the medium to a final concentration of 100 pM. Cells were preincubated with inhibitors for 15 min at 37°C.

To measure drug efflux, cells were "loaded" by incubating with CLB for 30 s, centrifuged at 1,100 x g for 1 min and then transferred to drug-free medium.

Quantititation of Cell-associated [14C]CLB. A modification of the method described by Zweiling et al. (18) was used. Aliquots (0.3 ml) of cell suspension were layered on 0.4 ml of Versilube F-50 silicone oil (General Electric, Waterford, NY) in 1.5 ml conical plastic Eppendorf tubes; after centrifugation at 4,400 x g for 1 min, the supernatant was decanted and the inside of the tube blotted dry. The tip was then severed and placed overnight in a counting vial containing 2 ml of 0.3 N KOH. Radioactivity was measured in a Beckman LS 7000 scintillation counter following the addition of 15 ml of acidified Aquassure (New England Nuclear, Boston, MA). We determined medium trapping with [3H]-inulin to be 0.6 µl/sample; this volume was corrected for in all calculations.
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Extraction of CLB for HPLC. The extraction procedure is a modification of the one described for plasma by Ahmed et al. (19). The cell suspension (0.3 ml) was layered on 0.4-ml Versilube F-50 in 3-ml Pyrex conical tubes and centrifuged at 4,000 x g for 1 min. The supernatant was decanted, the inside of the tube blotted dry, and 1 ml of water added. The cells were dispersed by agitation on a Vortex Genie (Scientific Industries, Bohemia, NY) and allowed to stand for 15 min at 4°C; 1.5 ml of acetonitrile containing 0.2% acetic acid was then added, the mixture agitated again and frozen on dry ice. The tubes were then centrifuged at 4,000 x g for 5 min. One ml of the organic phase was transferred to glass test tubes and taken to dryness in a Speed-Vac concentrator (Savant Instruments, Hicksville, NY). Each sample was redissolved in 80 μl of solvent B (see below), of which 50 μl was injected into the HPLC apparatus. Control experiments showed that recovery of CLB standards was between 95 and 105%. Medium trapping using [3H]inulin was quantified at approximately 2 μl/sample, accounting for 10–20% of the peak cell-associated CLB.

The technique described above yielded organic, aqueous and solid phases. In some experiments, this method was applied to cells incubated with radioactive drug, and a sample of each phase removed for scintillation counting, to assess the partitioning of label in the cells.

In selected experiments following centrifugation of the cell suspension through oil, 200 μl of medium supernatant were extracted with 400 μl of acetonitrile/0.2% acetic acid, using the same technique as for cell lysates, to quantify CLB concentration.

Quantitation of CLB by HPLC. All HPLC measurements were performed on a Spectra Physics SP8000B apparatus equipped with a 5-μm end-capped Nucleosil 4.5 x 250 mm column. Solvent A was acetonitrile:water:acetic acid, 125:375:1, v/v/v. Solvent B was acetonitrile:water:acetic acid, 375:125:1, v/v/v. The gradient was developed as follows: 100% A x 3 min; linear 100% A to 73% B x 2 min; linear 73% B to 100% B x 8 min; 100% B x 2 min; linear 100% B to 100% A x 2 min; 100% A x 3 min. Absorbance at 254 nm was measured. CLB had a retention time of 16 to 17 min under these conditions. A standard curve was linear over the range of 10 ng to 5 μg.

**Drug-DNA Interaction.** DNA from CLL lymphocytes incubated with [14C]CLB was extracted using the phenol/chloroform method (20). The DNA content was determined spectrophotometrically by the A260. The bound CLB was measured in a liquid scintillation counter. The DNA was applied to a 1 cm x 25 cm column of Sephadex G-25 and eluted with phosphate buffered saline.

DNA was digested sequentially with DNase I, snake venom phosphodiesterase and alkaline phosphatase, according to Tomasz et al. (21). The digest was filtered through G-25 as described above. HPLC analysis was carried out on a 1 cm x 25 cm semipreparative 5 μm C-18 column with a 1 cm x 5 cm guard column packed with the same 5-μm material. To release purine-drug adducts, DNA was heated in a boiling water bath as described (22), passed through a 0.2-μm Nalgene syringe filter and injected into the HPLC apparatus. As a control, an HPLC elution profile was obtained on [14C]CLB in aqueous medium and heated in the same way as the DNA.

Solvent C was acetonitrile:0.02 M potassium phosphate, pH 5.0, 8:92, v/v; Solvent D was acetonitrile:0.02 M potassium phosphate, pH 5.0, 50:50, v/v. The mobile phase was: 100% C x 25 min, linear 100% C to 100% D x 60 min, 100% D x 5 min, linear 100% D to 100% C x 5 min.

All experiments were performed at least twice.

**RESULTS**

**Drug Influx and Efflux.** CLB was found to be stable in RPMI 1640 containing 0.4 mg/ml HSA at 4°C, but not at 37°C, where the half-life was observed to be approximately 35 min (Fig. 1). Maximal cell-associated drug was observed within 30 s of incubation and was independent of temperature (Fig. 2). A rapid decrease in cellular drug occurred with time at 37°C; at 4°C, a small decline also took place, consistent with nonenzymatic catalysis. The pattern of drug uptake by cells from a patient with clinical CLB resistance was similar to that obtained with cells from untreated patients.

As shown in Table 1, 2-deoxyglucose, sodium azide, hemicholinium-3, and melphalan, had no effect on the maximal amount of cell-associated drug. These data suggest that CLB influx occurs via passive diffusion. This conclusion is further supported by the linear and unsaturable drug accumulation over
a wide concentration range (Fig. 3). There was nearly immediate, temperature- and energy-independent efflux of drug from cells transferred to drug-free medium after loading with CLB (Fig. 4). These results indicated that influx and efflux of CLB occur via passive diffusion without receptor mediation.

The peak cell-associated CLB was determined for the lymphocytes from 17 patients (four with stage 0, four with stage 1, seven with stage 2, one with stage 3, one with stage 4). There was no significant heterogeneity, including three patients with clinical drug resistance. Peak cell-associated drug in lymphocytes from previously untreated patients was 19.9 ± 6.2 pmol/10⁷ cells by HPLC (N = 11, mean ± SD), 15.0 ± 2.6 pmol/10⁷ by radioassay (N = 16), and 16.5 ± 3.0 pmol/10⁷ cells (N = 3) by radioassay for lymphocytes from subjects with drug-resistant CLL. The above values are normalized for average cell volume and corrected for initial medium CLB concentration.

Drug Metabolism. A newly developed HPLC assay was utilized to investigate the intracellular fate of CLB. When cell-associated drug was measured simultaneously by both methods (Fig. 5) similar values were obtained at 30 s but the amount of drug measured by radioassay exceeded that determined by HPLC at the later time points. This consistent discrepancy can be explained by the inability of the radioassay to discriminate between the parent compound and its metabolites and adducts.

Analysis of the organic phase by simultaneous measurement of A₂₅₄ and radioactivity during HPLC (Fig. 6) showed the concurrent formation of two radioactive derivatives in medium and cells. No other metabolites were detected at 5 min and 30 min (data not shown). The material in the peak designated A was analyzed by mass spectrometry and found to contain no chloride moieties (data not shown); it was therefore considered to be a nonreactive product of spontaneous hydrolysis of the chloroethyl groups. This characteristic pattern of metabolite formation was observed for all CLL cells studied, regardless of clinical resistance to CLB. These results suggest that metabolism occurs by simple chemical breakdown of the drug and that CLB resistance is not explained by differences in intracellular drug metabolism. Furthermore, as shown in Table 2, water-soluble metabolites and presumably drug-macromolecule complexes are generated, as indicated by the partitioning of radioactivity into the aqueous and solid phases after a 60 minute incubation at 37°C, but not at 4°C.

Drug-DNA Interaction. Since cellular DNA is commonly believed to be the target of alkylating drugs, the binding of CLB and/or a metabolite to DNA was investigated. Radioactivity bound to DNA increased over time in cells incubated for 2 h with [¹⁴C]CLB at 37°C but not at 4°C (Fig. 7). This pattern was in contrast to the time-dependent decrease in total cell-associated CLB seen in Fig. 2. Drug-DNA interaction at 60 min of incubation at 37°C was found to increase with CLB concentration (Fig. 8). No saturation was seen over a wide range, suggesting a large excess of sites susceptible to alkylation.

Further characterization of this interaction revealed that the radioactivity was closely associated with macromolecular DNA when eluted on a column of Sephadex G-25; however, after digestion of the DNA with DNase I, snake venom phosphodiesterase, and alkaline phosphatase, a different elution pattern was seen (Fig. 9). All of the radioactivity was eluted in two broad peaks in the internal volume of the column; two peaks of material with a high absorbance at 260 nm, corresponding to purines and pyrimidines, as determined by standards, were noted. Identical results were obtained using [¹⁴C]CLB labeled in the phenyl ring or the chloroethyl groups. The above data suggest that covalent binding occurs between DNA and CLB and/or its metabolites. This interaction is temperature-sensitive.

<table>
<thead>
<tr>
<th>Phase</th>
<th>0.5 min</th>
<th>60 min</th>
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<th>60 min</th>
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<tr>
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<td>97%</td>
<td>61%</td>
<td>98%</td>
<td>97%</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.5%</td>
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<tr>
<td>Solid</td>
<td>1.5%</td>
<td>21%</td>
<td>1%</td>
<td>2%</td>
</tr>
</tbody>
</table>

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Fig. 4. Efflux of CLB. Cells were exposed to [¹⁴C]CLB for 30 s, then centrifuged and resuspended in drug-free medium. Cellular radioactivity in cells resuspended at 37°C (● ● ●) and 4°C (○ ○ ○) is compared to control cells whose incubation was continued in the presence of [¹⁴C]CLB at 37°C (● ● ●) and 4°C (○ ○ ○). Results shown are representative of two experiments.

Fig. 5. Cell-associated CLB as a function of time. Cells were incubated at 37°C, and CLB as measured by HPLC (●) and radioassay (○). A representative experiment is shown.

Fig. 6. HPLC pattern of radioactivity and absorbance at 254 nm in medium and cell extracts. Top left, medium at 30 s; bottom left, cells at 30 s; top right, medium at 60 min; bottom right, cells at 60 min. Incubations were performed at 37°C. The mobile phase used is described in "Methods." Absorbance (full scale) = 0.040. The peak of absorbance at the extreme left was found in cells incubated without drug, and therefore was of no relevance to CLB metabolism.

Table 2. Partitioning of label in cell extracts

| After incubation with [¹⁴C]CLB as described in "Methods," cells were lysed and extracted with acetonitrile. Radioactivity in each phase is expressed as a percentage of the total. |
CLB UPTAKE AND DNA BINDING IN CLL LYMPHOCYTES

Fig. 7. Effect of temperature on drug-DNA binding. The amount of radioactivity found in DNA from cells incubated with [3H]CLB at 37°C (•) and 4°C (○). No significant drug-DNA binding occurred at 30 s and 37°C, as found in another experiment (△).

Fig. 8. Effect of CLB concentration on DNA binding. [14C]CLB associated with DNA from cells incubated for 60 min at 37°C with increasing concentrations of drug. Results of two experiments are shown.

Fig. 9. Characterization of drug-DNA binding. DNA was extracted from CLL lymphocytes incubated with [3H]CLB and eluted on Sephadex G-25 with PBS. Top, undigested DNA; bottom, the same DNA following digestion as described in “Methods.”

and both the ethyl groups and phenyl ring of the drug molecule are present in the resulting adduct.

DNA exposed to DNase I, snake venom phosphodiesterase, and alkaline phosphatase is degraded to nucleosides (21). If alkylated DNA is degraded, complexes between nucleosides and the alkylator moiety are released. HPLC analysis of the lymphocyte DNA after digestion showed two major and two minor peaks of radioactivity, most likely representing drug-nucleoside adducts (Fig. 10, top). The same pattern was obtained regardless of the location of label in the [3H]CLB. HPLC analysis was also performed on undigested DNA subjected to a boiling bath for 10 min in neutral buffer (△). The pattern obtained with [3H]CLB in aqueous medium treated in the same way is shown (○).

Table 3 summarizes the quantitation of drug-DNA interaction in cells from 20 patients. There was heterogeneity among these subjects; cells from patients with clinical resistance (Nos. 17, 18, 19, 20) had more radioactivity bound to the DNA than from untreated patients. In lymphocytes from untreated patients, no correlation was found between drug-DNA binding and clinical stage.

The above data demonstrate the presence of multiple adducts and indirectly indicate that most of these are purine-drug complexes.

Table 3

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
<th>Drug-DNA binding (ng CLB/mg DNA)*</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>1</td>
<td>32.6 ± 5.9 (N = 2)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>30.9 ± 3.3 (N = 2)</td>
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<tr>
<td>3</td>
<td>1</td>
<td>57.7 (N = 1)</td>
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<tr>
<td>4</td>
<td>1</td>
<td>34.5 ± 4.2 (N = 2)</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>68.4 ± 24.2 (N = 3, mean ± SD)</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>37.4 (N = 1)</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>47.0 ± 19.9 (N = 2)</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>36.8 ± 3.7 (N = 2)</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>61.3 ± 37.1 (N = 3, mean ± SD)</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>59.3 ± 13.4 (N = 2)</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>51.4 ± 2.1 (N = 2)</td>
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<td>12</td>
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</tr>
<tr>
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<td>0</td>
<td>42.2 (N = 1)</td>
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<td>14</td>
<td>0</td>
<td>29.7 (N = 1)</td>
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<tr>
<td>15</td>
<td>1</td>
<td>59.0 ± 2.6 (N = 2)</td>
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<tr>
<td>16</td>
<td>0</td>
<td>77.4 (N = 1)</td>
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<td>4</td>
<td>75.4 ± 18.1 (N = 6, mean ± SD)</td>
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<tr>
<td>19a</td>
<td>4</td>
<td>84.7 (N = 1)</td>
</tr>
<tr>
<td>20a</td>
<td>2</td>
<td>91.9 (N = 1)</td>
</tr>
</tbody>
</table>

* The amount of drug-DNA binding was measured in lymphocytes incubated 60 min at 37°C.

* Patient with clinically CLB-resistant CLL.
DISCUSSION

The results presented above characterize two aspects of the pharmacokinetics of CLB in CLL lymphocytes: drug uptake and interaction with DNA.

Several lines of evidence support the concept that CLB enters CLL lymphocytes by passive diffusion. Drug accumulation was immediate, unsaturable over a wide concentration range and temperature-independent. Neither sodium azide nor 2-deoxyglucose had any effect on CLB uptake. Cellular uptake of CLB is through a different mechanism than that of nitrogen mustard and melphalan, two other alkylating agents. It was not affected by hemicholinium-3, an inhibitor of the choline transport system by which nitrogen mustard is transported into L5178Y cells (23), nor by melphalan, a drug transported via the L amino acid system (17). These results are in keeping with previous reports about CLB uptake in Yoshida ascites sarcoma (6), CLL (7), and murine L5178Y cells (5). As could be anticipated from reports about CLB uptake in Yoshida ascites sarcoma (6), CLL lymphocytes was a time- and temperature-dependent process. These observations are in keeping with earlier reports about CLB uptake in Yoshida ascites sarcoma cells (6, 7). Aqueous hydrolysis was found to be the only pathway of drug metabolism. Similar elution patterns were observed with cell extracts from untreated subjects as well as from patients with CLB-resistant disease. This suggests that differences in cellular metabolism of CLB are not the basis for clinical drug resistance and contrasts with an earlier report of a correlation between more rapid intracellular breakdown of the phenyl ring and CLB resistance in CLL lymphocytes (7). Another study of CLB uptake in L5178Y cells used thin layer chromatography to discriminate between the labeled parent drug and its metabolites. A pattern similar to ours was observed with a time-dependent formation of a metabolite simultaneously in cells and in medium (5).

We found that the binding of CLB to DNA in CLL lymphocytes was a time- and temperature-dependent process. These observations are in keeping with earlier reports about tritiated CLB in Yoshida ascites sarcoma cells (8, 9). The amount of drug-DNA binding was proportional to the starting CLB concentration. However, drug-DNA binding increased with time at 37°C, even though total cell-associated drug had been shown to steadily decrease over time. Therefore, the amount of drug-DNA binding is more than a simple reflection of total cellular drug at any given time. It appears that an activation step maybe necessary. The failure of drug-DNA binding to occur at 4°C also supports the concept that a chemical or enzymatic activation step may be involved.

Our results suggesting that the association between CLB and DNA is through covalent binding are based on the following evidence: (a) the radioactivity remained associated with the DNA after phenol/chloroform extraction; (b) radioactivity coeluted with DNA in the void volume of a Sephadex G-25 column with a shift to the internal volume after enzymatic digestion of the DNA. The elution pattern of radioactivity on G-25 and HPLC after digestion of the DNA indicates the presence of multiple adducts. The fact that the identical pattern was obtained with CLB labeled in the phenyl ring or chloroethy groups is consistent with the generally accepted notion that both moieties are present in the adducts. The chloroethy groups are thought to attack susceptible sites on target molecules, forming cross-links. This type of mechanism has been demonstrated for several other alkylating agents (12, 14–15).

By utilizing the neutral thermal hydrolysis technique described by Beranek, et al. (22), we found that most of the radioactivity in the DNA appeared to be present in purine adducts. This is analogous to the case of busulfan and mitomycin C (14, 15), two other alkylating agents.

In the survey of CLL patients, we found a higher amount of drug-DNA binding in cells from patients with clinically resistant disease compared to untreated patients. This finding is in contrast with results in Yoshida ascites sarcoma cells selected for CLB resistance (8). The cause for this unexpected result is unknown. If, as our data suggest, metabolic activation is necessary for adduct formation, the above result could be explained if the lymphocytes from patients with more advanced disease contained a higher percentage of metabolically active cells. Also, cytotoxicity has been linked to cell cycle for several alkylating agents (24–26). It is conceivable that populations of cells isolated from subjects with CLB-resistant CLL vary from the others in the proportion of lymphocytes in each phase of the cell cycle, accounting for the differences in drug-DNA binding.

ACKNOWLEDGMENTS

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REFERENCES


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